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

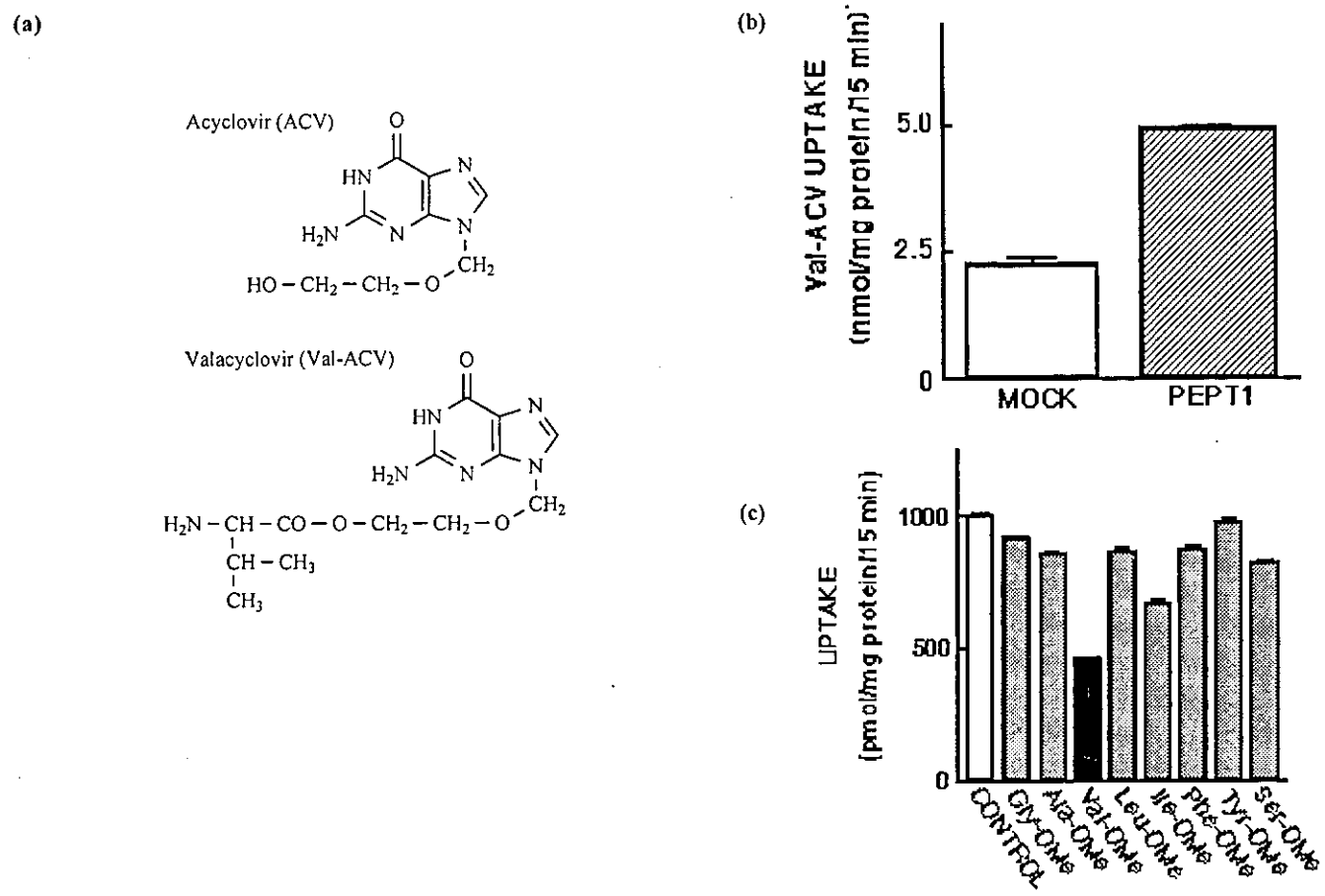

Fig. (3). (a) Chemical structures of acyclovir (ACV) and valacyclovir (Val-ACV). (b) Val-ACV uptake by PEPT1-expressing cells. The amount of Val-ACV taken up by the cells was determined by HPLC, and more than 90% of Val-ACV in the cells was converted to ACV. (c) Effect of L-amino acid methyl esters on [^{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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Received January 17, 2004; accepted March 15, 2004

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 LipofectAMINE 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).

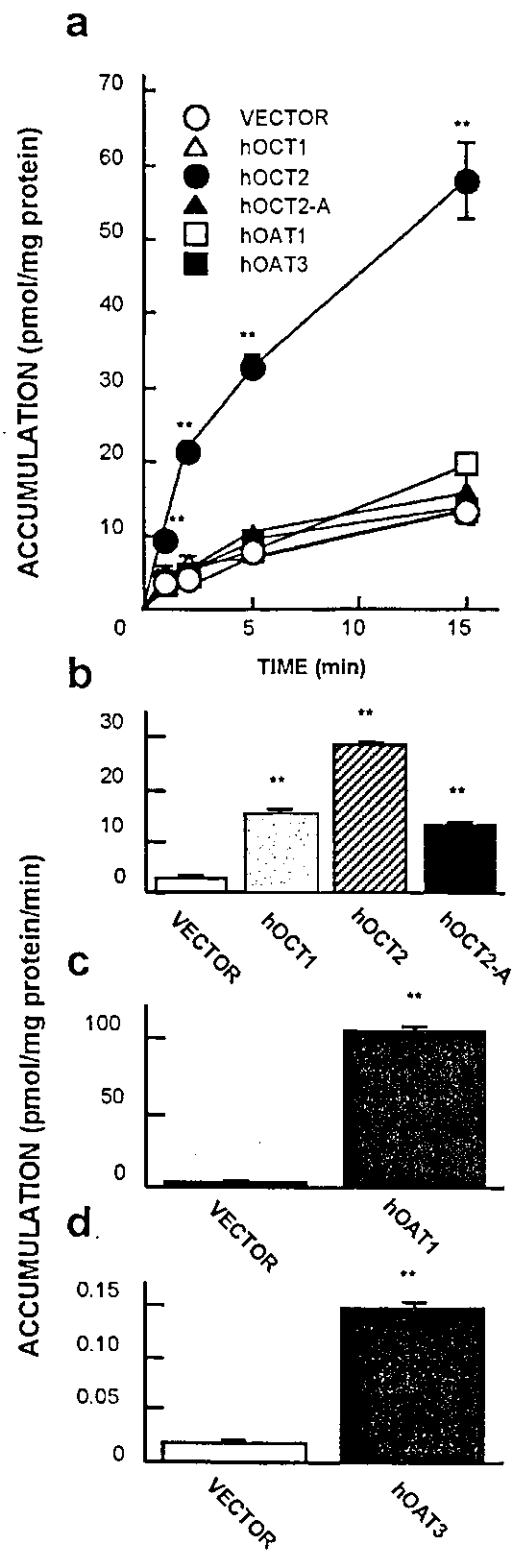

Fig. 1. Transport activity for [^{14}C]creatinine by HEK293 cells transiently expressing human organic ion transporters. (a) HEK293 cells transfected with hOCT1 (△), hOCT2 (●), hOCT2-A (▲), hOAT1 (□), hOAT3 (■), or pCMV6-XL4 vector (○) were incubated for the specified periods at 37°C with 5 μM [^{14}C]creatinine. 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).

