Table 1 *MDR1* genetic variants in 24 Japanese nephrectomized patients

Location	Position	Allele	Effect	Frequency	Genotype	Frequency	Goto et al.[a]
Intron 1	Exon 2-1	G	Initiation of translation?	100	G/G	100	100
		A		0	G/A	0	0
					A/A	0	0
Exon 2	cDNA 61	A	21 Asn	100	A/A	100	100
		G	21 Asp	0	A/G	0	0
					G/G	0	0
Exon 5	cDNA 307	T	103 Phe	100	T/T	100	100
		C	103 Leu	0	T/C	0	0
					C/C	0	0
Intron 6	Exon 6+139	C	?	33.3	C/C	8.3	10.3
		T		66.7	C/T	50	52.9
					T/T	41.7	36.8
Exon 11	cDNA 1199	G	400 Ser	100	G/G	100	100
		A	400 Asn	0	G/A	0	0
					A/A	0	0
Exon 12	cDNA 1236	C	Wobble	33.3	C/C	8.3	10.1
		T		66.7	C/T	50	49.3
					T/T	41.7	40.6
Intron 12	Exon 12+44	C	?	100	C/C	100	100
		T		0	C/T	0	0
					T/T	0	0
Intron 16	Exon 17-76	T	?	68.8	T/T	45.8	42.6
		A		31.3	T/A	45.8	51.5
					A/A	8.3	5.9
Exon 21	cDNA 2677	G	893 Ala	35.4	G/G	0	22.9
		T	893 Ser	45.8	G/A	29.2	5.8
		A	893 Thr	18.8	G/T	41.7	39.1
					T/A	8.3	15.9
					T/T	20.8	15.9
					A/A	0	1.4
Exon 26	cDNA 3435	C	Wobble	47.9	C/C	25	30.4
		T		52.1	C/T	45.8	50.7
					T/T	29.2	18.8

[a]These values are from our previous study with Japanese recipients of living-donor liver transplantation

variation of drug absorption and disposition (Goto et al. 2002; Hashida et al. 2001; Schuetz et al. 1995). A nuclear receptor, pregnane X receptor (termed as steroid and xenobiotic receptor), predominantly expressed in the liver and small intestine, was reported to regulate MDR1 expression in these tissues as a part of the regulatory mechanisms by various compounds including endogenous steroids and xenobiotics (Synold et al. 2001). Therefore, the nuclear receptor may contribute, at least in part, to the large interindividual variability of the expression level of MDR1 in the liver and small intestine. However, there is no information predicting the expression regulation of renal Pgp. The elucidation of the nuclear receptors and SNPs in the transcriptional regulatory region of the *MDR1* gene would clarify interindividual variation of renal Pgp content.

During the last few decades, the incidence of RCC has steadily increased (Chow et al. 1999). Obesity, hypertension, gender, smoking, and several drugs such as diuretics, phenacetin, and aspirin are suggested to be associated with RCC (Dhote et al. 2000). Furthermore, various genetic polymorphisms were also reported to be related to the disease (Nakamura et al. 2002; Tanaka et al. 2002). Recently, Siegsmund et al. (2002) reported that the frequency of T/T genotype at MDR1 cDNA 3435 was significantly higher in patients with RCC than in the control Caucasians, suggesting that this SNP would be a risk factor for RCC in Caucasian. In this study, the T/T was observed in seven of 24 RCC patients (29.2%). Since this frequency is not significantly different from healthy Japanese (20%) in the data reported by Schaeffeler et al.(2001) with χ^2 statistics ($P > 0.540$), the T/T genotype at MDR1 cDNA 3435 might not be a risk factor for RCC in Japanese. Further research is needed to elucidate the association between the cDNA 3435 SNP and RCC in Japanese. Chow et al. (1999) reported the incidence of RCC in black subjects was higher than in Caucasian subjects. On the other hand, the frequency of the T/T genotype at the cDNA 3435 is reported to be markedly lower in black subjects than in Caucasian and Japanese subjects (Schaeffeler et al. 2001). The T/T frequency in black subjects with RCC should be interested. Siegsmund et al. (2002) also represented that Pgp expression levels in renal noncancerous segments were significantly lower with the T/T genotype at cDNA 3435 than with the C/C genotype by using the quantitative immunohistochemistry method. This suggests that renal Pgp expression levels influence susceptibility to the development of renal epithelial cancers. Our work illustrates that MDR1 levels were not affected by the cDNA 3435 T/T genotype. In the future, not only to determine the predominant factor(s)/material(s) developing RCC but also to clarify its renal handling, including the contribution of Pgp, are necessary for

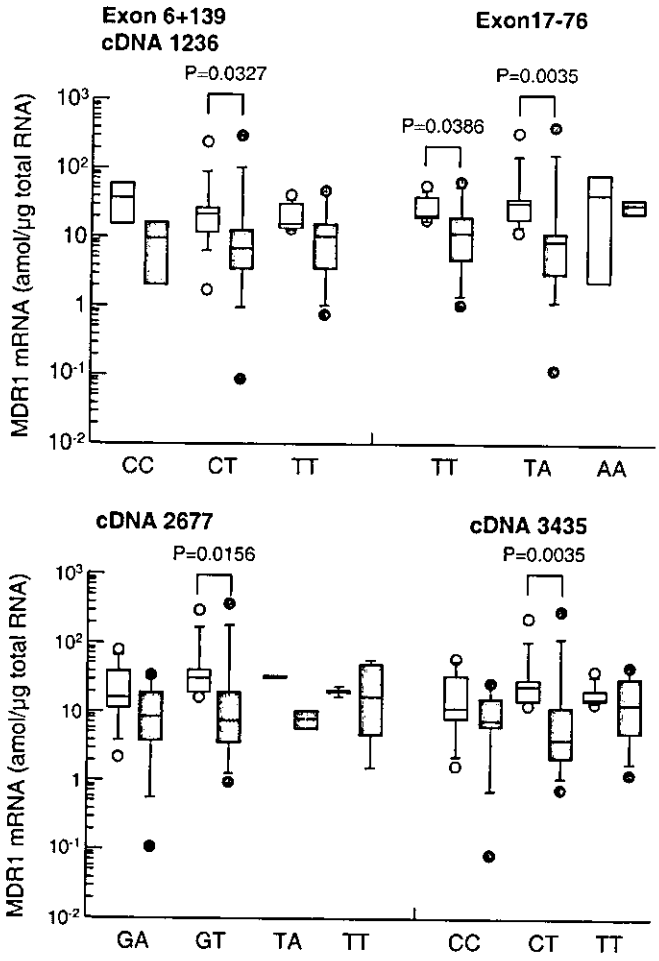

Fig. 3 Comparison of mRNA levels in the normal kidney cortex, renal cell carcinoma (RCC) segments, or their variation by carcinogenesis with the *MDR1* genotype. The MDR1 mRNA levels in the normal kidney cortex (*open box*) and RCC segments (*gray box*) were compared with the five polymorphic SNPs. Simultaneously, the variation of the expression levels of MDR1 mRNA were assessed in comparison with the *MDR1* genotypes between normal and RCC segments. After statistical analyses by Mann-Whitney *U* test, *P* values less than 0.05 were shown

analysis of the relationship between RCC and the SNP of the *MDR1* gene.

RCC displays an intrinsically high degree of resistance to chemotherapy (Hartmann and Bokemeyer 1999). Fojo et al. (1987) and Kakehi et al. (1988) represented that the resistance of RCC against anticancer drug vinblastine was associated with MDR1 expression using cell lines. The present study clarified that MDR1 expression levels showed a downward tendency by malignant transformation to RCC and that expression in the RCC was still relatively high. These results are compatible with other groups (Fojo et al. 1987; Kakehi et al. 1988). Taken together with the fact that various anticancer drugs are substrates of Pgp, it is suggested that this phenomenon is, at least in part, involved in the chemoresistance of RCC. The downregulation of MDR1 mRNA in the kidney cortex by transformation to RCC was dependent on the T allele at exon 17–76 (Fig. 3). Despite the small number of patients carrying the A/A genotype at exon 17–76 ($n=2$), the mRNA expression of MDR1 in the kidney might be downregulated by carcinogenesis in patients with the T allele. Therefore, further studies would clarify the effect of SNPs in the *MDR1* gene on the reducing rate of its mRNA level in renal tissue by carcinogenesis. In addition to MDR1, glutathione S-transferase, topoisomerase II, and MDR-associated protein are suggested to contribute to the chemoresistance of RCC (Volm et al. 1993; Kim et al. 1996). Elucidation of not only renal handling of anticancer agents but also function and expression levels of these proteins in RCC, and of their relations with SNPs, give information for the adequate selection of anticancer agents for individual RCC.

In summary, we estimated the copy number of MDR1 mRNA in the human kidney cortex and RCC and found that there is a wide interindividual variation in renal MDR1 expression levels and that MDR1 mRNA levels tend to decrease by malignant transformation to RCC in the human kidney. In addition, the ten common polymorphisms of the *MDR1* gene were examined, and the effect of SNPs on expression levels of the transporter mRNA was not observed in the normal kidney cortex and RCC. To our knowledge, this is the first report representing the measurement of renal MDR1 mRNA and correlation of the expression and SNPs of *MDR1*.

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Peptide Transporters: Structure, Function, Regulation and Application for Drug Delivery

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Abstract: Proton-coupled peptide transporters, localized at brush-border membranes of intestinal and renal epithelial cells, play important roles in protein absorption and the conservation of peptide-bound amino nitrogen. These transporters also have significant pharmacological and pharmacokinetic relevance to the transport of various peptide-like drugs such as β-lactam antibiotics. The identification and molecular characterization of H^+/peptide cotransporters (PEPT1 and PEPT2) have facilitated the clarification of many aspects of these transporters such as the structure/function relationship and regulation. Recent findings that intestinal PEPT1 can transport L-valine ester prodrugs such as valacyclovir provided a major step forward toward the development of novel drug delivery systems. It has been demonstrated that peptide transporters, which have a similar substrate specificity to PEPT1 and PEPT2, but possess other distinct functional properties, are localized at basolateral membranes of intestinal and renal epithelial cells. This review highlights the recent advances in our knowledge of the cellular and molecular nature of PEPT1, PEPT2 and the basolateral peptide transporters.

