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Transport of guanidine compounds by human organic cation transporters, hOCT1 and hOCT2

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ABSTRACT

Although some guanidine compounds were reported as superior substrates for organic cation transporter (OCT)2 than OCT1, it was unclear whether this guanidino group was an important factor in determining the specificity of hOCT1 and hOCT2. Using HEK293 cells transfected with human (h)OCT1 or hOCT2 cDNA, we assessed the role of hOCT1 and/or hOCT2 in the transport of guanidine compounds such as uremic toxins and therapeutic agents. Guanidine, creatinine and aminoguanidine more markedly inhibited the uptake of [^{14}C]tetraethylammonium (TEA) by hOCT2 than by hOCT1. [^{14}C]TEA uptake by hOCT2, but not hOCT1, was *trans*-stimulated by unlabeled guanidine, methylguanidine, creatinine, aminoguanidine and phenylguanidine. In patients with renal failure, the impairment of hOCT2 might decrease the excretion of guanidine, methylguanidine, and creatinine as uremic toxins. The uptake of aminoguanidine, a candidate for an anti-diabetic agent, was enhanced by hOCT2 with the Michaelis constant (K_m) of 4.10 ± 0.35 mM. Metformin, which was also an anti-diabetic agent, and creatinine more potently inhibited the uptake of [^{14}C]aminoguanidine by hOCT2 than that by hOCT1. Aminoguanidine had little impact on the uptake of [^{14}C]metformin by hOCT1, but inhibited that by hOCT2 with the IC_{50} of 1.49 ± 0.14 mM. These results indicated that the specificity of hOCT1 and hOCT2 was not determined simply by guanidino group. Among guanidine compounds, aminoguanidine was identified as a new superior substrate for hOCT2.

1. Introduction

Organic cation transporters (OCTs) play an important role in the tissue distribution of a wide variety of positively charged molecules, including drugs and endogenous substrates. Human organic cation transporter 1 (hOCT1) is preferentially expressed in the liver, and mediates hepatic uptake of cationic compounds [1,2]. In contrast, hOCT2 is specifically expressed in the renal proximal tubules, and is considered to mediate the renal uptake of cationic compounds [3,4]. Functional studies suggested that these transporters were often similar in substrate specificity [4,5], but in recent years, the compounds with a guanidino group such as guanidine, creatinine, and metformin were reported to be better substrates for OCT2 than OCT1 in rat and/or human [6–8]. However, it was unclear whether a guanidino group was important in determining the affinity of the two transporters.

Some guanidine compounds have been known as uremic toxins [9–14]. Other guanidine compounds have been reported as anti-diabetic agents, in particular, aminoguanidine is positively charged at physiological pH, and its renal clearance was more than twice the glomerular filtration rate (GFR), suggesting the contribution of tubular secretion [15–17].

The aim of this study was to compare the specificity of hOCT1 and hOCT2 for several guanidine compounds, including uremic toxins and aminoguanidine (Fig. 1).

2. Materials and methods

2.1. Materials

[Ethyl-1-^{14}C]tetraethylammonium bromide (55 mCi/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). [^{14}C]aminoguanidine (51 mCi/mmol) and [biguanidine-^{14}C]metformin hydrochloride (26 mCi/mmol) were purchased from Moravek Biochemicals, Inc. (Brea, CA). [^{3}H]1-methyl-4-phenylpyridium acetate (MPP) (2.7 TBq/mmol) was purchased from PerkinElmer Life and Analytical Sciences Waltham, MA). Creatinine and guanidine hydrochloride were obtained from Nacalai Tesque (Kyoto, Japan). N_α-Acetyl-L-arginine, aminoguanidine bicarbonate salt, 1-butylguanidine sulfate, creatine anhydrous, 1,1-dimethylguanidine sulfate salt, guanidinoacetic acid, guanidinosuccinic acid, guanidinovaleric acid hemihydrate, methylguanidine hydrochloride, N-propylguanidine sulfate, phenylguanidine carbonate salt, 1,1,3,3-tetramethylguanidine, and 1-