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-

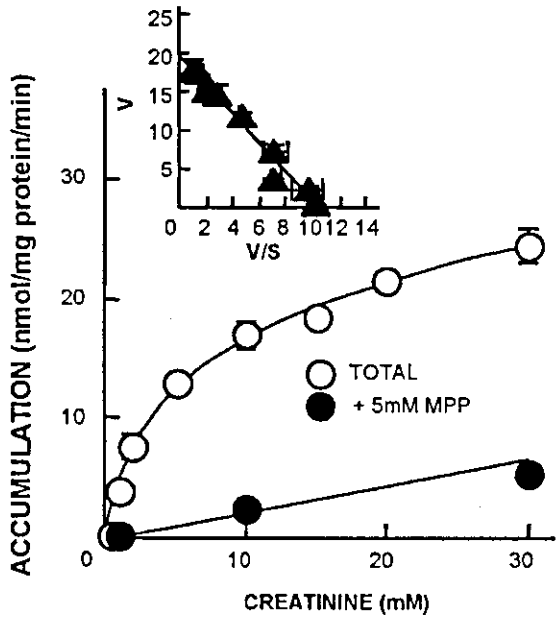

Fig. 2. Concentration-dependence of [^{14}C]creatinine uptake by HEK293 cells stably expressing hOCT2. hOCT2 transfectants were incubated at 37°C for 2 min with 5 μM [^{14}C]creatinine in the absence (○) or presence (●) of 5 mM MPP (pH 7.4). Each point represents the mean ± SE for three monolayers from a typical experiment. Inset: Eadie-Hofstee plots of creatinine uptake after a correction for nonsaturable 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.

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

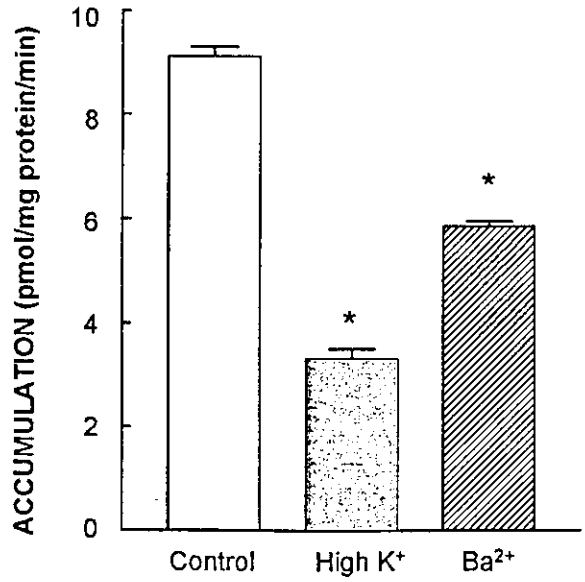

Fig. 3. Effect of membrane potential on [^{14}C]creatinine uptake by HEK293 cells stably expressing hOCT2. HEK293 cells transfected with hOCT2 were incubated with respective buffers at 37°C with 4.5 μM [^{14}C]creatinine. Each column represents the mean ± SE of three monolayers from a typical experiment.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

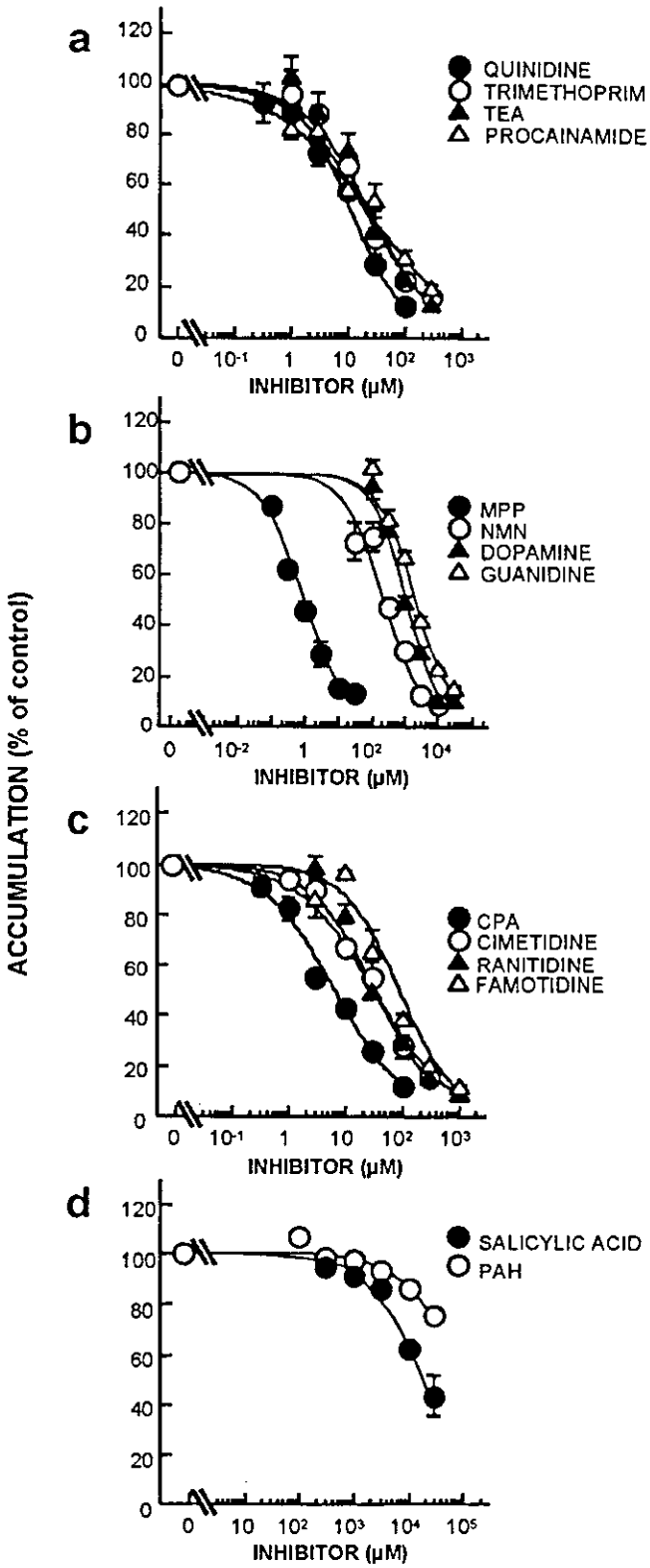

Fig. 4. Effects of cationic and anionic compounds on [^{14}C]creatinine uptake by the hOCT2-transfectants. HEK293 cells transfected with hOCT2 were incubated at 37°C for 2 min with 5 μM [^{14}C]creatinine (pH 7.4) in the presence of (a) quinidine (●), trimethoprim (○), TEA (▲), or procainamide (△); (b) 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.