1. INTRODUCTION

Dietary protein undergoes a series of degradative steps, resulting in a mixture of free amino acids and small peptides. These products are then taken up by the intestinal epithelial cells and delivered into the circulation [1]. Similarly in the kidney, filtered amino acids and small peptides are efficiently reabsorbed from the proximal tubular cells for conservation of amino acid nitrogen [2].

A large number of studies have provided evidence that the absorption of protein digestion products in the small intestine occurs primarily in the form of small peptides [3]. The transport pathways for small peptides are called peptide transporters. Peptide transporters can accept di- and tripeptides as physiological substrates, indicating that they have a much broader substrate specificity than other nutritional transporters. Consequently, foreign compounds structurally resembling small peptides such as oral β-lactam antibiotics, are recognized by the peptide transporters. Therefore, peptide transporters work not only as nutritional transporters but also as drug transporters (Fig. **1**).

Currently, the peptide transporters are divided into two types; i.e., those localized at the brush-border membranes of epithelial cells, and those localized at the basolateral membranes. About a decade ago, two brush-border type peptide transporters were identified, and designated PEPT1 and PEPT2. There are several excellent reviews documenting the molecular nature of PEPT1 and PEPT2 from biochemical aspects to physiological and pharmacological significance [4-9]. In contrast to the brush-border type peptide transporters, little attention has been paid to the basolateral peptide transporters. However, our recent studies have provided unequivocal evidence that the basolateral peptide transporters, which are functionally distinguishable from PEPT1 and PEPT2, are expressed in the intestine and kidney [10-16]. This review deals with the current progress in cellular and/or molecular studies of PEPT1, PEPT2 and the basolateral peptide transporters, as well as with their brief historical background and physiological and pharmacokinetic roles.

2. DRUG TRANSPORT BY PEPTIDE TRANSPORTERS

More than 20 years ago, the clarification of absorption mechanisms for oral β-lactam antibiotics was a very attractive issue in the field of pharmacokinetic research. This is because oral β-lactam antibiotics are efficiently absorbed from the small intestine, although they are ionized at physiological pH and have very low lipid solubility. This completely contradicts the traditional pH-partition theory of drug absorption. Using tissue preparation techniques, various investigators tried to solve this puzzle, and found that these drugs were absorbed by a carrier-mediated system [17-19]. However, it was not clear which carrier was responsible for their absorption. Finally, using intestinal brush-border membrane vesicles, we first provided direct evidence that the orally active β-lactam antibiotics are transported *via* the H^+-coupled peptide transporter [20-21]. Subsequently, transport studies using brush-border mem-brane vesicles and the human intestinal cell line Caco-2, have demonstrated that many peptide-like drugs are absorbed by the H^+-coupled peptide transporter. For example, the anti-cancer agent Bestatin [22], renin inhibitors [23], and several angiotensin converting enzyme (ACE) inhibitors [24] were all reported to be recognized by the peptide transporters.

The transport of peptide-like drugs has also been reported in the kidney. The aminocephalosporin cephalexin was the

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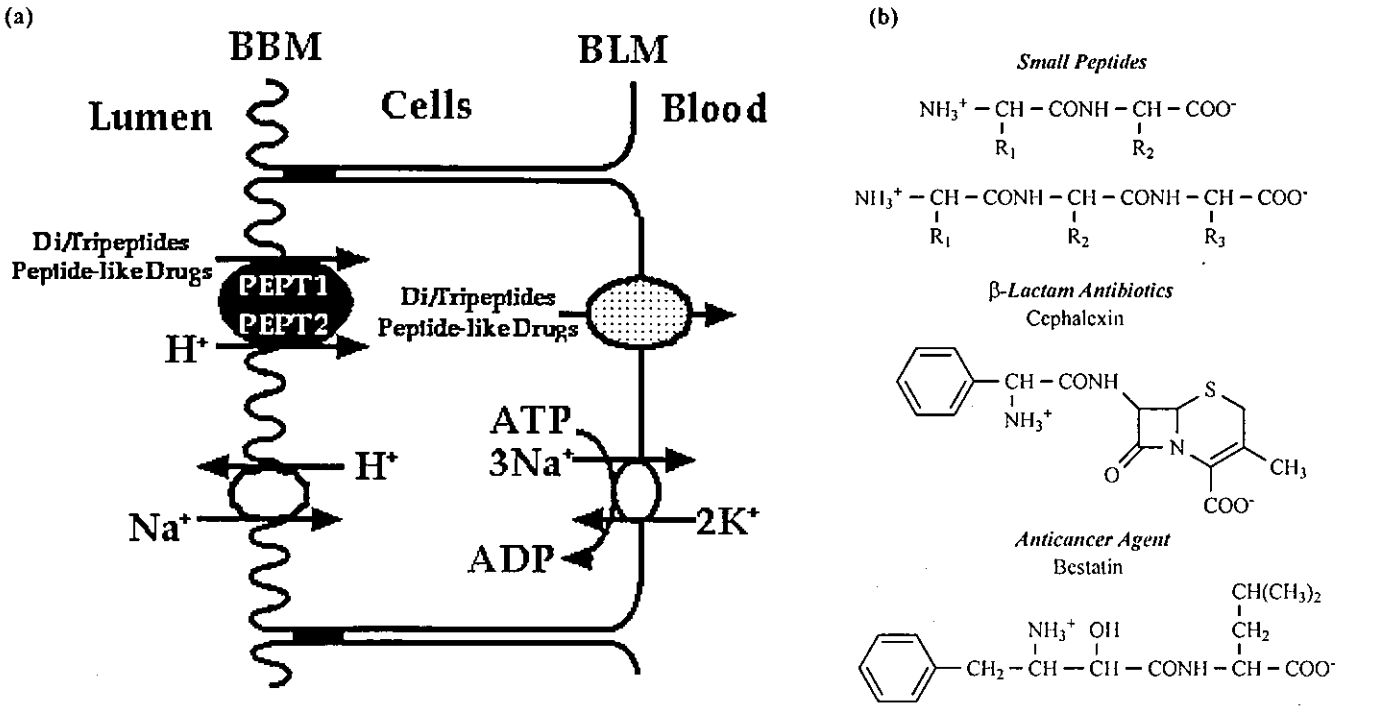

Fig. (1). (a) Peptide transporters in the epithelial cells. BBM, brush-border membranes; BLM, basolateral membranes. (b) Chemical structures of small peptides and peptide-like drugs. Peptide-like drugs structurally resemble di- or tripeptides.

first peptide-like drug whose transport was mediated *via* the renal H^+/peptide cotransporter [25-26]. However, unlike in the intestine, the transport of β-lactam antibiotics in the kidney was mediated by at least two distinct H^+/peptide cotransporters; namely, high affinity-low capacity and low affinity-high capacity transport systems [27-28]. The peptide transport system in the kidney is suggested to be involved in the active transport of these antibiotics from the glomerular filtrate, and to increase their half-life in the circulation.

3. CLONING OF PEPTIDE TRANSPORTERS PEPT1 AND PEPT2

3.1. Structure

A cDNA encoding the H^+/peptide cotransporter (PEPT1) was initially identified by expression cloning using a rabbit small intestinal cDNA library [29]. Homologous cDNAs were then found in human, rat, mouse, cow and chicken [30-34]. As an isoform of the intestinal peptide transporter PEPT1, the renal peptide transporter PEPT2 cDNA has been isolated from human, rabbit, rat and mouse [35-38]. PEPT1 and PEPT2 consist of 707-710 and 729 amino acid residues, respectively, with several putative glycosylation and phosphorylation sites. Hydropathy analysis and an epitope-insertion approach [39] suggested that peptide transporter proteins contain 12 transmembrane domains, with both the C- and N-terminal localized inside the cell. Overall amino acid identity between PEPT1 and PEPT2 is approximately 50%, and the amino acid sequence in the intra- or extracellular loops is more divergent than that in the putative transmembrane regions. The characteristics and putative secondary structure of PEPT1 and PEPT2 are shown in Fig. (2).

3.2. Gene Organization

The human PEPT1 gene is located at chromosome 13q24-q33, consisting of 23 exons, whereas the human PEPT2 gene is located at 3q13.3-q21, consisting of 22 exons. Although there is no report concerning functional promoter analysis of the human PEPT1 and PEPT2 genes, such analyses have been performed using rat [40] and mouse [32] PEPT1 genes and the mouse PEPT2 gene [38]. In the mouse PEPT1 gene, the promoter region upstream of the transcription start site does not contain the TATA box, but possesses three GC boxes. Functional promoter analysis demonstrated that essential promoter/enhancer elements are present within 1,140 base pairs (bp) upstream of the transcription start site. The mouse PEPT2 gene also possesses a TATA-less promoter, and functional promoter analysis revealed that the core promoter region was located between 432 and 286 bp upstream from the translation start site.

It has been reported that single nucleotide polymorphisms of drug transporter genes can affect the pharmacokinetic profiles of drugs [41]. Several polymorphic variants were discovered in the human PEPT1 gene, and suggested the possibility of susceptibility to bipolar disorders [42], but currently, there are no reports on human PEPT1 and PEPT2 gene polymorphism affecting transporter function.

3.3. Tissue Distribution and Membrane Localization

PEPT1 mRNA is mainly expressed in the small intestine and at low levels in the liver and kidney [29-31]. Immunological studies demonstrated that PEPT1 protein is localized to brush-border membranes of the absorptive epithelial cells of the small intestine, and that this protein

(a)

	PEPT1	*PEPT2*
Amino Acids	708~710	729
Identity	100	~50
Tissue Distribution	Small Intestine, Kidney, Liver	Kidney, Brain, Lung, Mammary Gland
Substrates	Di and Tripeptides Peptide-like Drugs Valacyclovir etc.	Di and Tripeptides Peptide-like Drugs δ-Amino Levulinic Acid etc.
Affinity (Km for Gly-Sar)	Low (~1.0 mM)	High (~0.1 mM)
Regulation	Diet, Hormons, Development, Diurnal Rhythm etc.	Chronic Renal Failure etc.