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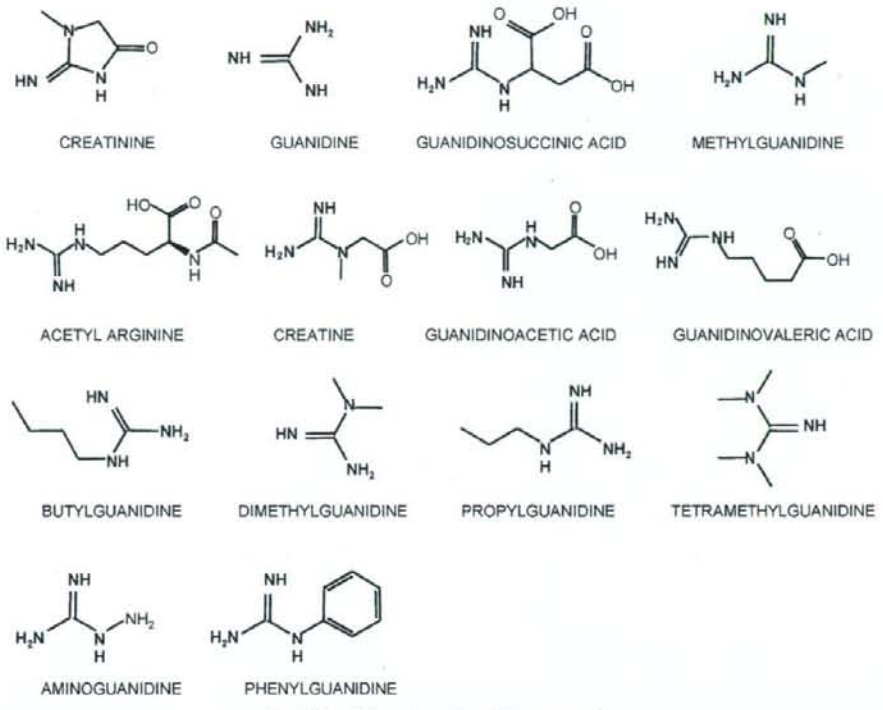

Fig. 1. Chemical structures of guanidine compounds.

methyl-4-phenylpyridium iodide were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). All other compounds used were of the highest purity available.

2.2. Cell culture

HEK 293 cells (ATCC CRL-1573, American Type Culture Collection, Manassas, VA) 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, and used as host cells. The transfectant stably expressing hOCT1 and hOCT2 were established as described previously [7,8]. The HEK293 cells transiently transfected with pCMV6-XL4 plasmid vector DNA (OriGene Technologies, Rockville, MD) containing hOCT1, hOCT2 or hOCT3-cDNA were prepared as described previously [7,18]. The cell monolayers were used at day 3 of culture for uptake experiments. In the present study, cells were used between the 78th and 90th passages.

2.3. Uptake experiments

The cellular uptake of cationic compounds was measured with monolayer cultures of HEK293 cells grown on poly-D-lysine-coated 24-well plates [7,19]. 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) with bovine-globulin as a standard. For the *cis*-inhibition study, the uptake of [^{14}C]tetraethylammonium (TEA), [^{14}C]aminoguanidine, or [^{14}C]metformin was achieved by adding various concentrations of unlabeled inhibitors to the incubation medium. IC_{50} values were calculated from the inhibition plots based on the equation, $V = V_0/[1 + ([I]/IC_{50})^n]$, by a nonlinear least square regression analysis with Kaleidagraph Version 4.00 (Synergy Software, Reading, PA, USA). V and V_0 were the uptake rates of [^{14}C]TEA, [^{14}C]aminoguanidine, or [^{14}C]metformin in the presence and absence of inhibitor, respectively. [I] is the concentration of inhibitor, and n is the Hill coefficient. For the

Table 1
The apparent IC_{50} values of guanidine compounds for [^{14}C]TEA uptake by hOCT1 and hOCT2.