ACKNOWLEDGMENTS

This work was supported in part by the Smoking Research Foundation, by a grant-in-aid for Scientific Research on Human Genome, Tissue Engineering and Food Biotechnology from Ministry of Health, Labor and Welfare of Japan (H12-Genome-019), by a grant-in-aid for Scientific Research from the Ministry of Education, Science, Culture and Sports of Japan, and by the 21st Century COE program "Knowledge Information Infrastructure for Genome Science."

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Kidney International, Vol. 66 (2004), pp. 29–45

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.

Received for publication August 31, 2003
and in revised form December 14, 2003, and January 16, 2004
Accepted for publication February 4, 2004

renal failure. Therefore, we adopted the digital expression profiling to identify up- or down-regulated genes simultaneously in progressive renal failure.

Recently we have identified an Na^+-dependent glucose transporter (rNaGLT1) from a rat kidney cDNA library by the digital expression profiling [20]. rNaGLT1 is abundantly expressed and considered to be critical for glucose and fructose reabsorption in renal proximal tubules [20, 21]. Thus, this method would be useful for cloning novel genes, which are abundantly expressed and are physiologically important. Applying this method to disease models may be beneficial for identifying some genes related to pathophysiologic status. In this study, we constructed a cDNA database of 5/6 nephrectomized and sham-operated rat kidneys 2 weeks after 5/6 nephrectomy by this method. The aim of this study was to construct a subtractive mRNA expression database for the 5/6 nephrectomized kidney, and find novel genes up- or down-regulated by subtotal nephrectomy.

METHODS

Materials

Vancomycin and cisplatin were purchased from Shionogi Co. (Osaka, Japan) and Bristol-Myers Squibb Co. (Tokyo, Japan), respectively. Protease inhibitor cocktail was obtained from Calbiochem (San Diego, CA, USA). Antirat osteopontin antibody was from Immuno Biological Laboratories Co. (Gunma, Japan). Antirat cathepsin B antibody and antimouse Na^+-K^+ATPase α1 subunit antibody were from Upstate Biotechnology Co. (Lake Placid, NY, USA). All other chemicals were of the highest purity available.

Animals

Male Wistar albino rats were purchased from SLC Animal Research Laboratories, and cared for in accordance with the *Guidelines for Animal Experiments of Kyoto University*. The animals were fed normal pellet food ad libitum, and given water freely. To induce 5/6 nephrectomy, 7-week-old male rats weighing 200 to 220 g were surgically operated as previously described [9, 10]. Briefly, the right kidneys were removed, and the posterior and anterior apical segmental branches of the left renal artery were individually ligated. Two weeks after surgery, the rats were placed in metabolic cages for the collection of 24-hour urine samples used for measurements of urinary creatinine and *N*-acetyl-β-D-glucosaminidase (NAG) excretion. Blood was collected from a cervical vein for the measurement of plasma creatinine and blood urea nitrogen (BUN). Creatinine was measured using the Jaffé method with commercial kits (Wako Pure Chemical Industries, Osaka, Japan). BUN was measured with i-STAT® Portable Clinical Analyzer (i-STAT Co., East Windsor, NJ, USA) as described elsewhere [22]. NAG was measured using commercial kits (Shionogi Co.). Urinary albumin was assessed using an enzyme-linked immunosorbent assay (ELISA) kit (Nephrat II) (Exocell, Inc., Philadelphia, PA, USA).

To induce nephrotoxicity with cisplatin or vancomycin, 9-week-old male rats weighing 290 to 310 g were administered cisplatin intraperitoneally (6 mg/kg) or vancomycin intravenously (500 mg/kg) [23]. Vehicle control rats were administered saline intravenously (10 mL/kg). Two days after the administration, the rats were placed in metabolic cages, and biochemical parameters of blood and urine were measured as described above.

Construction of a rat kidney cDNA library

Rat kidney total RNA was extracted from eight 5/6 nephrectomized or four sham-operated rats by guanidine isothiocyanate-CsCl ultracentrifugation, 2 weeks after nephrectomy. The poly(A)$^+$ RNA was purified with oligo(dT)-cellulose (Stratagene, La Jolla, CA, USA) affinity column chromatography, as described previously [24]. The creatinine clearance of 5/6 nephrectomized rats used in the construction of the cDNA library was significantly lower than that of sham-operated rats (5/6 nephrectomized rats 2.0 ± 0.2 mL/min/kg, sham-operated rats 4.1 ± 0.5 mL/min/kg, mean ± SE of eight and four rats, respectively). After confirmation of induction of the appropriate nephropathy by creatinine clearance, the poly(A)$^+$ RNA isolated from sham-operated or 5/6 nephrectomized rat kidneys was used to construct the cDNA libraries. The libraries were constructed using the λZAP Express cDNA Synthesis Kit (Stratagene) according to the manufacturer's instructions. Briefly, cDNA was synthesized using a linker primer, and digested by the restriction enzyme *Xho*I. To remove excess adaptor and to concentrate the complete length of cDNA, the cDNA was size-fractionated with cDNA Size Fraction Columns (Invitrogen Life Technology Co., Carlsbad, CA, USA). The cDNA was ligated in λZAP expression vector and excised out of the phage in the form of the pBK-cytomegalovirus (CMV) phagemid vector. The phagemid vectors with the cDNA inserts were transferred into *Escherichia coli* (strain XLOLR). From these cDNA libraries, 1033 (5/6 nephrectomized kidney) and 1015 (sham-operated kidney) randomly selected colonies were sequenced using a Multi-Capillary DNA Sequencing System RISA-384 (Shimadzu Co., Kyoto, Japan).