(b)

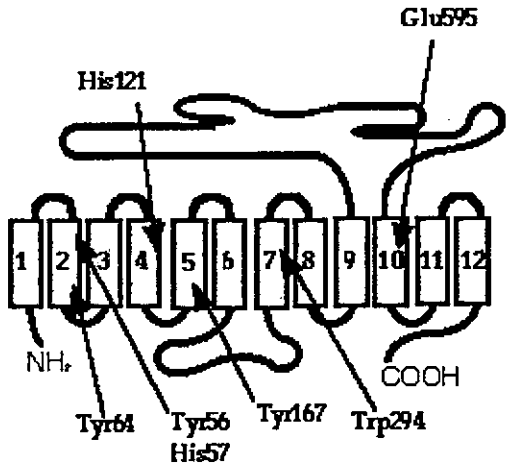

Fig. (2). (a) Summary of PEPT1 and PEPT2. (b) 2nd structure of PEPT1 and PEPT2. The amino acids indicated were putative essential amino acid residues reported by mutagenesis analysis.

was abundant at the tip of the villus and was decreased at the crypt base [43]. In the kidney, PEPT1 protein was localized at the brush-border membranes of the S1 segment of proximal tubules [44]. In the liver, PEPT1 protein is localized at the apical membrane of cholangiocytes of the extrahepatic biliary duct [45]. The physiological and pharmacological significance of PEPT1 in biliary epithelium is currently unclear.

PEPT2 mRNA is predominantly expressed in the kidney, but also in the brain, lung, spleen and mammary gland [35-37]. PEPT2 protein is localized at the brush-border membranes of the S3 segment of proximal tubules [44]. The different intrarenal distributions of PEPT1 and PEPT2 may contribute to the efficient reabsorption of small peptides, and reflects the previous finding that transport of β-lactam antibiotics in the kidney was mediated by at least two distinct H^+/peptide cotransporters [27-28]. In the brain, *in situ* hybridization studies have demonstrated that PEPT2 mRNA is expressed by astrocytes, subependymal cells, ependymal cells and epithelial cells of the choroid plexus [46]. Immunological analysis showed that PEPT2 protein is expressed in satellite glial cells surrounding the ganglionic neurons [47] and present at the apical membranes of choroidal epithelial cells [48]. In the lungs, PEPT2 protein was expressed in alveolar type II pneumocytes, bronchial epithelium, and endothelium of small vessels [49]. In the mammary gland, PEPT2 protein was expressed in epithelial cells of the gland and duct [50]. The function of PEPT2 in the choroid plexus, lung and mammary gland was also confirmed by using various tissue preparation techniques [48-50], and hence PEPT2 has been suggested to play important roles in drug delivery and disposition in these tissues. Very recently, Shen *et al.* [51] have developed *PEPT2*-deficient mice, which were viable and without obvious kidney or brain abnormalities. Using isolated choroid plexus from $PEPT2^{-/-}$ mice, they have clearly demonstrated that $PEPT2^{-/-}$ mice show impaired uptake of dipeptide in the choroid plexus, and have suggested that *PEPT2* is the primary member of the peptide transporter family responsible for the trafficking of peptides/mimetics at the blood-cerebrospinal fluid barrier [51].

4. FUNCTIONAL CHARACTERISTICS OF PEPT1 AND PEPT2

4.1. Substrate Specificity

Both PEPT1 and PEPT2 can transport di- and tripeptides with different molecular sizes and charges, but not free amino acids and tetrapeptides [29, 36]. Pharmacologically active peptide-like drugs such as β-lactam antibiotics, Bestatin and ACE inhibitors have been also reported to be transported by PEPT1 and PEPT2 [31, 37, 52-54]. Recently, it has been demonstrated that the bacteria-derived chemotactic peptide (*N*-formylmethionyl-leucyl-phenylalanine (fMLP)) is transported by intestinal PEPT1, and suggested that fMLP transport by PEPT1 induces intestinal inflammation [55-57].

It is believed that the presence of peptide-bond(s) is the most important factor in the recognition of substrates by peptide transporters. However, structural requirements of PEPT1 and PEPT2 were re-evaluated (most studies were performed in PEPT1), and it was demonstrated that even compounds without peptide bond(s) can be accepted as substrates. For example, 4-aminophenylacetic acid [58], δ-amino levulinic acid [59], ω-amino fatty acid [60], amino

acid aryl amide [61], and valacyclovir [62] can be accepted as substrates. From the pharmaceutical standpoint, the finding of valacylcovir transport by PEPT1 can provide new strategies for drug delivery, and this topic will be discussed in Kunta *et al.* (this issue). Interestingly, some peptide mimetic drugs such as the ACE inhibitor quinapril and the anti-diabetic agent glibenclamide showed noncompetitive inhibition of PEPT1 and PEPT2 [63-65]. These findings and data obtained in different expression systems have been used for modeling to obtain a template for the interaction within the substrate-binding domain [66].

4.2. Substrate Affinity

Functional expression studies have clearly established that PEPT1 is a low-affinity transporter, whereas PEPT2 is a high-affinity transporter. For example, PEPT2 showed higher affinity for chemically diverse dipeptides and tripeptides compared to PEPT1 [67]. PEPT2 also exhibited higher affinity for amino β-lactam antibiotics [68]. These differences in substrate affinity are not necessarily limited to substrates with peptide bond(s). Nonpeptidic compounds such as valacyclovir and δ-amino levulinic acid are preferentially recognized by PEPT2 rather than PEPT1 [69]. Notably, anionic β-lactam antibiotics such as ceftibuten [68] and 8 amino-octanoic acid [69] showed higher affinity for PEPT1 than for PEPT2. A feature of these compounds is the lack of α- or β-amino carbonyl function, thus, it has been suggested that the α- or β-amino carbonyl function is a key structure in exhibiting higher affinity for PEPT2 than for PEPT1 [69-70].

4.3. Stoichiometry

PEPT1 and PEPT2 can transport a wide variety of substrates in an electrogenic mode as a consequence of H^+ and substrate cotransport. These electrogenic characteristics of PEPT1 and PEPT2 are commonly observed, but it has been unclear how carrier proteins can transport differently charged substrates in the same general transport mode.

Several studies have shown that the transport of zwitterionic dipeptides by PEPT1 is electrogenic, and this occurred at a proton-to-substrate flux coupling ratio of 1:1 [29, 71-73]. Cationic dipeptides can be transported in neutral and positively-charged forms, resulting in excess transport current as compared to neutral substrates [71, 73]. On the other hand, in the case of anionic dipeptides, the situation is more complicated, and several hypotheses have been suggested as follows. 1) Electrogenic transport of anionic dipeptides may be due to the cotransport of one substrate molecule together with two protons [72-74], or 2) it could result from the cotransport of one proton per substrate molecule and a simultaneous countertransport of one negatively-charged counterion such as OH^- or HCO_3^- [71]. 3) An alternative explanation could be the preferential transport of only the zwitterionic form of the substrate with one proton [75-76]. So, the stoichiometry of anionic dipeptides to H^+ is still controversial, and may be caused by these mixed effects. In the case of PEPT2, a proton to substrate stoichiometry of 3:1 was proposed for the influx of dipeptides [77]. As many studies have been performed using dipeptides, there is limited information available about the stoichiometry of peptide-like drugs to H^+ [75].

4.4. Structure/Function Relationship Analysis

To fully exploit PEPT1 (also PEPT2) for optimizing drug delivery, it will be necessary to understand its substrate recognition mechanisms. After the cloning of PEPT1 and PEPT2 cDNAs, several approaches have been used to clarify their substrate binding domains by protein engineering methods and computer modeling analyses.

Using a site-directed mutagenesis technique, it has been shown that the conserved histidine (His57 and His121) and tyrosine (Tyr56, Tyr64 and Tyr167) residues in the second, fourth and fifth transmembrane domain (TMD) of PEPT1 and PEPT2 are essential for the transport activity and/or substrate binding [78-81] (Fig. 2). One histidine residue was suggested to be the binding site for an α-amino group of substrates [82], and another was an H^+-binding site [81-82]. As described below, based on computational modeling, Bolger *et al.* [83] reported that the mutation of Trp294 or Glu595 in human PEPT1 reduced glycylsarcosine (Gly-Sar) uptake by 80 and 95%, respectively (Fig. 2).

The second approach is the analysis of chimeric PEPT1/PEPT2 proteins. Döring *et al.* [84] first constructed a chimeric peptide transporter with rabbit PEPT1 and PEPT2 and compared its functions with those of the parent transporters. They demonstrated that the phenotypic characteristics of PEPT2 were determined by its amino-terminal region (TMD1~9), suggesting that the large extracellular loop between TMD9 and TMD10, comprising one-third of the transporter protein, might not be responsible for substrate binding [84]. Following PEPT1/PEPT2 chimera studies from various groups, narrower segments responsible for substrate binding and other functional properties have been identified [85-86]. According to the recent report of Döring *et al.* [87], the first 59 amino acid residues up to the second TMD may form an important part of the substrate-binding domain in peptide transporters.

The third approach is computer modeling. Through the iterative process (from computational modeling to functional assay), it was predicted that the substrate binding domain of human PEPT1 was composed of Tyr12 and Glu26 from TMD1, Tyr91 from TMD3; Tyr167 from TMD5; Trp294 and Arg282 from TMD7; Asp341 from TMD8 and Tyr588 and Glu595 from TMD10 [83].

All three approaches provide important information about the structure/function relationship of peptide transporters. However, it should be noted that all of the functional residues and domains described here are not completely correlated with each other. Further studies will be needed to clarify how PEPT1 and PEPT2 recognize and transport various substrates.

4.5. Regulation

It has been reported that intestinal PEPT1 activity is physiologically regulated by various factors including dietary conditions [40, 88-89], hormones (insulin, leptin and thyroid hormone) [90-93], growth factor (epidermal growth factor) [94], development [95-97] and diurnal rhythm [98]. As well as physiological factors, PEPT1 is regulated by pharmacological agents such as σ-receptor ligand (+)-pentazocine [99] and α_2-adrenergic agonists [100]. The

regulation of the apical Na^+/H^+ exchanger has been shown to indirectly modulate PEPT1 function [101-102]. Among these factors, the dietary regulation of intestinal PEPT1 has been extensively investigated. For example, we previously demonstrated that short-term starvation markedly increased the amount of PEPT1 protein, whereas dietary administration of amino acids reduced the amount [89].