Guanidine compounds	IC_{50} values for [^{14}C]TEA uptake (mM)	
	hOCT1	hOCT2
Uremic toxins[a]		
Creatinine	N/A	6.06 ± 0.98
Guanidine	N/A	3.03 ± 0.42
Guanidinosuccinic acid	1.54 ± 0.15	1.47 ± 0.20
Methylguanidine	2.36 ± 0.06	1.53 ± 0.31
Acetyl arginine	N/A	N/A
Creatine	N/A	N/A
Guanidinoacetic acid	N/A	N/A
Guanidinovaleric acid	0.66 ± 0.03	1.18 ± 0.14*
Butylguanidine	0.21 ± 0.02	0.12 ± 0.01*
Dimethylguanidine	0.54 ± 0.09	0.36 ± 0.02
Propylguanidine	0.36 ± 0.04	0.29 ± 0.02
Tetramethylguanidine	0.48 ± 0.08	0.78 ± 0.13
Aminoguanidine	N/A	0.80 ± 0.11
Phenylguanidine	0.23 ± 0.03	0.26 ± 0.02

See experimental conditions in the legend of Fig. 2. The apparent IC_{50} values were calculated from inhibition plots (Fig. 2) by nonlinear regression analysis as described in Section 2. The data represent the mean ± S.E. of three independent experiments. N/A, not available. *$P < 0.05$, significantly different from the IC_{50} value of hOCT1.

[a] [18].

trans-inhibition study, the cells were preincubated with either the incubation medium (control) or the incubation medium plus the indicated concentration of unlabeled compounds for 30 min. The cells were rinsed twice with 1 mL of ice-cold incubation medium before the uptake experiments. The concentration dependence of the transport of aminoguanidine by hOCT1 and hOCT2 was analyzed using the Michaelis–Menten equation: $V = V_{max}\cdot[S]/(K_m + [S]) + K_{diff}\cdot[S]$, where V is the transport rate, V_{max} is the maximum transport rate, [S] is the concentration of aminoguanidine, K_m is the Michaelis constant and K_{diff} is a diffusion constant. The accumulation of [^{14}C]aminoguanidine by hOCT1- and hOCT2-HEK293 cells was measured at various concentrations (0.1–10 mM) for 2 min at 37 °C (pH 7.4).

2.4. Statistical analysis

Data were expressed as the mean ± S.E. Data were analyzed statistically using the non-paired Student's *t* test or one-way analysis of variance (ANOVA) and Dunnett's multiple comparison procedure.

3. Results

3.1. Inhibitory effects of guanidine compounds on TEA uptake by hOCT1 and hOCT2

To compare the specificity of hOCT1 and hOCT2, the inhibitory effects of several guanidine compounds on the uptake of [^{14}C]TEA