Data analysis

The isolated clones were partially sequenced (500–550 bp) from the 5′ end using T3 primer, and the resulting sequences were searched against GenBank, EMBL, DDBJ, and PDB using the BLAST program. If the Expect value (E value) was less than 10^{-50} (1e-50), the clone was regarded as representing the corresponding gene. A clone with an E value of more than 1e-50 was identified as

a "novel gene." The novel genes were further sequenced from the 3′ end using T7 primer. The poly(A)$^+$ tail was eliminated from the sequencing data. If overlapping regions had more than 90% homology, the two sequences were considered identical. The subtractive database of the 5/6 nephrectomized kidney and the sham-operated kidney was created in silico. The criteria of a significant difference in the subtractive difference was defined as follows; a frequency in one database was threefold higher than that in the other database and the frequency in the subtractive database was required at least two.

Western blotting

The rat whole kidney was homogenized in sucrose buffer (250 mmol/L sucrose, 5 mmol/L Tris-Hepes, pH 7.4, and 1% protease inhibitor cocktail). The tissue lysate was sonicated and clarified by centrifugation [lysis buffer 50 mmol/L Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L ethyleneglycol tetraacetate (EGTA), and 1% protease inhibitor cocktail]. The crude membrane fractions of the rat kidney were prepared as described [25]. The kidney lysate was used for analysis of cathepsin B, and the membrane fraction was used for osteopontin, OAT1, and the Na^+-K^+ATPase α1 subunit. The lysate (20 μg) or the membrane fraction (25 μg) was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Hybond®-PVDF) (Amersham Biosciences, Piscataway, NJ, USA) by semidry electroblotting for 35 minutes. The blots were blocked, washed, and incubated with the primary antibodies overnight at 4°C. The bound antibody was detected on x-ray film by enhanced chemiluminescence (ECL) with a horseradish peroxidase-conjugated antirabbit or antimouse IgG antibody and cyclic diacylhydrazides (Amersham Biosciences).

Northern blotting

The rat kidney total RNA was extracted using RNeasy® kit (Qiagen, Inc., Hilden, Germany), according to the manufacturer's directions. Five micrograms of total RNA was electrophoresed in a 1% denaturing agarose gel containing formaldehyde and transferred onto Hybond® N^+ nylon membranes (Amersham Biosciences). The transferred RNAs were linked to the nylon membrane by an ultraviolet cross linker. The quality of RNA was assessed by ethidium bromide staining. After transfer, the blots were hybridized under high stringency conditions [50% formamide, 5 × sodium chloride sodium phosphate EDTA buffer (SSPE)] [20 × SSPE; 3 mol/L NaCl, 0.2 mol/L NaH_2PO_4, and 0.02 mol/L ethylenediaminetetraactetic acid (EDTA), pH 7.4], 5 × Denhardt's solution, 0.1% SDS, and 10 μg/mL of herring sperm DNA at 42°C) with each cDNA insert labeled with [α-^{32}P] deoxycytidimine triphosphate (dCTP) (29.6 TBq/mmol) (Amersham Biosciences). After hybridization, the blots were washed three times with 2 × sodium chloride sodium citrate buffer (SSC) (20 × SSC; 3 mol/L NaCl and 0.3 mol/L sodium citrate, pH 7.0) containing 0.1% SDS at 42°C for 10 minutes, and then twice with 0.5 × SSC/0.1% SDS at 42°C for 30 minutes. The dried membranes were exposed to the imaging plates of Fujix BIO-imaging Analyzer BAS-2000 II (Fuji Photo Film Co., Tokyo, Japan).

As nonspecific hybridization was observed in the detection of 4F2hc mRNA using full-length probe, Northern blotting of 4F2hc was performed with a digoxigenin (DIG)-labeled probe corresponding to nucleotide positions 660–1074. The probe was labeled with DIG using PCR DIG probe synthesis kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Hybridization was performed as described above. After hybridization, the blots were washed twice with 2 × SSC/0.1% SDS for 10 minutes, and then three times with 0.5 × SSC/0.1% SDS for 20 minutes. DIG-labeled nucleic acids were detected using a DIG luminescent detection kit (Roche Diagnostics), according to the manufacturer's directions.

Microdissection of rat nephron segments

The rat nephron was microdissected as described previously [26]. Briefly, the left kidneys of 5/6 nephrectomized and sham-operated rats 2 weeks after subtotal nephrectomy were perfused and removed. Slices were cut along the medullary axis and incubated with collagenase. The tubules were microdissected to obtain the following structures: glomerulus, proximal convoluted tubule (PCT), proximal straight tubule (PST), medullary thick ascending limb (MAL), cortical thick ascending limb (CAL), cortical collecting duct (CCD), outer medullary collecting duct (OMCD), and inner medullary collecting duct (IMCD). After microdissection, 20 glomeruli and 8 mm of each dissected tubule segment were transferred into tubes to isolate total RNA using RNeasy® minikit (Qiagen).

RT-PCR analysis

Total RNA from the dissected tubules and poly(A)$^+$ RNA from rat tissues (brain, heart, lung, liver, small intestine, spleen, kidney cortex, and kidney medulla) was reverse-transcribed with random hexamers, using Superscript II reverse transcriptase (Invitrogen), followed by RNase H (Invitrogen) digestion. These single-stranded DNA fragments were amplified with primer sets specific for the five unknown genes.

Analysis of novel or unknown genes

There were 343 mRNAs in which the nucleic acid and amino acid sequences have been clarified with little

information available concerning their functions and tissue distributions (about 17% of 2048 clones sequenced). We identified them as "unknown genes." There were 18 novel or unknown genes appearing twice either in the 5/6 nephrectomized or in the sham-operated kidney. Among them, five genes were fully sequenced because significant differences in expression were shown between the 5/6 nephrectomized and sham-operated kidney by Northern blotting. To analyze the 5′ upstream region, a 5′ rapid amplification of cDNA ends (RACE) was performed using Marathon-Ready cDNA kits (Clontech Laboratories, Inc., Palo Alto, CA, USA). The motifs of their deduced amino acid sequences were searched against Smart (Simple Modular Architecture Research Tool, *smart.embl-heidelberg.de/*) and Pfam (Protein families database of alignments and HMMs, *pfam.wustl.edu/*) libraries. In addition, the tissue distribution, the localization along the nephron, the expression in the earlier phases of 5/6 nephrectomized rats, and the expression in acute nephropathy induced by cisplatin or vancomycin of these five unknown genes were examined.

Statistical analysis

Data are expressed as the mean ± SEM. Data were analyzed statistically by the unpaired Student *t* test, or by a one-way analysis of variance (ANOVA) followed by Fisher's *t* test, when multiple comparisons against the control were needed. Significance was set at $P < 0.05$.