Although there is little data available on the regulation of PEPT2, some significant information has been recently accumulated. Using 5/6 nephrectomyzed rats, we found that renal PEPT2 was selectively upregulated with regards to its expression and function in chronic renal failure [103]. As clinical background, ACE inhibitors have been reported to reduce renal injury in patients with kidney disease [104]. Furthermore, it has been suggested that peptide-like drugs at therapeutic concentrations interacted predominantly with PEPT2 in the kidney [105]. Taking the transport of ACE inhibitors *via* PEPT2 [54] into consideration, the upregulation of PEPT2 may contribute to prevention of the urinary loss of ACE inhibitors by enhanced reabsorption, thereby preventing progression of renal failure. Another example is the regulation of mammary PEPT2. In mammary epithelial cells, the expression of PEPT2 mRNA is increased about 30-fold in the lactating state as compared with the nonlactating state [106]. The authors suggested that PEPT2 expression in the lactating mammary epithelium has an important function as a scavenger uptake system for the short-chain peptide products of milk protein hydrolysis.

Most studies have shown that the expression and/or transport activity of peptide transporters are regulated by various factors, but it is unclear whether these factors also affect the pharmacokinetics of peptide-like drugs. Furthermore, it is also not clear how intracellular signaling events occur after various stimulations, and which transcription factors are involved in the constitutive and regulatory expression of peptide transporters. The next step for a molecular understanding of the regulatory aspects of PEPT1 and PEPT2 will be to resolve these issues.

5. INTESTINAL BASOLATERAL PEPTIDE TRANSPORTER

The absorption of peptide-like drugs through the intestinal epithelium requires the crossing of two distinct membranes; i.e., uptake by epithelial cells from the lumen across the brush-border membranes, followed by transfer to the blood across the basolateral membranes. Although orally active β-lactam antibiotics are efficiently absorbed from the intestine, they are difficult to move across the basolateral membranes by passive diffusion because of their physicochemical properties. Based on these findings, we hypothesized that the peptide transporter is also expressed in the basolateral membranes of intestinal epithelial cells. Through the characterization of peptide-like drug transport via the basolateral membranes of Caco-2 cells grown on microporous membrane filters, we have demonstrated that the peptide transporter, which is distinguished from PEPT1, is expressed in the basolateral membranes of intestinal epithelial cells [10-13, 15]. In this chapter, we will introduce the functional characteristics of the intestinal basolateral peptide transporter.

5.1. Transport Mechanisms

We have examined the transport mechanisms of the intestinal basolateral peptide transporter using various substrates, and we present the data regarding studies using Bestatin below [11]. The accumulation of Bestatin from the apical side was greater than that from the basolateral side. The ratio of the intracellular to extracellular concentration of Bestatin at equilibrium was 4.2 for apical uptake, indicating that PEPT1 is an active transporter. In contrast, the ratio of basolateral uptake was almost 1.0, suggesting the basolateral transporter is a facilitative transporter. The transport of Bestatin across the basolateral membranes was shown to be less sensitive to the medium pH. These findings are also observed for other substrates such as cephradine [10], ceftibuten [12] and Gly-Sar [13]. It is, therefore, suggested that the basolateral peptide transporter in Caco-2 cells is an H^+-independent facilitative transporter. However, it is noted that there have been reports that the peptide transporter in the basolateral membranes was H^+-dependent [107-108] and an active transporter [109].

5.2. Substrate Specificity

The basolateral peptide transporter can transport di- and tripeptides and most peptide-like drugs, but not amino acids [13]. This transporter also recognizes nonpeptidic compounds, but the specificity is somewhat different from that of PEPT1 [15]. Namely, small alkyl valine esters, such as valine methyl ester showed inhibitory effects on Gly-Sar uptake by PEPT1, but not by the basolateral peptide transporter. Considering that valine benzyl ester and valacyclovir had inhibitory effects on Gly-Sar uptake both by transporters, the ability to recognize molecular size might differ between PEPT1 and the basolateral peptide transporter. Although such a difference was observed, it can be concluded that the basolateral peptide transporter and PEPT1 have a similar substrate specificity.

5.3. Substrate Affinity

We compared the inhibition constant (*Ki*) values of various substrates including small peptides, peptide-like drugs and nonpeptidic compounds between the basolateral peptide transporter and PEPT1. All of these substrates showed much higher affinity for PEPT1 than for the basolateral peptide transporter [13].

These differences may be relevant for different physiological situations. In other words, the intracellular concentration of substrates taken by PEPT1 may be higher than that in the intestinal lumen, because PEPT1 mediates the active accumulation of its substrates. Consequently, the basolateral peptide transporter is required to work at a higher substrate concentration. It is possible that the lower substrate affinity of the basolateral peptide transporter may allow for normal activity in such an environment. Furthermore, the facilitative transport mechanism of the basolateral peptide transporter is energetically favorable due to this concentration gradient. Similarly, Na^+-glucose cotransporter (SGLT1) and facilitated glucose transporter (GLUT2) were shown to be localized at brush-border and basolateral membranes of small intestinal epithelial cells, respectively, and the apparent *Km* values of

D-glucose have been reported to be 0.8 mM for SGLT1 and 15-20 mM for GLUT2 [110].

5.4. Transport Direction

The physiological roles of transporters located at basolateral membranes of epithelial cells are generally believed to be divided into two categories. One is the efflux of substrates from the cells into circulating blood to accomplish the transepithelial (re)absorption. The other is the transport of substrates into the cells from peritubular capillaries for cellular metabolism and/or regulation. Although it is easily speculated that the intestinal basolateral peptide transporter plays the former role, our experimental data supported this hypothesis [11, 16]. Namely, substrate efflux studies indicated that the intestinal basolateral peptide transporter mediated the expulsion of substrate from the cells. In addition, transcellular transport studies showed that apical-to-basolateral transport, corresponding to the direction of intestinal absorption, was markedly faster than in the opposite direction. Taken together, the intestinal basolateral peptide transporter may mediate the efflux of substrate from the intracellular space, and the cooperation of PEPT1 with the basolateral peptide transporter enables the unidirectional transport through intestinal epithelial cells.

6. RENAL BASOLATERAL PEPTIDE TRANSPORTER

The kidney, as well as the small intestine, plays an important role in the homeostasis of small peptides [3]. Several studies using isolated perfused kidney suggest that peritubular uptake, in addition to luminal transport, contributes to the total renal clearance of dipeptides [111-112]. It was hypothesized that the renal basolateral peptide transporter was involved in the peritubular clearance of dipeptides, but the nature of this transporter has been little understood. Recently, using rat renal cortical slices, we confirmed that Gly-Sar transport through the renal basolateral membranes was mediated by a specific transporter [14]. In addition, using Madin-Darby canine kidney (MDCK) cells, we performed extensive functional characteristic analysis of the renal basolateral peptide transporter [14, 16].

6.1. Transport Mechanisms

The uptake of Gly-Sar by renal cortical slices and *via* the basolateral membranes of MDCK cells was increased in a time-dependent manner, but no accumulation in the steady-state occurred against the concentration gradient [14]. It is, therefore, suggested that the renal basolateral peptide transporter is also a facilitative transporter. Gly-Sar uptake by the renal basolateral peptide transporter was decreased in accordance with the decreases in pH from 7.4 to 5.0 [14]. This pH-profile is distinct from that of the intestinal basolateral peptide transporter, and also that of PEPT1 and PEPT2.

6.2. Substrate Specificity

The renal basolateral peptide transporter can recognize di- and tripeptides, most peptide-like drugs and nonpeptidic compounds such as valacyclovir, but not amino acids and tetrapeptide [14, 16]. Thus, in terms of substrate specificity, it is similar to other peptide transporters.

6.3. Substrate Affinity

Km values of Gly-Sar for the renal basolateral peptide transporters (55 μM in rat renal cortical slices and 71 μM in MDCK cells) were much smaller than the value for the intestinal basolateral peptide transporter of Caco-2 cells (2.1 mM). Comparison of *Ki* values of various substrates between the renal and intestinal basolateral peptide transporter demonstrated that all substrates tested showed higher affinity for the renal basolateral peptide transporter. Thus, it can be concluded that the renal basolateral peptide transporter has much higher affinity than the intestinal basolateral peptide transporter.

6.4. Transport Direction

In MDCK cells, Gly-Sar accumulation was greater on the basolateral side than the apical side, and there was little net transcellular transport [16]. In addition, the efflux of Gly-Sar from MDCK cells was negligible to both sides. These findings are in contrast to those in Caco-2 cells, and suggest that the renal basolateral peptide transporter does not mediate the efflux of substrate from the cells. Alternatively, this transporter may contribute to the cellular uptake of substrates from the extracellular space.

MDCK cells retain the basic characteristics of cells from distal tubules or collecting ducts [113]. In these segments, the luminal small peptide concentrations are quite low; therefore, reabsorption of small peptides may not be significant. So, physiologically, the basolateral peptide transporter expressed in these segments may mediate the cellular uptake of small peptides from the blood, and contribute to various cellular protein metabolisms. The high affinity of this transporter appears to mediate the efficient uptake of plasma small peptides despite low concentrations. The pharmacokinetic and pharmacological relevance of this transporter is not clear at present. Table **1** describes the functional features of intestinal and renal basolateral peptide transporters.

7. APPLICATION TO DRUG DELIVERY

The intestinal peptide transporters, especially PEPT1, have been a key target molecule for prodrug approaches [114]. According to this approach, prodrugs, which are appropriately designed in the form of di- or tripeptide analogs, can be absorbed across the intestinal brush-border membranes via PEPT1, and may be absorbed intact or hydrolyzed intracellularly by peptidases or esterases prior to exit from the cell.

L-α-Methyldopa is a poorly absorbed antihypertensive agent and amino acid analog. Its absorption is mediated by amino acid transporters. Amino acid transporters are structurally restrictive [1], and this is thought to be the main reason for the poor intestinal absorption of L-α-methyldopa. However, when L-α-methyldopa was converted to dipeptidyl derivatives, these prodrugs showed a several-fold increase in permeability because dipeptidyl derivatives serve as substrates for the intestinal peptide transporter [115-117]. Hydrolysis of the dipeptidyl prodrugs was observed in intestinal cell homogenates *in vitro*, suggesting liberation of the parent compound after intestinal uptake. To minimize the

Table 1. Functional Features of Intestinal and Renal Basolateral Peptide Transporters

	Intestinal Basolateral Peptide Transporter	Renal Basolateral Peptide Transporter
Transport mode	Facilitative	Facilitative
Substrates	Dipeptides, Tripeptides Peptide-like drugs β-Lactam antibiotics Bestatin δ-Amino levulinic acid etc.	Dipeptides, Tripeptides Peptide-like drugs β-Lactam antibiotics Bestatin δ-Amino levulinic acid etc.
Substrate affinity (Km value for Gly-Sar: mM)	Low (2.1)	High (0.07)
pH-sensitivity	Insensitive	Sensitive
Transport direction	Cell to extracellular space	Extracellular space to cell

extensive metabolism of L-dopa in the gut wall, a tripeptide prodrug of L-dopa, p-Glu-L-dopa-Pro, was designed to be absorbed via the peptide transporter and converted to L-dopa by peptidases [118].