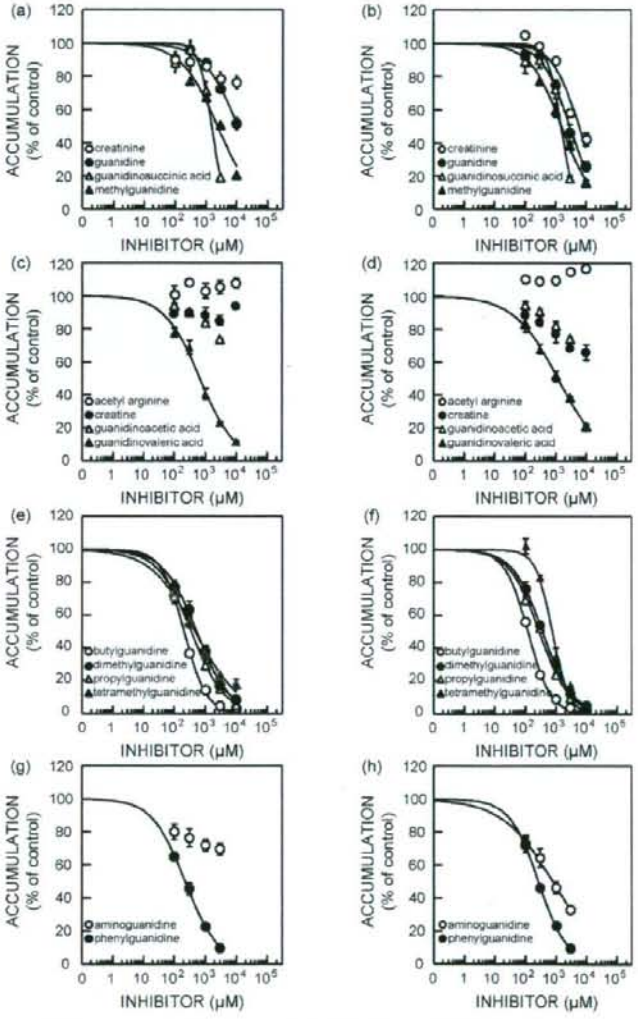

Fig. 2. Effects of guanidine compounds on [^{14}C]TEA uptake by hOCT1 (a, c, e and g) and hOCT2 (b, d, f and h). HEK293 cells transfected with hOCT1 and hOCT2 were incubated at 37 °C for 2 min with 5 μM [^{14}C]TEA (pH 7.4) in the presence of (a and b); creatinine (open circle), guanidine (closed circle), guanidinosuccinic acid (open triangle), or methylguanidine (closed triangle), (c and d); acetyl arginine (open circle), creatine (closed circle), guanidinoacetic acid (open triangle), or guanidinovaleric acid (closed triangle), (e and f); butylguanidine (open circle), dimethylguanidine (closed circle), propylguanidine (open triangle), or tetramethylguanidine (closed triangle), (g and h); aminoguanidine (open circle), or phenylguanidine (closed circle). Each point represents the mean ± S.E. of three independent experiments.

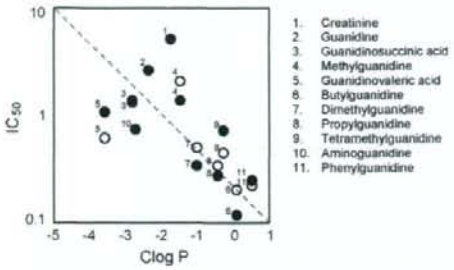

Fig. 3. Relationship between the calculated hydrophobicity (*C* log *P*) of guanidine compounds and IC_{50} values for inhibition of [^{14}C]TEA uptake by hOCT1 and hOCT2. The relationship between the calculated hydrophobicity (*C* log *P*) of guanidine compounds and the measured IC_{50} values for inhibition of [^{14}C]TEA uptake in hOCT1-HEK293 (open circle) and hOCT2-HEK293 (closed circle) cells. See experimental conditions in the legend of Fig. 2. The apparent IC_{50} values were calculated from inhibition plots (Fig. 2) by nonlinear regression analysis as described in Section 2. Octanol/water partition coefficients (log *P*) were calculated using Chem Draw Ultra 7.0 software.

(a typical substrate for the organic cation transporter) were examined (Table 1, Fig. 2). The inhibitory effects of guanidinosuccinic acid and methylguanidine were comparable between hOCT1 and hOCT2. Guanidine and creatinine had stronger inhibitory effects on [^{14}C]TEA uptake by hOCT2 than by hOCT1, whereas guanidinovaleric acid inhibited hOCT1 more than hOCT2. Guanidinoacetic acid tended to inhibit the uptake of [^{14}C]TEA by both hOCT1 and hOCT2, while creatine tended only to inhibit the hOCT2. Acetyl arginine did not inhibit [^{14}C]TEA uptake by either transporters. The inhibition curves of alkyl guanidine compounds for [^{14}C]TEA uptake showed that alkyl guanidine compounds had potent inhibitory effects on both hOCT1 and hOCT2, and that only butylguanidine had moderately higher affinity for hOCT2 than hOCT1. Phenylguanidine had an inhibitory effect on [^{14}C]TEA uptake by both hOCT1 and hOCT2, while aminoguanidine had a much greater inhibitory effect on hOCT2 than hOCT1. Fig. 3 plots the relationship between the log of the measured IC_{50} values (Table 1) and the calculated log *P* values (*C* log *P*) of guanidine compounds.