RESULTS

Gene expression profiles and in silico subtraction

In the 5/6 nephrectomized kidney, there were 41 genes highly expressed (more than three times), 84 genes expressed twice, and 663 genes expressed once. In total, 788 genes were found. In the sham kidney, there were 46 genes highly expressed, 79 genes expressed twice, and 594 genes expressed once, a total of 729. These isolated genes were classified according to function and localization. The results are shown in Table 1. In the 5/6 nephrectomized kidney, the growth factor-related mRNA and the mRNA encoding cytoskeletal or membrane proteins were increased compared with those in the sham-operated kidney. In contrast, the genes related to the transporter were decreased in the 5/6 nephrectomized kidney.

In the 5/6 nephrectomized kidney, 853 clones were identical to previously reported genes, but 168 clones among them were classified as unknown genes. There were 180 clones for which the E value of the BLAST homologue analysis was more than 1e-50 and thought to be novel genes. Among them, 12 clones were considered to be duplicated, as their sequences at the 3′ end were identical. As a result, we obtained 174 novel genes in the 5/6 nephrectomized kidney. In the sham-operated kidney, 851 clones were identical to previously reported genes, but 175 clones among them were unknown genes. There were 164 clones classified as novel genes. Among them, 14 clones were judged to be duplicated, based on the sequence of the 3′ end. As a result, 157 novel genes were observed in the sham-operated kidney.

Table 1. Classification of mRNA in the 5/6 nephrectomized and sham-operated kidney according to function or localization

Category	5/6 Nephrectomy frequency	%	Sham-operated frequency	%
Cytoskeletal, membrane protein	73	7.1	37	3.6
Growth factor–related	15	1.5	4	0.4
Ribosome	27	2.6	19	1.9
Heat shock protein	5	0.5	7	0.7
Metabolic enzyme	29	2.8	31	3.1
Enzyme	183	17.7	227	22.4
Protease	29	2.8	27	2.7
Nuclear factor	66	6.4	54	5.3
Mitochondria	53	5.1	46	4.5
Signal transduction	54	5.2	52	5.1
Transporter	24	2.3	37	3.6
Receptor channel	17	1.6	14	1.4
Endocytosis	15	1.5	15	1.5
Other	95	9.2	106	10.4
Unknown; function unknown	168	16.3	175	17.2
Unknown; novel	180	17.4	164	16.2
Total	1033	100.0	1015	100.0

Frequency and percentages of clones classified into 16 categories are listed. "Other" means a clone not classified into the above 13 categories.

In silico subtraction of the 5/6 nephrectomized and the sham-operated kidney databases was performed. Sixty-three genes were increased (Table 2) and 59 decreased in the 5/6 nephrectomized kidney (Table 3).

Confirmation of the database

To confirm whether the in silico subtractive database of 5/6 nephrectomized kidney reflected the actual expression of mRNA, the expression of 12 genes which was considered to be common in the kidney was examined by Northern blotting in five 5/6 nephrectomized and sham-operated rats at 2 weeks after surgery. A summary of the 12 genes is described in Table 4. Because the mRNA database to reflect not only mRNA expression level but also protein expression level is important to elucidate the mechanisms of progressive nephropathy, the expressional changes of cathepsin B, osteopontin, OAT1, and the Na^+-K^+ATPase α_1 subunit, whose specific antibodies were available, were also examined by Western blotting. The blood and urine biochemical parameters and kidney weights are shown in Table 6. Decreases in creatinine clearance and albuminuria were found in the 5/6 nephrectomized rats, but urinary NAG excretion, an indicator of proximal tubule injury, was not significantly changed.

Table 2. The mRNAs up-regulated in the 5/6 nephrectomized (NR) kidney database by in silico subtraction

Frequency NR	Sham-operated	Gene name	Category	Accession number
5	0	Rat liver aldolase B mRNA, complete cds	Enzyme	M10149
5	0	Rattus norvegicus cytochrome b, mRNA	Mitochondria	AF295545
4	0	Rat mRNA for sulfated glycoprotein 2	Other	AB013455
4	0	Rattus norvegicus cathepsin B (Ctsb), mRNA	Protease	X13231
4	0	Rattus norvegicus mRNA for NaPi-2 gamma, complete cds	Transporter	NM_022597
4	1	Rattus norvegicus cytochrome P450, 4a10 (Cyp4a10), mRNA	Metabolic enzyme	NM_031605
4	1	Rattus norvegicus eukaryotic translation elongation factor 2 (Eef2), nRNA	Nuclear	NM_017245
3	0	Rattus norvegicus CD24 antigen (Cd24), mRNA	Other	NM_013457
3	0	Rattus norvegicus kidney injury molecule-1 (KIM-1), mRNA, complete cds	Other	BC006660
3	0	Rattus norvegicus 3-hydroxyanthranilate 3,4-dioxygenase (Haao), mRNA	Enzyme	NM_020076
3	0	Rattus norvegicus glutamate-cysteine ligase regulatory, mRNA	Enzyme	NM_012752
3	0	Mus musculus adducin 1 (alpha) (Add1), mRNA	Cytoskeletal, membrane protein	AF219904
3	0	Rattus norvegicus folate binding protein, mRNA, complete cds	Cytoskeletal, membrane protein	NM_017305
3	0	Rattus norvegicus Sialoprotein (osteopontin) (Spp1), mRNA	Cytoskeletal, membrane protein	AF035963
3	0	Rattus norvegicus ubiquitin C (Ubc), mRNA	Protease	NM_017341
3	0	Rattus norvegicus similar to NADH-ubiquinone oxidoreductase 75 kD subunit	Mitochondria	NM_012881
3	1	Rat leukemia virus gag (gag), polymerase, and envelope protein genes, complete cds	Enzyme	AK004990
3	1	Rat mRNA for preprocathepsin D (EC 3.4.23.5)	Protease	M77194
3	1	Rattus norvegicus similar to claudin 2	Cytoskeletal, membrane protein	XM236535
2	0	Mus musculus alpha-2 type IV collagen, mRNA, complete cds	Other	J04695
2	0	Mus musculus interferon regulatory factor 3 (Irf3), mRNA	Other	NM_016849
2	0	Mus musculus procollagen, type VI, alpha 1 (Col6a1), mRNA	Other	NM_009933
2	0	Rattus norvegicus peroxiredoxin 3 (Prdx3), mRNA	Other	NM_022540
2	0	Mus musculus, ELK3, member of ETS oncogene family, clone MGC:11528	Other	BC005686
2	0	HSD IV = peroxisome proliferator-inducible gene [rats, F344, liver, mRNA partial, 2480 nt]	Enzyme	S83279
2	0	Mus musculus serine hydroxymethyltransferase, mRNA, complete cds	Enzyme	AF237702
2	0	Rat mRNA for glutamine synthetase (EC 6.3.1.2.)	Enzyme	X07921
2	0	Rattus norvegicus cysteine desulfurase (NifS), mRNA, complete cds	Enzyme	AF336041
2	0	Rattus norvegicus D-dopachrome tautomerase (Ddt), mRNA	Enzyme	NM_024131
2	0	Rattus norvegicus kynurenine aminotransferase II (Kat2), mRNA	Enzyme	NM_017193
2	0	Rattus norvegicus mRNA for H(+)-transporting ATPase, complete cds	Enzyme	D10874
2	0	Rattus norvegicus NG,NG dimethylarginine dimethylaminohydrolase (Ddah1), mRNA	Enzyme	D86041
2	0	Rattus norvegicus ornithine aminotransferase (Oat), mRNA	Enzyme	NM_022521
2	0	Rattus norvegicus protein disulfide isomerase, mRNA	Enzyme	NM_012998
2	0	Rattus norvegicus insulin-like growth factor binding protein 1 (Igfbp1), mRNA	Growth factor–related	NM_013144
2	0	Mus musculus destrin (Dsn-pending), mRNA	Cytoskeletal, membrane protein	NM_019771
2	0	Mus musculus presenilin-1 gene, alternatively spliced transcripts, complete cds	Cytoskeletal, membrane protein	AF007560
2	0	Rat brain calbindin-d28k (CaBP28K), mRNA	Cytoskeletal, membrane protein	M27839
2	0	Rattus norvegicus mRNA for neuronal beta-catenin like protein (ORF1)	Cytoskeletal, membrane protein	AJ301634
2	0	Rattus sp. liver tricarboxylate carrier, mRNA	Mitochondria	S70011