This strategy has been extended to nonpeptidyl prodrugs, i.e., amino acid ester prodrugs, for polar nucleosides. Acyclovir (ACV), an antiviral agent, is poorly absorbed from the intestine, but its valyl ester prodrug valacyclovir (Val-ACV) dramatically improves intestinal absorption of ACV [119]. Han *et al.* [62, 120] clearly demonstrated that this improved absorption was caused by PEPT1-mediated Val-ACV transport, and that Val-ACV taken up by the cells was rapidly converted to ACV by intracellular hydrolysis. We also confirmed Val-ACV uptake by PEPT1 using our experimental system (Fig. 3). It was also demonstrated that Val-ACV was about 3-fold more permeable across the rabbit corneal epithelium than ACV, suggesting that this effect was caused by the peptide transporter [121]. These results provide a new rational design for targeting peptide transporters with great flexibility in structural modification.

Beauchamp *et al.* [122] evaluated the bioavailability of 18 ester compounds, including amino acid ester compounds, of ACV during the course of developing prodrugs for ACV. They found that the L-valyl ester provided the best ACV bioavailability, followed by the L-isoleucyl, L-alanyl, glycyl and L-leucyl esters. We found similar inhibitory effects of L-amino acid methyl esters on Gly-Sar uptake by PEPT1 [123] (Fig. 3). Therefore, the findings of Beauchamp *et al.* [122] may reflect the affinities of these compounds to intestinal PEPT1. Taken together, the degree of interaction of L-amino acid ester compounds with PEPT1 is dependent on L-amino acids, and L-valine is suggested to be a preferable L-amino acid for this purpose. Similar strategies have been applied to other antiviral agents such as zidovudine [62] and gancyclovir [124]. Thus, L-valyl esterification of poorly absorbed drugs will be a promising strategy to improve their intestinal absorption.

In addition to PEPT1, the intestinal basolateral transporter has been demonstrated to have the ability to transport nonpeptidic compounds such as δ-amino levulinic acid (δ-ALA) [15]. δ-ALA is a precursor of porphyrins and heme, and plays an important role in the production of heme-containing proteins. Recently, there has been growing interest in the transport and metabolism of δ-ALA, because this compound has been successfully used in treating various tumors by photodynamic therapy [125-127]. When δ-ALA was administered orally, it showed relatively high oral bioavailability (approximately 60% in a human study) and there was a rapid increase in the circulating plasma level [128]. We reported that the transport of δ-ALA by PEPT1 and the basolateral peptide transporter can explain the good bioavailability of δ-ALA [15]. Although this example does not show the prodrug approaches, this finding suggests that the intestinal basolateral peptide transporter can be utilized as a new drug delivery target.

Another application of peptide transporters to drug delivery is the tissue or cellular specific targeting of peptide-like drugs using the selective expression of peptide transporters. For example, as PEPT2 is expressed in alveolar type II pneumocytes and bronchial epithelium, pulmonary delivery of peptides and peptide mimetics has been proposed [49]. The low proteolytic activity and bypassed hepatic metabolism are benefits for pulmonary delivery. Nakanishi *et al.* [129-130] reported that the dipeptide transport system, which is similar but not identical to peptide transporters PEPT1 and PEPT2, exists in fibroblast-derived tumor cells but not in normal cells, and demonstrated the potential tumor-selective delivery of dipeptides or peptide-mimetic drugs. Although it is necessary to confirm the selective expression of this transporter in various tumor cells but not in normal cells, this could be a novel strategy for the specific delivery of peptide-like anticancer drugs into tumor cells. Recently, from the same laboratory group, peptide-like drug delivery into the liver has been demonstrated using adenovirus-mediated hepatic expression of PEPT1 [131].

8. SUMMARY AND PERSPECTIVE

Peptide transporters PEPT1 and PEPT2 have been demonstrated to play important physiological and nutritional roles, and also to have pharmacokinetic and pharmacological significance. Further molecular clarification of the drug recognition mechanisms of PEPT1 and PEPT2 will provide

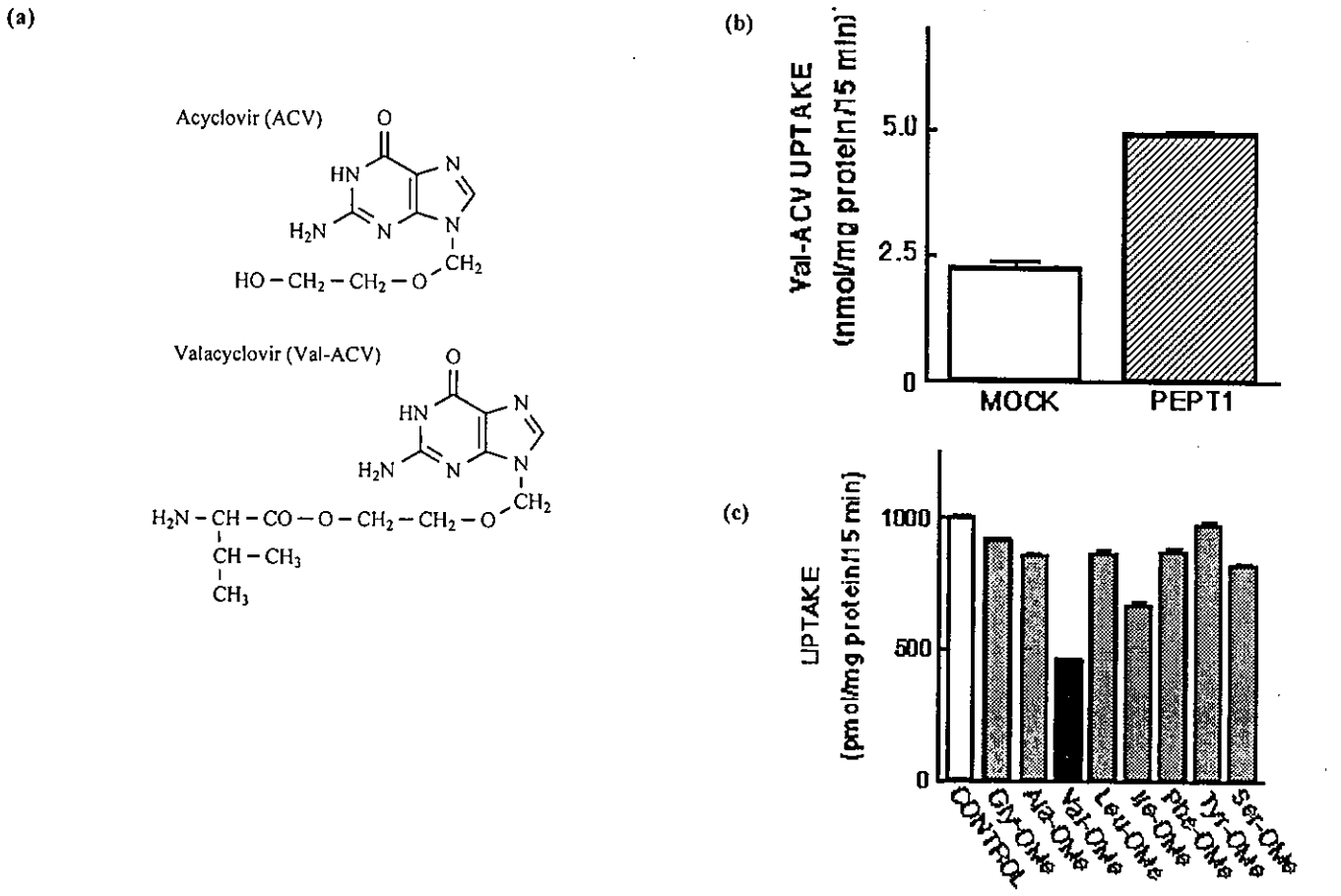

Fig. (3). (a) Chemical structures of acyclovir (ACV) and valacyclovir (Val-ACV). (b) Val-ACV uptake by PEPT1-expressing cells. The amount of Val-ACV taken up by the cells was determined by HPLC, and more than 90% of Val-ACV in the cells was converted to ACV. (c) Effect of L-amino acid methyl esters on [^{14}C]Gly-Sar uptake by PEPT1-expressing cells. L-Valine methyl ester showed the most potent inhibitory effect.

useful information for drug design and delivery systems to improve the efficiency of drug therapy. From the pharmaceutical perspective, species differences in the substrate specificity, tissue distribution, and level of expression of transporters should be explored to aid in the prediction of *in vivo* kinetic profiles of drugs from *in vitro* data. In addition, considering the overall handling of peptide-like drugs, efforts must be directed toward the identification and characterization of basolateral peptide transporters [132]. Lastly, the evaluation of genetic polymorphism in PEPT1 and PEPT2 could have clinical and pharmacological importance, and in the near future, the information obtained could be used for establishing appropriate medications for individual patients.

ABBREVIATIONS

PEPT	=	H^+/peptide cotransporter
FMLP	=	*N*-formylmethionyl-leucyl-phenylalanine
Gly-Sar	=	Glycylsarcosine
TMD	=	Transmembrane domain
SGLT	=	Na^+/glucose cotransporter
GLUT	=	Facilitated glucose transporter
MDCK cells	=	Madin-Darby canine kidney cells
ACV	=	Acyclovir
Val-ACV	=	Valacyclovir
δ-ALA	=	δ-amino levulinic acid

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Creatinine Transport by Basolateral Organic Cation Transporter hOCT2 in the Human Kidney

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Purpose. Creatinine is excreted into urine by tubular secretion in addition to glomerular filtration. The purpose of this study was to clarify molecular mechanisms underlying the tubular secretion of creatinine in the human kidney.