3.2. *Trans*-stimulation effects of guanidine compounds on TEA uptake by hOCT1 and hOCT2

To examine whether these guanidine compounds were substrates of hOCT1 and hOCT2, *trans*-stimulation experiments were performed. The transfectants were preincubated with a concentration equivalent to approximately 3-fold the IC_{50} value of the unlabeled guanidine compounds, or else with 10 mM if the IC_{50} value was not available (Table 1) [5]. Then, the [^{14}C]TEA uptake by the preincubated transfectants was measured. Fig. 4 shows the

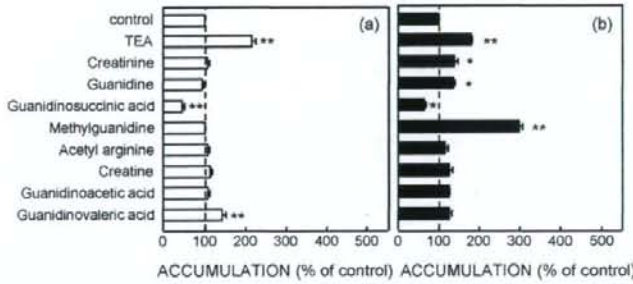

Fig. 4. *Trans*-stimulation effects of guanidine compounds as uremic toxins on [^{14}C]TEA uptake by hOCT1 (a) and hOCT2 (b). HEK-hOCT1 and HEK-hOCT2 cells were incubated for 2 min at 37 °C with 5 μM [^{14}C]TEA after preincubation with incubation medium (control) or incubation medium containing TEA (5 mM), creatinine (10 mM, hOCT1; 20 mM, hOCT2), guanidine (10 mM, hOCT1; 9 mM, hOCT2), guanidinosuccinic acid (5 mM, hOCT1; 4 mM, hOCT2), methylguanidine (7 mM hOCT1; 5 mM, hOCT2), acetyl arginine (10 mM), creatine (10 mM), guanidinoacetic acid (10 mM), and guanidinovaleric acid (2 mM, hOCT1; 4 mM, hOCT2) for 30 min at 37 °C, respectively. Data are expressed as a percentage of the control value. Control values for HEK-hOCT1 and HEK-hOCT2 were 25.6 ± 1.4 and 14.5 ± 1.2 pmol/mg protein/2 min, respectively. Each column represents the mean ± S.E. of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from the control.

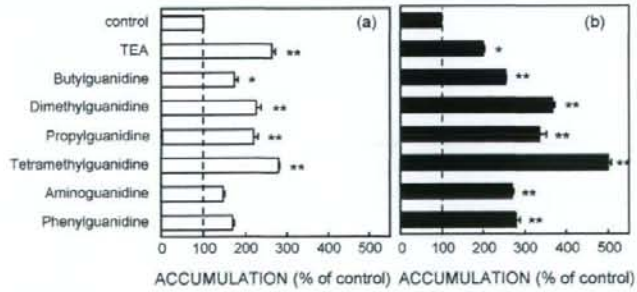

Fig. 5. *Trans*-stimulation effects of alkyl guanidine compounds, aminoguanidine and phenylguanidine on [^{14}C]TEA uptake by hOCT1 (a) and hOCT2 (b). HEK-hOCT1 and HEK-hOCT2 cells were incubated for 2 min at 37 °C with 5 μM [^{14}C]TEA after preincubation with incubation medium (control) or incubation medium containing TEA (5 mM), butylguanidine (0.6 mM, hOCT1; 0.4 mM, hOCT2), dimethylguanidine (2 mM hOCT1; 1 mM, hOCT2), propylguanidine (1 mM, hOCT1; 0.9 mM, hOCT2), tetramethylguanidine (1 mM, hOCT1; 2 mM, hOCT2), aminoguanidine (10 mM, hOCT1; 2 mM hOCT2) and phenylguanidine (0.7 mM, hOCT1; 0.8 mM, hOCT2) for 30 min at 37 °C, respectively. Data are expressed as a percentage of the control value. Control values for HEK-hOCT1 and HEK-hOCT2 were 25.9 ± 1.4 and 11.0 ± 0.2 pmol/mg protein/2 min, respectively. Each column represents the mean ± S.E. of three independent experiments. $^{*}P < 0.05$. $^{**}P < 0.01$, significantly different from the control.