Table 2. Continued.

Frequency NR	Sham-operated	Gene name	Category	Accession number
2	0	Mus musculus adult male kidney cDNA, RIKEN fclone:0610007F03	Mitochondria	AK018712
2	0	Mus musculus histone deacetylase 5 (Hdac5), mRNA	Nuclear	NM_010412
2	0	Rattus norvegicus mRNA for ribonucleoprotein F, complete cds	Nuclear	NM_022397
2	0	Human DNA sequence from clone 486I3 on chromosome 6q22.1–22.3	Nuclear	AL050331
2	0	Homo sapiens 5-HT receptor, mRNA, complete cds	Receptor channel	AF251055
2	0	Mus musculus chloride intracellular channel 4 (mitochondrial) (Clic4), mRNA	Receptor channel	NM_013885
2	0	Homo sapiens cDNA FLJ13989 fis, clone Y79AA1002083	Ribosome	AK024051
2	0	Homo sapiens, clone IMAGE:2820942, mRNA, partial cds	Ribosome	BC006474
2	0	Mus musculus N-myc downstream regulated 3 (Ndr3), mRNA	Signal transduction	NM_013865
2	0	Mus musculus pyruvate kinase 2 (pkm2), mRNA	Signal transduction	NM_011099
2	0	Rat casein kinase I delta, mRNA, complete cds	Signal transduction	L07578
2	0	Rattus norvegicus 14-3-3-zeta isoform (Prkcz), mRNA	Signal transduction	U37252
2	0	Rattus norvegicus mRNA for inositol hexakisphosphate kinase, complete cds	Signal transduction	AB049151
2	0	Rattus norvegicus rap7a (Rap7a), mRNA	Signal transduction	NM_022526
2	0	R.norvegicus mRNA for monocarboxylate transporter	Transporter	X86216
2	0	Mus musculus 10, 11 days embryo cDNA, RIKEN clone:2810413P16	Unknown	BC011420
2	0	Mus musculus 10 days embryo cDNA, RIKEN clone:2610315D18	Unknown	AK012030
2	0	Rattus norvegicus 4 BAC CH230-5L13	Unknown	AC126722
2	0	Rattus norvegicus similar to hypothetical protein MGC14421	Unknown	AC126722
2	0	Mus musculus ES cells cDNA, RIKEN clone:2410083E08	Unknown	AK010737
2	0	Novel gene 1	Novel	
2	0	Novel gene 2	Novel	
2	0	Novel gene 3	Novel	

The mRNAs, which were more frequent in the 5/6 nephrectomized kidney database compared with sham-operated kidney database, were listed. Significant difference was defined when the frequency of 5/6 nephrectomized kidney database was threefold higher than that of sham-operated kidney database and the subtractive difference was more than two.

The residual kidneys, which were removed necrotic region, were actually enlarged twofold in the 5/6 nephrectomized rats, as the weights of the 5/6 nephrectomized kidney were about one third those of the sham-operated kidneys despite 5/6 ablations.

The results of Northern blotting and Western blotting are shown in Figure 1. The mRNA expression of cathepsin B, osteopontin, kidney injury molecule-1 (KIM-1), and insulin-like growth factor binding protein-1 (IGFBP1) was significantly increased in the 5/6 nephrectomized kidney (Fig. 1A). The mRNA expression of OAT1, Na^+-K^+ATPase α1 subunit, elongation factor 1α, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and aquaporin 2 was maintained in the 5/6 nephrectomized kidney (Fig. 1B). However, the mRNA expression of phenylalanine hydroxylase, 4F2hc, and OCTN2 was significantly decreased in the 5/6 nephrectomized kidney (Fig. 1C). These results were well comparable with the in silico subtractive database (Tables 2 and 3). In addition, the protein expression of cathepsin B, osteopontin, OAT1, and Na^+-K^+ATPase α1 subunit were also comparable with the in silico subtractive database (Fig. 1).

Novel or unknown genes differentially expressed between the 5/6 nephrectomized and sham-operated kidneys

There were eight novel or unknown genes appearing twice in the 5/6 nephrectomized and 0 times in the sham-operated kidney database (Table 2). Among them, the expression of two genes designated UR-NR (up-regulated in the 5/6 nephrectomized kidney) #1 (GenBank Accession No. AB108667) and #2 (GenBank Accession No. AB108668) (the nucleotide sequences reported in this article have been submitted to GenBank with accession numbers between AB108667 and AB108671) was significantly increased in the 5/6 nephrectomized kidney as determined by Northern blotting (Fig. 2A and B). There were 10 novel or unknown genes appearing twice in the sham-operated and 0 times in the 5/6 nephrectomized kidney database (Table 3). Among them, three genes designated DR-NR (down-regulated in the 5/6 nephrectomized kidney) #1 (GenBank Accession No. AB108669), #2 (AB108670), and #3 (AB108671) had a significantly decreased expression in the 5/6 nephrectomized kidney as shown by Northern blotting (Fig. 2C to E).