Methods. Transport of [^{14}C]creatinine by human organic ion transporters (SLC22A) was assessed by HEK293 cells expressing hOCT1, hOCT2, hOCT2-A, hOAT1, and hOAT3.

Results. Among the organic ion transporters examined, only hOCT2 stimulated creatinine uptake when expressed in HEK293 cells. Creatinine uptake by hOCT2 was dependent on the membrane potential. The Michaelis constant (K_m) for creatinine transport by hOCT2 was 4.0 mM, suggesting low affinity. Various cationic drugs including cimetidine and trimethoprim, but not anionic drugs, markedly inhibited creatinine uptake by hOCT2.

Conclusion. These results suggest that hOCT2, but not hOCT1, is responsible for the basolateral membrane transport of creatinine in the human kidney.

KEY WORDS: creatinine; glomerular filtration rate; hOCT2; organic cation transporter; tubular secretion.

INTRODUCTION

In the proximal tubules of mammalian kidney, organic ion transporters limit or prevent the toxicity of organic anions and cations by actively secreting these substances from the circulation into the urine (1–5). We isolated a second member of the organic cation transporter (OCT) family, rat (r) OCT2 (6), showing 67% amino acid identity to rOCT1 (7). Functional studies using *Xenopus* oocytes (6–10) and transfected mammalian cells (11–13) as expression systems suggested that rOCT1 and rOCT2 transport various structurally unrelated cations in a voltage-dependent fashion. rOCT1 and rOCT2 possess similar but not identical specificities for various cationic compounds. Both rOCT1 and rOCT2 protein were localized in the basolateral membrane of renal tubular cells (14,15), although the distributions of these transporters along the nephron were distinct (13).

To date, three distinct genes encoding human organic cation transporters have been identified including hOCT1, hOCT2, and hOCT3 (5). In addition, we identified hOCT2-A, an alternatively spliced variant of hOCT2, expressed in the human kidney, with different transport characteristics from that of hOCT2 (16). We also demonstrated that the mRNA level of hOCT2 was the highest in the human kidney among organic cation transporters examined, suggesting hOCT2 to be the dominant organic cation transporter in the human kidney (17). In contrast, hOCT1 is mainly transcribed in the liver, suggesting that hOCT1 is responsible for the hepatic uptake of organic cations (18–19). Although characterization of organic cation transport by hOCT2 have been done, intrinsic roles of hOCT2 in the disposition of physiological substances have not been clarified.

It is established that creatinine, a catabolic product of creatine, is eliminated predominantly into urine. Creatinine can also be secreted via the renal tubules in addition to the glomerular filtration, however, the molecules mediating tubular secretion of creatinine in the human kidney have not been identified. Because organic ion transporters recognize a wide variety of ionic compounds, thereby mediate tubular secretion of organic ions, we measured creatinine transport by organic ion transporters (SLC22A), hOCT1, hOCT2, hOCT2-A, hOAT1, and hOAT3, to assess the involvement of these transporters in the tubular secretion of creatinine.

MATERIALS AND METHODS

Cell Culture

HEK293 cells (ATCC CRL-1573), a transformed cell line derived from human embryonic kidney, were cultured in complete medium consisting of Dulbecco's modified Eagle's medium with 10% fetal bovine serum in an atmosphere of 5% CO_2/95% air at 37°C. For uptake experiments, the cells were seeded onto poly-D-lysine-coated 24-well plates at a density of 2.0×10^5 cells per well. The cell monolayers were used at day 3 of culture for uptake experiments. In this study, HEK293 cells between the 68th and 89th passages were used.

Transfection

pCMV6-XL4 plasmid vector (OriGene Technologies, Rockville, MD, USA) DNA containing hOCT1, hOCT2, hOCT2-A, hOAT1, and hOAT3 cDNA, and pBK-CMV vector (Stratagene, La Jolla, CA, USA) were purified using Marligen High Purity Plasmid-Prep Systems (Invitrogen, Carlsbad, CA, USA). The day before the transfection, HEK293 cells were seeded onto poly-D-lysine-coated 24-well plates at a density of 2.0×10^5 cells per well. The cells were transfected with 0.8 μg of total plasmid DNA per well using Lipofect-AMINE 2000 (Invitrogen) according to the methods described previously (16). At 48 h after transfection, the cells were used for uptake experiments. To construct a transfectant stably expressing hOCT2, HEK293 cells were transfected with 0.8 μg of total plasmid DNA (pCMV6-XL4: pBK-CMV vector = 2:1) per well. At 24 h after transfection, the cells split between 1:15 and 1:30 were cultured in complete medium containing G418 (0.5 mg/ml) (Wako Pure Chemical, Osaka, Japan). Then 14 to 21 days after transfection, single colonies were picked out. G418-resistant colonies were analyzed by RT-PCR for the expression of hOCT2 mRNA.

Uptake Experiments Using HEK293 Transfectants

Cellular uptake of cationic and anionic compounds using HEK293 cells was measured with monolayer cultures grown

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ABBREVIATIONS: hOCT, human organic cation transporter; GFR, glomerular filtration rate; MPP, 1-methyl-4-phenylpyridinium; NMN, N^1-methylnicotinamide; PAH, *p*-aminohippuric acid; TEA, tetraethylammonium.

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on poly-D-lysine-coated 24-well plates (16). The cells were preincubated with 0.2 ml of incubation medium for 10 min at 37°C. The medium was then removed, and 0.2 ml of incubation medium containing [^{14}C]creatinine, [^{14}C]TEA, [^{14}C]PAH, or [^{3}H]estrone sulfate was added. The composition of the incubation medium was as follows (in mM): 145 NaCl, 3 KCl, 1 $CaCl_2$, 0.5 $MgCl_2$, 5 D-glucose, and 5 HEPES (pH 7.4). The composition of high K^+ incubation medium was as follows (in mM): 3 NaCl, 145 KCl, 1 $CaCl_2$, 0.5 $MgCl_2$, 5 D-glucose, and 5 HEPES (pH 7.4). When indicated, 9.2 mM $BaCl_2$ was added to the incubation medium. The medium was aspirated off at the end of the incubation, and the monolayers were rapidly rinsed twice with 1 ml of ice-cold incubation medium. The cells were solubilized in 0.5 ml of 0.5 N NaOH, and then the radioactivity in aliquots was determined by liquid scintillation counting. The protein content of the solubilized cells was determined by the method of Bradford (20), using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) with bovine γ-globulin as a standard. For the cis-inhibition study, the uptake of [^{14}C]creatinine was achieved by adding various concentrations of unlabeled inhibitors to the incubation medium. Concentration dependence of creatinine transport by hOCT2 was analyzed using Michaelis-Menten equation; $V = V_{max}[S]/(K_m + [S]) + K_d[S]$, where V is transport rate, V_{max} is the maximal transport rate, [S] is the concentration of creatinine, K_m is Michaelis constant, and K_d is a diffusion constant. The apparent IC_{50} values were calculated from inhibition plots based on the equation, $V = V_0/[1 + (I / IC_{50})^n]$ by a nonlinear least-squares regression analysis with Kaleidagraph Version 3.5 (Synergy Software, Reading, PA, USA) (13). V and V_0 are the uptake of [^{14}C]creatinine in the presence and absence of inhibitor, respectively. I is the concentration of inhibitor, and n is the Hill coefficient.

Materials

[2-^{14}C]Creatinine hydrochloride (55 mCi/mmol) and [ethyl-1-^{14}C] tetraethylammonium (TEA) bromide (55 mCi/mmol) were purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). *p*-[Glycyl-^{14}C]aminohippuric acid (PAH) (50.4 mCi/mmol) and [6,7-^{3}H(*N*)]estrone sulfate ammonium salt (43.5 Ci/mmol) were obtained from Perkin Elmer Life Science Products (Boston, MA, USA). Creatinine, tetraethylammonium bromide, dopamine hydrochloride, guanidine hydrochloride, cimetidine, and (±)-chlorpheniramine maleate were obtained from Nacalai Tesque (Kyoto, Japan). N^1-Methylnicotinamide (NMN) iodide and 1-methyl-4-phenylpyridinium (MPP) iodide were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other compounds used were of the highest purity available.

Statistical Analyses

Data were analyzed statistically by one-way analysis of variance followed by Dunnett's test or non-paired Student's *t* test. p values of less than 0.05 were considered to be significant.

RESULTS

[^{14}C]Creatinine Uptake by HEK293 Cells Expressing Human Organic Ion Transporters

First, we evaluated the uptake of [^{14}C]creatinine by HEK293 cells transfected with hOCT1, hOCT2, hOCT2-A, hOAT1, and hOAT3 cDNA. As shown in Fig. 1a, the uptake of [^{14}C]creatinine was markedly stimulated in hOCT2-transfected HEK293 cells. In contrast, the uptake of [^{14}C]creatinine by hOCT1-, hOCT2-A-, hOAT1-, and hOAT3-transfected cells was comparable to that by null vector-transfected cells. In these experiments, the functional expression of hOCTs, hOAT1, and hOAT3 in the corresponding batches of the transfected cells was verified by the transport activity of [^{14}C]TEA, [^{14}C]PAH, and [^{3}H]estrone sulfate, respectively (Figs. 1b, 1c and 1d).

Concentration Dependence of [^{14}C]Creatinine Uptake by hOCT2

To examine characteristics of creatinine transport by hOCT2, we constructed HEK293 cells stably expressing hOCT2. Figure 2 shows the concentration-dependence of [^{14}C]creatinine uptake in HEK293 cells stably expressing hOCT2. The uptake of creatinine by these cells was saturated at high concentrations (Fig. 2). The uptake by hOCT2-transfected cells increased time-dependently, and its uptake was linear for up to 2 min (data not shown). The apparent K_m value of the creatinine uptake by hOCT2-transfected cells estimated from three separate experiments using three monolayers was 4.0 ± 0.3 mM. The V_{max} value of the creatinine uptake by hOCT2-transfected cells was 23.5 ± 5.2 nmol·mg protein^{-1}·min^{-1}. Eadie-Hofstee plots were linear (inset of Fig. 2), suggesting absence of endogenous transport system for creatinine in HEK293 cells.