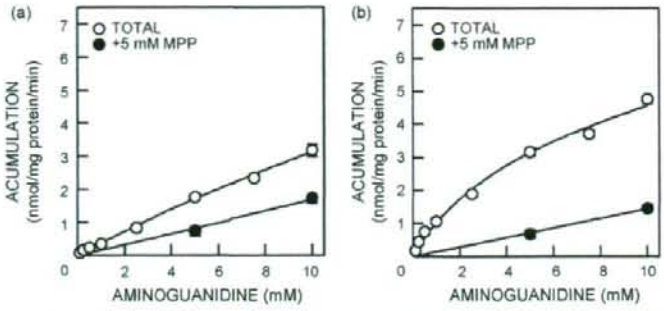

Fig. 6. Concentration dependence of [^{14}C]aminoguanidine transport by hOCT1 (a) and hOCT2 (b). hOCT1 and hOCT2 transfectants were incubated at 37 °C for 2 min with various concentrations of [^{14}C]aminoguanidine (0.1, 0.25, 0.5, 1, 2.5, 5, 7.5 and 10 mM) in the absence (open circle) or presence (closed circle) of 5 mM 1-methyl-4-phenylpyridinium (pH 7.4). Each point represents the mean ± S.E. of three independent experiments.

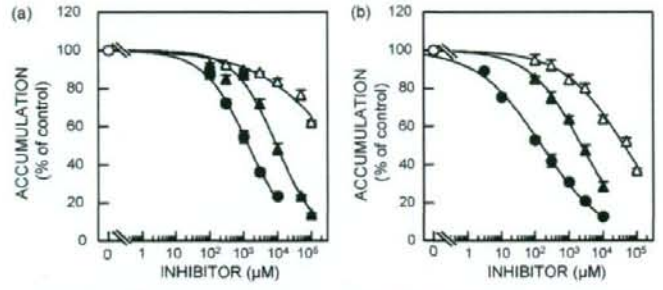

Fig. 7. Effects of TEA, creatinine and metformin on [^{14}C]aminoguanidine transport by hOCT1 (a) and hOCT2 (b). HEK 293 cells transfected with hOCT1 and hOCT2 were incubated at 37 °C for 2 min with 10 μM [^{14}C]aminoguanidine (pH 7.4) in the absence (open circle) or presence of TEA (closed circle), creatinine (open triangle), or metformin (closed triangle). Each point represents the mean ± S.E. of three independent experiments.

trans-stimulation effects of endogenous guanidine compounds as uremic toxin. Preincubation with unlabeled guanidine, methylguanidine, and creatinine significantly increased the uptake of [^{14}C]TEA by hOCT2 but not hOCT1. Meanwhile, preincubation with guanidinovaleric acid increased the [^{14}C]TEA uptake by hOCT1 but not hOCT2. As shown in Fig. 5, we examined the *trans*-stimulation effects of the other guanidine compounds. Preincubation with unlabeled butylguanidine, propylguanidine, dimethylguanidine, and tetramethylguanidine increased the uptake of [^{14}C]TEA by both transfectants. On the other hand, the preincubation with aminoguanidine and phenylguanidine significantly enhanced the [^{14}C]TEA uptake by hOCT2 but not hOCT1.