Table 3. The mRNAs down-regulated in the 5/6 nephrectomized (NR) kidney database by in silico subtraction

Frequency NR	Sham-operated	Gene name	Category	Accession number
0	7	Rattus norvegicus phenylalanine hydroxylase (Pah), mRNA	Enzyme	NM_012619
2	7	Rattus norvegicus urmodulin (Tamm-Horsfall protein) (Umod), mRNA	Other	NM_011044
1	7	Mus musculus phosphoenolpyruvate carboxykinase 1, cytosolic (Pck1), mRNA	Enzyme	NM_017082
2	6	Rattus norvegicus cytochrome P450 arachidonic acid epoxygenase, mRNA	Metabolic enzyme	U04733
0	5	Rattus norvegicus intestinal type II membrane glycoprotein 4F2hc, mRNA	Transporter	U59324
1	5	Mus musculus, similar to glutathione S-transferase theta 1, clone MGC:6769, mRNA	Enzyme	BC003903
0	4	Rattus norvegicus acyl-coenzyme A dehydrogenase, C-4 to C-12 straight-chain, mRNA	Enzyme	NM_013113
0	4	Rattus norvegicus pyruvate dehydrogenase kinase 2 subunit p45 (PDK2) (Pdk2), mRNA	Signal transduction	NM_016986
0	4	Rattus norvegicus ATPase Na+/K+ transporting beta 1 polypeptide (Atp1b1), mRNA	Transporter	NM_030872
1	4	Rat mRNA for cerebellar Ca-binding protein, spot 35 protein	Cytoskeletal, membrane protein	NM_031984
0	3	Rattus norvegicus calreticulin (Calr), mRNA	Other	NM_022399
0	3	Rattus norvegicus spp-24 precursor, mRNA, partial cds	Other	NM012570
0	3	Rattus norvegicus glutamate dehydrogenase (Glud1), mRNA	Enzyme	U19485
0	3	Rattus norvegicus thyroxine deiodinase, type I (Dio1), mRNA	Enzyme	NM_012653
1	3	Mus musculus, similar to NADH dehydrogenase Fe-S protein 2, clone IMAGE:3600832	Enzyme	BC003898
1	3	Rat mRNA for gamma-glutamyl transpeptidase (EC 2.3.2.2)	Enzyme	X15443
1	3	Rattus norvegicus methylmalonate semialdehyde dehydrogenase gene (Mmsdh), mRNA	Enzyme	AF359355
1	3	Rattus norvegicus retinol dehydrogenase type II, mRNA, complete cds	Enzyme	NM_031057
1	3	Rattus norvegicus superoxide dimutase 3 (Sod3), mRNA	Enzyme	U33500
1	3	Rattus norvegicus membrane-bound aminopeptidase P, mRNA, complete cds	Protease	NM_012880
0	2	Rattus norvegicus (rsec6), mRNA, complete cds	Other	U32575
0	2	Rattus norvegicus transcobalamin II precursor (Tcn2p), mRNA	Other	NM_022534
0	2	Rattus norvegicus prosaposin (sulfated glycoprotein, sphingolipid hydrolase activator)	Other	NM_013013
0	2	Homo sapiens, calcium binding atopy-related autoantigen 1, clone MGC:4521, mRNA	Other	BC004216
0	2	Mus musculus interferon alpha responsive gene, 15 kD (Ifrg15-pending), mRNA	Other	NM_022329
0	2	Mus musculus Mpv17-like protein, mRNA, complete cds	Other	AF305634
0	2	Rattus norvegicus src family associated phosphoprotein 2 (Scap2), mRNA	Other	NM_130413
0	2	Mus musculus, similar to autocrine motility factor receptor, clone IMAGE:3500628, mRNA	Receptor channnel	BC003256
0	2	Rattus norvegicus hydroxysteroid dehydrogenase, 11 beta type 1 (Hsd11b1), mRNA	Enzyme	NM_017080
0	2	Rattus norvegicus pyruvate carboxylase, mRNA, complete cds	Enzyme	U36585
0	2	Hprt = hypoxanthine phosphoribosyltransferase [rats, mRNA, 853 nt]	Enzyme	S79292
0	2	Rattus sp. mRNA for argininosuccinate lyase, complete cds	Enzyme	D13978
0	2	Rattus norvegicus tissue-type transglutaminase (TgaseII), mRNA, complete cds	Enzyme	AF106325
0	2	Rattus norvegicus cysteine-sulfinate decarboxylase (Csad), mRNA	Enzyme	NM_021750
0	2	Rattus norvegicus Glutamate oxaloacetate transaminase 2, mitochondrial (Got2), mRNA	Enzyme	NM_013177
0	2	Homo sapiens heat shock protein 75 (TRAP1), mRNA	HSP	NM_016292
0	2	Rattus norvegicus vesicle-associated membrane protein 7 (Vamp7) mRNA, complete cds	Cytoskeletal, membrane protein	AF281632
0	2	Homo sapiens, Similar to membrane protein of cholinergic synaptic vesicles, mRNA	Cytoskeletal, membrane protein	XM_213485
0	2	Rat mRNA for mitochondrial long-chain 3-ketoacyl-CoA thiolase beta-subunit	Mitochondria	D16479
0	2	Mus musculus SMT3 (supressor of mif two, 3) homolog 1 (S. cerevisiae) (Smt3h1), mRNA	Nuclear	NM_019929
0	2	Mus musculus NUDE-like protein mRNA, complete cds	Nuclear	AF323918
0	2	Mus musculus microtubule-associated protein 7 (Mtap7), mRNA	Nuclear	NM_008635
0	2	Mus musculus proteaseome (prosome, macropain) 28 subunit, 3 (Psme3), mRNA	Protease	NM_011192
0	2	Rattus mRNA for ribosomal protein S2	Ribosome	X57432
0	2	Homo sapiens sorting nexin 17 (SNX17), mRNA	Signal transduction	NM_014748
0	2	Rattus norvegicus solute carrier family 25, member 1 (Slc25a1), mRNA	Transporter	NM_172577
0	2	Rattus norvegicus solute carrier family 12, member 1 (Slc12a1), mRNA	Transporter	NM_019134
0	2	Rattus norvegicus organic cation/carnitine transporter (OCTN2) mRNA	Transporter	AF110416
0	2	Rattus norvegicus Glutathione-S-transferase, alpha type 2 (Gsta2), mRNA	Enzyme	NM_017013
0	2	Rattus norvegicus similar to hypothetical protein FLJ20356, mRNA	Unknown	XM_223527
0	2	Mus musculus RIKEN cDNA 2210404O07 gene, mRNA	Unknown	BC036156
0	2	Mus musculus adult male liver cDNA, RIKEN clone:1300002B20, full insert sequence	Unknown	AK004845
0	2	Mus musculus ES cells cDNA, RIKEN clone:2410074K14, full insert sequence	Unknown	AK010721
0	2	Mouse DNA sequence from clone RP23-22K4 on chromosome 2, complete sequence	Unknown	AL663077
0	2	Homo sapiens hypothetical protein DKFZp564B1162 (DKFZP564B1162), mRNA	Unknown	NM_031305
2	0	Mus musculus 13 days embryo head cDNA, RIKEN clone:3110030P16	Unknown	XM_055115
0	2	Mus musculus NN5H6H tumor-related protein, mRNA, complete cds	Unknown	AY035213
0	2	Novel gene 4	Novel	
0	2	Novel gene 5	Novel	