Effect of Membrane Potential on [^{14}C]Creatinine Uptake by hOCT2

Next, we examined the effect of membrane potential on [^{14}C]creatinine uptake by hOCT2-expressing HEK293 cells (Fig. 3). With this approach, increasing the concentration of K^+ in the uptake buffer depolarized the cell membrane potential. The uptake of creatinine decreased in the presence of high K^+ buffer. Furthermore, the accumulation of creatinine decreased in the presence of Ba^{2+}, a nonselective K^+ channel blocker. These results suggest that the transport of creatinine by hOCT2 is dependent on the membrane potential, consistent with the characteristics of hOCT2 (16).

Effect of Organic Cations and Anions on [^{14}C]Creatinine Uptake by hOCT2

To determine the substrate affinity of hOCT2 for cationic compounds, we examined the inhibitory effects of various cationic and anionic compounds on the uptake of creatinine by the hOCT2 transfectants and calculated the apparent IC_{50} values using the equation described in "Materials and Methods" (Fig. 4 and Table I). Cationic drugs (Fig. 4a), neurotoxin and endogenous cations (Fig. 4b) inhibited the uptake of creatinine by the hOCT2 transfectants in a dose-dependent manner. MPP had the most potent inhibitory effect on the uptake of creatinine by hOCT2 among the compounds tested (Table I). Furthermore, hOCT2 showed higher affinities for cationic drugs, H_1- and H_2-receptor antagonists, and endogenous cations, in comparison with the affinity for creatinine. Salicylic acid and PAH had weak inhibitory effects on the uptake of creatinine by hOCT2 at high concentrations (Fig. 4d).

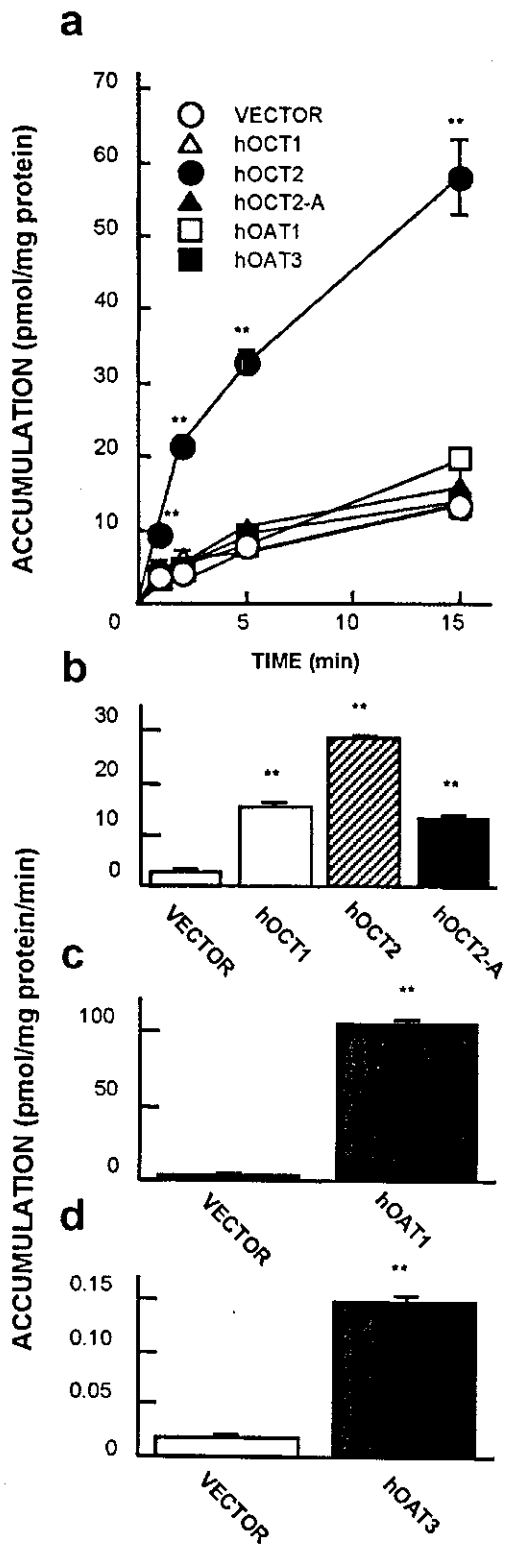

Fig. 1. Transport activity for [^{14}C]creatinine by HEK293 cells transiently expressing human organic ion transporters. (a) HEK293 cells transfected with hOCT1 (△), hOCT2 (●), hOCT2-A (▲), hOAT1 (□), hOAT3 (■), or pCMV6-XL4 vector (○) were incubated for the specified periods at 37°C with 5 μM [^{14}C]creatinine. Each point represents the mean ± SE for three monolayers. (b) HEK293 cells transfected with hOCT1 (shaded column), hOCT2 (hatched column), hOCT2-A (closed column), or null vector (open column) were incubated at 37°C for 1 min with 5 μM [^{14}C]TEA. (c) HEK293 cells transfected with hOAT1 (shaded column), or null vector (open column) were incubated at 37°C for 1 min with 10 μM [^{14}C]PAH. (d) HEK293 cells transfected with hOAT3 (shaded column), or null vector (open column) were incubated at 37°C for 1 min with 19 μM [^{3}H]estrone sulfate. Each column represents the mean ± SE for three monolayers. **$p < 0.01$ vs. null vector-transfected HEK293 cells by Dunnett's test (Figs. 1a and 1b) and Student's *t* test (Figs. 1c and Figs. 1d).

DISCUSSION

Creatinine clearance, calculated from serum and urine creatinine concentrations, is often used for the estimation of glomerular filtration rate (GFR). However, creatinine clearance usually exceeds GFR because of the tubular secretion of creatinine (21,22). In addition, overestimation of GFR by means of creatinine clearance has been marked in patients with renal disease, especially in those with glomerular disorders (23-27).

The mechanisms underlying the tubular secretion of creatinine have been controversial; Berglund *et al.* (28), Burgess *et al.* (29), and van Acker *et al.* (30) suggested base-secreting pathways for creatinine secretion based on the findings that concomitant cimetidine or trimethoprim blocked the tubular secretion of creatinine. However, Crawford (31) and Burry and Dieppe (32) demonstrated inhibition of creatinine clearance by exogenous organic anions. Because cimetidine is a good substrate for hOCT2 (13,16,33), and hOCT2 is a predominant organic cation transporter in the human kidney lo-

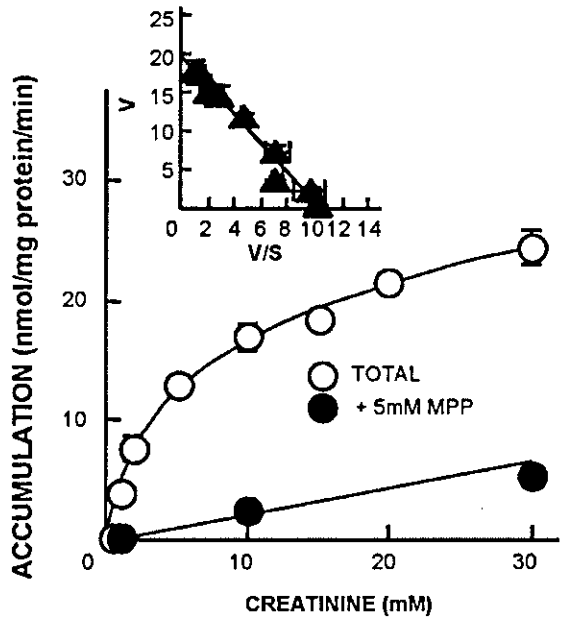

Fig. 2. Concentration-dependence of [^{14}C]creatinine uptake by HEK293 cells stably expressing hOCT2. hOCT2 transfectants were incubated at 37°C for 2 min with 5 μM [^{14}C]creatinine in the absence (○) or presence (●) of 5 mM MPP (pH 7.4). Each point represents the mean ± SE for three monolayers from a typical experiment. Inset: Eadie-Hofstee plots of creatinine uptake after a correction for non-saturable components. V, uptake rate (nmol·mg protein^{-1}·min^{-1}); S, creatinine concentration (mM). Unlabeled creatinine was added to [^{14}C]creatinine to give the final concentrations indicated.

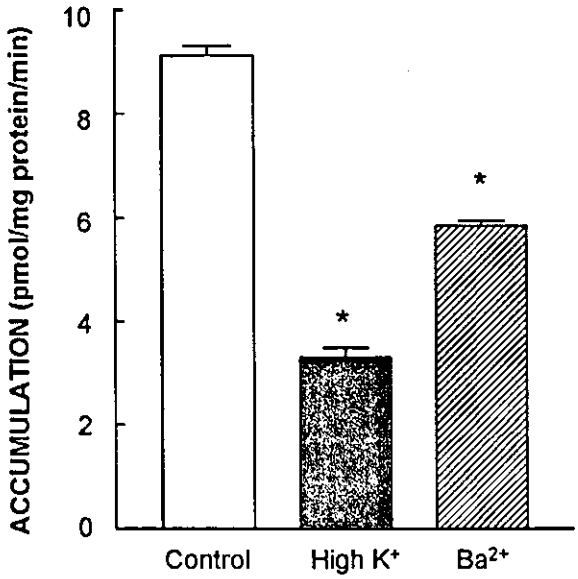

Fig. 3. Effect of membrane potential on [^{14}C]creatinine uptake by HEK293 cells stably expressing hOCT2. HEK293 cells transfected with hOCT2 were incubated with respective buffers at 37°C with 4.5 μM [^{14}C]creatinine. Each column represents the mean ± SE of three monolayers from a typical experiment. **p < 0.01 vs. control by Dunnett's test.

calized at the basolateral membranes of the proximal tubules (17), we supposed hOCT2 to be a responsible transporter mediating tubular secretion of creatinine. In the current study, hOCT2 was the only transporter mediating creatinine transport among several organic ion transporters examined (Fig. 1), suggesting hOCT2 to be the responsible transporter regulating creatinine uptake at the basolateral membranes of renal proximal tubules. We also found much higher Michaelis constant of creatinine for hOCT2 (K_m: 4.0 ± 0.3 mM) than physiological (about 45–85 μM for male and 30–60 μM for female) and even pathophysiological concentrations of creatinine in human serum, suggesting that hOCT2 could function as creatinine transporter without saturation. We speculate that this low affinity transport of creatinine by hOCT2 would be beneficial for the efficient extrusion of creatinine from circulation even in the patients with decreased glomerular filtration.