3.3. Uptake of aminoguanidine by hOCT1 and hOCT2

To obtain more information about the substrate specificity of hOCT2, the transport characteristics of [^{14}C]aminoguanidine was compared between hOCT1 and hOCT2. Fig. 6 shows the concentration dependence of [^{14}C]aminoguanidine uptake by hOCT1 and hOCT2. The uptake by hOCT2 was greater than that by hOCT1. The uptake was saturated at high concentrations in hOCT2-expressing cells, although no such saturation was observed in hOCT1-expressing cells. The apparent Michaelis–Menten constant (K_m) for the uptake of [^{14}C]aminoguanidine by hOCT2 was 4.10 ± 0.35 mM. The maximal uptake rate (V_{max}) in hOCT2-expressing cells was 4.40 ± 0.42 nmol/mg protein/min (mean ± S.E. of three separate experiments). Next, we examined the inhibitory effects of TEA, creatinine and metformin on the uptake of [^{14}C]aminoguanidine by hOCT1 and hOCT2 (Fig. 7). Fig. 7a and b shows the inhibition curves of TEA, creatinine and metformin in hOCT1- and hOCT2-expressing cells, respectively. Although TEA, creatinine and metformin inhibited the uptake of [^{14}C]aminoguanidine by both hOCT1 and hOCT2 in a dose-dependent manner, the uptake by hOCT2 was more inhibited. We calculated the IC_{50} values of these cationic compounds from the inhibition plots as described in Section 2 (Table 2). We also examined the inhibitory effect of aminoguanidine on the uptake of [^{14}C]metformin (Fig. 8). Aminoguanidine had little impact on the uptake of [^{14}C]metformin by hOCT1, and the IC_{50} value of aminoguanidine was not estimated (Fig. 8a). However, aminoguanidine inhibited the [^{14}C]metformin uptake by hOCT2 with the IC_{50} of 1.49 ± 0.14 mM (Fig. 8b).

To confirm aminoguanidine as a new substrate selective for hOCT2, the influence of *cis*-inhibition and *trans*-stimulation of aminoguanidine on the [^{3}H]MPP transport by hOCT3 was

Table 2
The apparent IC_{50} values of cationic compounds for [^{14}C]aminoguanidine uptake by hOCT1 and hOCT2.

Inhibitors	IC_{50} values for [^{14}C]aminoguanidine uptake (mM)	
	hOCT1	hOCT2
TEA	1.39 ± 0.06	0.16 ± 0.02
Creatinine	N/A	42.4 ± 2.6
Metformine	9.48 ± 0.56	2.37 ± 0.20

See experimental conditions in the legend of Fig. 7. The apparent IC_{50} values were calculated from inhibition plots (Fig. 7) by nonlinear regression analysis as described in Section 2. The data represent the mean ± S.E. of three independent experiments. N/A, not available.

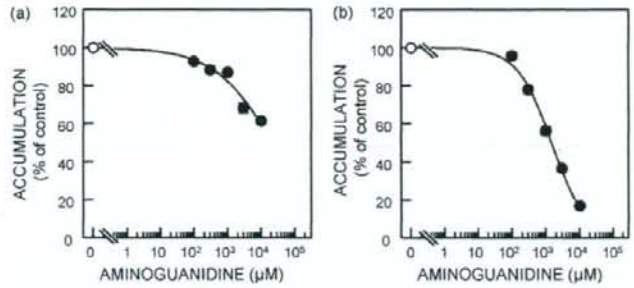

Fig. 8. Effects of aminoguanidine on [^{14}C]metformin transport by hOCT1 (a) and hOCT2 (b). HEK 293 cells transfected with hOCT1 and hOCT2 were incubated at 37 °C for 2 min with 10 μM [^{14}C]metformin (pH 7.4) in the absence (open circle) or presence of aminoguanidine (closed circle). Each point represents the mean ± S.E. of three independent experiments.