The mRNAs, which were less frequent in the 5/6 nephrectomized kidney database compared with sham-operated kidney database, are listed. Significant difference was defined when the frequency of 5/6 nephrectomized kidney database was threefold lower than that of sham-operated kidney database and the subtractive difference was more than two.

Table 4. The list of genes subjected to confirmation of the database

	Gene name	Frequency NR	Frequency Sham	Category	Accession number
NR > sham	Cathepsin B	4	0	Protease	NM022597
	Osteopontin	3	0	Cytoskeletal, membrane protein	NM012881
	KIM-1	3	0	Cytoskeletal, membrane protein	AF035963
	IGF binding protein1	2	0	Growth factor–related	NM013144
NR = sham	OAT1	2	4	Transporter	AF008221
	Na^+-K^+ATPase α1	2	2	Transporter	NM012504
	Elongation factor -1α	12	14	Ribosome	X61043
	GAPDH	4	6	Enzyme	NM017008
	Aquaporin 2	2	2	Transporter	D13906
NR < sham	Pah	0	7	Enzyme	NM 012619
	4F2hc	0	5	Transporter	U59324
	OCTN2	0	2	Transporter	AF110416

Abbreviations are: Accession number, number in Genbank database; frequency, times appeared in 1033 or 1015 sequences; KIM-1, kidney injury molecule-1; IGF, insulin like growth factor; OAT-1, organic anion transporter 1; Pah, phenylalanine hydroxylase; OCTN2, novel organic cation transporter 2. The expressions of four genes which were more frequent in the 5/6 nephrectomized (NR) than sham-operated (sham) kidney, five genes which were equally frequent in the 5/6 nephrectomized and sham-operated kidney, and three genes which were less frequent in the 5/6 nephrectomized than sham-operated kidney, were examined by Northern blotting or Western blotting. Nucleotide positions of probes are from the sequences published in GenBank/EBI Data Bank.

To obtain more information about these five genes, the expression levels in the earlier phases of 5/6 nephrectomized kidney, the tissue distribution, the localization along the microdissected nephron segments, and the expression levels in the drug-induced acute nephropathy models were examined. Blood and urine biochemical parameters and kidney weights are shown in Table 5. Creatinine clearance of 5/6 nephrectomized rats decreased to 2.1 mL/min/kg at 3 days after the operation, and this level was maintained until 2 weeks. On the other hand, compensatory hypertrophy was not observed in 5/6 nephrectomized kidneys 3 days after the operation, and kidney weight was increased sequentially according to the compensatory hypertrophy thereafter. Figure 2 shows the mRNA expression levels of the five unknown genes in sham-operated and 5/6 nephrectomized kidneys 3 days, 1 week, and 2 weeks after subtotal nephrectomy. The mRNA expression of UR-NR #1 of 5/6 nephrectomized kidney was more markedly increased at 1 week than at 2 weeks after the operation (Fig. 2A). The mRNA expression of UR-NR #2 of 5/6 nephrectomized kidney was maintained at 3 days after the operation but increased sequentially thereafter (Fig. 2B). The mRNA expression of DR-NR #1 and DR-NR #3 of 5/6 nephrectomized kidney was maintained at 3 days and 1 week after the operation and decreased at 2 weeks after the operation (Fig. 2C and E). The mRNA expression of DR-NR #2 of 5/6 nephrectomized kidney was equally decreased at 1 week and 2 weeks after the operation (Fig. 2D).

Figure 3 shows the mRNA distribution of the five unknown genes in eight tissues. UR-NR #1 mRNA was detected in all tissues examined except for the small intestine and spleen. UR-NR #2 and DR-NR #1 mRNAs were strongly detected in the spleen and kidneys, but weakly detected in other tissues. DR-NR #2 mRNA existed predominantly in the kidneys. DR-NR #3 mRNA was detected only in the kidney and liver.

mRNA localization of the five unknown genes along the nephron segments in sham-operated (Fig. 4A) and 5/6 nephrectomized kidneys (Fig. 4B) was examined. UR-NR #1 mRNA was localized at IMCD in the sham-operated kidneys. It was detected not only in IMCD but also in glomerulus, PCT, and PST in 5/6 nephrectomized kidneys. UR-NR #2 mRNA was not detected either in sham-operated or 5/6 nephrectomized renal nephron segments, and was thought to exist in the interstitium or blood vessels in the kidneys. DR-NR #1 mRNA was detected ubiquitously both in sham-operated and 5/6 nephrectomized renal nephron segments except in MAL. DR-NR #2 mRNA was localized at glomerulus, PCT, and the collecting duct in sham-operated kidneys, but was only localized at PCT and IMCD in 5/6 nephrectomized kidneys. DR-NR #3 mRNA was detected predominantly in the proximal tubules in sham-operated kidneys, and its localization shifted to glomerulus or MAL in 5/6 nephrectomized kidneys.

The mRNA expression levels of the five unknown genes were also examined in acute renal insufficiency induced by the administration of cisplatin or vancomycin. Blood and urine biochemical parameters and kidney weights are shown in Table 6. In the cisplatin nephropathy, a marked reduction of renal function, decrease in creatinine clearance, increases in BUN and urinary NAG excretion, and albuminuria were observed. However, there was no change in kidney weight. In contrast to the cisplatin nephropathy, there was no change in creatinine clearance and only a slight increase of BUN in the vancomycin nephropathy. Although urinary NAG excretion was increased, albuminuria did not appear in the vancomycin nephropathy. The kidney weight of the animals with vancomycin nephropathy was markedly increased. The results of Northern blotting are shown in Figure 5. The mRNA expression of UR-NR #1 was significantly increased in the vancomycin but not the cisplatin