In general, organic ion transporters are multispecific (polyspecific) and thereby share common substrates. In the current study, however, we found that creatinine is specifically transported by hOCT2, but not by any other organic cation and anion transporters examined. To our knowledge, this is the first demonstration that creatinine, an endogenous organic cation, is a specific substrate for hOCT2. Because hOCT1 is dominantly expressed in the liver, but not in the kidney (18,19), it is reasonable that renal hOCT2 would regulate the kidney-specific secretion of creatinine.

Several reports have emerged to date that cimetidine inhibits the tubular secretion of creatinine in humans without altering GFR (29,30). Unlike cimetidine, ranitidine, another H_2-receptor antagonist, does not inhibit the tubular secretion of creatinine (34). The therapeutic range of cimetidine is about 6- to 10-fold higher than that of ranitidine, and 20- to 50-fold higher than that of famotidine (35). In the current study, the order of the affinity of H_2-receptor antagonists for

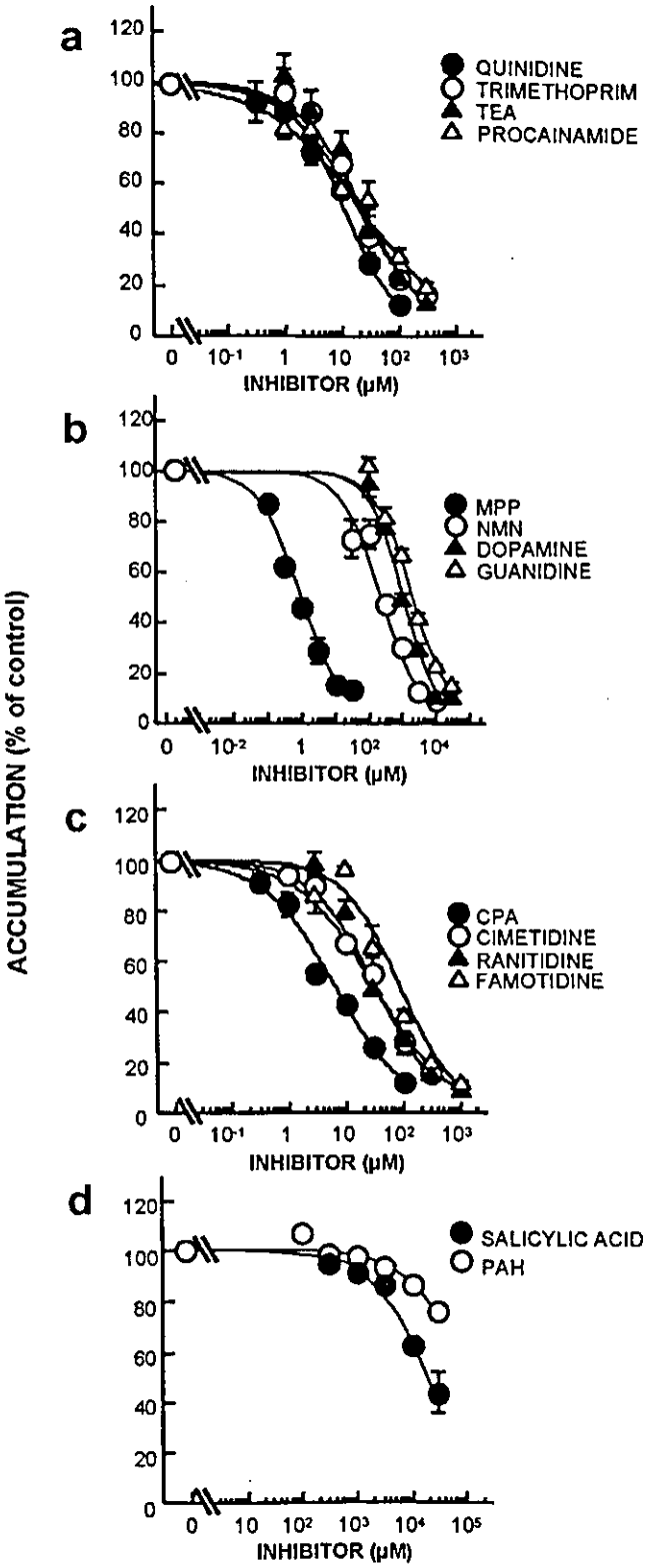

Fig. 4. Effects of cationic and anionic compounds on [^{14}C]creatinine uptake by the hOCT2-transfectants. HEK293 cells transfected with hOCT2 were incubated at 37°C for 2 min with 5 μM [^{14}C]creatinine (pH 7.4) in the presence of (a) quinidine (●), trimethoprim (○), TEA (▲), or procainamide (△); (b) MPP (●), NMN (○), dopamine (▲), or guanidine (△); (c) chlorpheniramine (CPA) (●), cimetidine (○), ranitidine (▲), or famotidine (△); (d) salicylic acid (●) or PAH (○). Each point represents the mean ± SE for three monolayers from a typical experiment.

Table I. The Apparent IC_{50} Values of Various Cationic and Anionic Compounds for [^{14}C]Creatinine Uptake by hOCT2

Compounds	Apparent IC_{50} values for [^{14}C]creatinine uptake (μM)
MPP	1.1 ± 0.2
Chlorpheniramine	6.0 ± 0.3
Quinidine	10 ± 1
Trimethoprim	21 ± 2
TEA	24 ± 6
Cimetidine	27 ± 6
Procainamide	28 ± 10
Ranitidine	38 ± 5
Famotidine	70 ± 8
NMN	310 ± 70
Dopamine	1400 ± 100
Guanidine	2200 ± 100
Salicylic acid	14000 ± 3000

See experimental conditions in the legend of Fig. 4. The apparent IC_{50} values were calculated from inhibition plots (Fig. 4) by nonlinear regression analysis as described in "Materials and Methods." The data represent the mean ± SE for three independent experiments. MPP, 1-methyl-4-phenylpyridinium; TEA, tetraethylammonium; NMN, N'-methylnicotinamide.

the uptake of creatinine by hOCT2 was cimetidine ~ ranitidine > famotidine (Fig. 4C and Table I). These findings indicate that at therapeutic concentrations, cimetidine would moderately inhibit creatinine uptake via hOCT2, whereas ranitidine and famotidine would exert almost no influence. We speculate that the stronger inhibitory effect of cimetidine on the tubular secretion of creatinine is likely to be associated with the high affinity binding of cimetidine to hOCT2 as well as the higher therapeutic range of cimetidine compared with other H_2-receptor antagonists.

In conclusion, hOCT2 mediates basolateral membrane transport of creatinine in the human kidney. Unlike hOCT1, hOCT2 should be responsible for the kidney specific disposition of creatinine.

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Gene expression variance based on random sequencing in rat remnant kidney

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Gene expression variance based on random sequencing in rat remnant kidney.

Background. Several examinations have been performed to identify the genes involved in chronic renal failure using 5/6 nephrectomized rats. Recently, many systematic techniques for examining molecular expression have been developed. They might also be effective in elucidating the molecular mechanism of progressive renal failure. In this study, digital expression profiling was carried out to construct a subtractive mRNA expression database for the 5/6 nephrectomized kidney.

Methods. One thousand clones were randomly sequenced from 5/6 nephrectomized and sham-operated rat kidney cDNA libraries, respectively, and defined by BLAST search. In silico subtractive analysis was performed to search for genes up- or down-regulated in the 5/6 nephrectomized kidney.

Results. The growth factor-related mRNAs and the mRNAs encoding cytoskeletal or membrane proteins were up-regulated, but the transporter-related mRNAs were down-regulated in the 5/6 nephrectomized kidney database. In silico subtraction revealed that 63 mRNAs were increased and 59 were decreased in the 5/6 nephrectomized kidney. To confirm whether the in silico subtractive database reflected the actual expression of mRNA or protein, 12 known genes were examined by Northern blotting or immunoblotting, respectively. The actual expression of the 12 genes was comparable with the results of in silico subtraction. In addition, we successfully isolated five unknown genes, two up-regulated and three down-regulated in the 5/6 nephrectomized kidney.

Conclusion. We constructed a subtractive mRNA expression database for 5/6 nephrectomized kidney, which reflects the actual alterations in mRNA expression after subtotal nephrectomy. This database may be useful for elucidation of the molecular mechanism of progressive renal failure.

Five-sixth nephrectomized rats are widely used as a model of progressive renal failure [1, 2]. There are several findings in which specific genes have been up-regulated or down-regulated in the 5/6 nephrectomized kidney. One to 4 weeks after 5/6 nephrectomy, the genes related to hypertrophy, development of glomerular sclerosis, or vascular tone, such as transforming growth factor β (TGF-β), insulin-like growth factor (IGF) and so on, are up-regulated in the remnant kidney [3–8]. On the other hand, organic ion transporters OAT-K and OCT-2 in proximal tubules were down-regulated in the 5/6 nephrectomized kidneys [9, 10]. However, the kidney is a heterogeneous tissue with many types of cell and the progression of chronic renal failure (CRF) involves many factors. Therefore, analysis of overall genes should be done to understand further the molecular mechanisms of progressive CRF.

To date, many systematic techniques have been developed to analyze molecular expression. Polymerase chain reaction (PCR)-coupled representational difference analysis (RDA-PCR) [11], differential display [12], cDNA microarray [13, 14], and so on are examples. Digital expression profiling is one of the methods of large-scale gene expression analysis in which the mRNA population in a given tissue is assessed quantitatively by sequencing randomly selected clones from a 3′-directed cDNA library [15, 16]. Recently, Takenaka et al [17, 18] constructed cDNA databases for mouse proximal tubules and collecting ducts, and successfully obtained information on the expression profile of normal kidney.

The isolation of genes up-regulated in 5/6 nephrectomized mouse kidneys using RDA-PCR was previously reported by Zhang et al [19]. Ten known genes and nine novel genes were isolated in that study. Although this method is advantageous for screening apparently up-regulated genes, it is difficult to isolate all up-regulated genes unless thousands of clones are screened [19]. In addition, the relative expression level could not be determined by this method. Taken together, RDA-PCR would certainly be effective for searching for up-regulated genes; however, it would be insufficient for screening for massive physiologic variation in progressive

Key words: expression profiling, in silico subtraction, database, chronic renal failure, 5/6 nephrectomized rats.

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