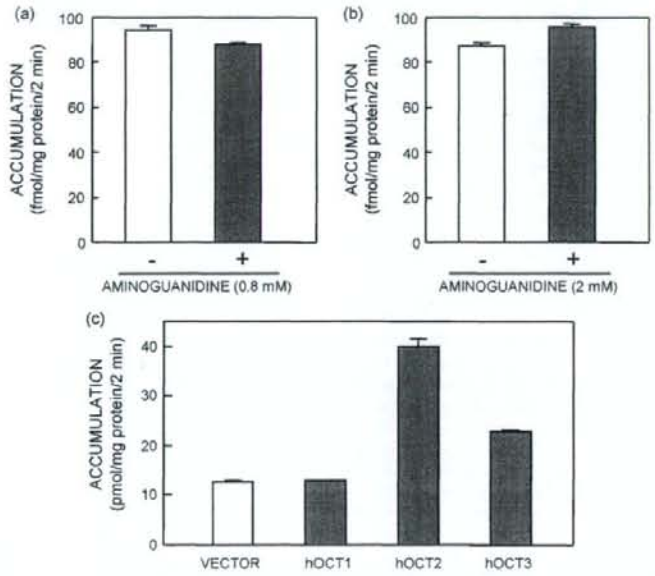

Fig. 9. Influence of *cis*-inhibition (a) and *trans*-stimulation (b) of aminoguanidine on the [^{3}H]MPP transport by hOCT3, and [^{14}C]aminoguanidine transport by hOCT1, hOCT2 and hOCT3 (c). (a) HEK293 cells transiently expressing hOCT3 were incubated at 37 °C for 2 min with 13.7 nM [^{3}C]MPP (pH 7.4) in the absence (−) or presence (+) of aminoguanidine (0.8 mM). (b) HEK293 cells transiently expressing hOCT3 were incubated for 2 min at 37 °C with 13.7 nM [^{3}H]MPP after preincubation with incubation medium (−) or incubation medium containing aminoguanidine (2 mM) (+) for 30 min at 37 °C. (c) HEK293 cells transfected with empty vector, hOCT1, hOCT2 or hOCT3 were incubated for 2 min at 37 °C with 5 μM [^{14}C]aminoguanidine (pH 7.4). Each column represents the mean ± S.E. of three monolayers.

examined in comparison with hOCT2 (Fig. 9a and b). The hOCT3-mediated uptake of [^{3}H]MPP was little affected by aminoguanidine in both conditions of *cis*-inhibition and *trans*-stimulation. In addition, the transport of [^{14}C]aminoguanidine by hOCT2 was the highest among three OCT isoforms (Fig. 9c).

4. Discussion

Previous reports suggested that guanidine and creatinine, which had a guadinino group, were predominantly transported by OCT2 rather than OCT1 [6,7]. We tested the hypothesis that the guanidino group was a decisive factor in being recognized by hOCT2, but could not found such selectivity simply by this group. At the same time, we discovered that aminoguanidine was a new superior substrate for hOCT2 than hOCT1.

Several guanidine compounds were reported to accumulate in blood with renal insufficiency, some being described as uremic toxins [9–14]. Guanidinosuccinic acid and methylguanidine had the two highest scores for the uremic concentration (C_U)/normal concentration (C_N) ratio, and there were also significant differences between the C_U and C_N of guanidine and creatinine [14]. The plasma concentrations of many cationic drugs increase with renal failure. It has been considered that the tubular secretion of organic cations is impaired and the elevated plasma level of alpha1 acid glycoprotein prevents the renal excretion in renal failure [21–25]. Based on the present results, it is also possible that the uremic guanidine compounds inhibit the excretion of cationic drugs mediated by hOCT.

Fig. 3 shows the relationship between the inhibitory patterns and the *C log P* values of guanidine compounds. In guanidine

compounds, hydrophobicity was not the major factor in determining the affinity for hOCT as it was, for example, in n-tetraalkylammonium [5,26,27].

In the *trans*-stimulation study, we showed that the [^{14}C]TEA uptake by hOCT2, but not hOCT1, were increased by preincubation with unlabeled guanidine, methylguanidine, creatinine, aminoguanidine and phenylguanidine. Possibly, these compounds are transported by hOCT2 and the dysfunction of hOCT2 with renal failure decreases the excretion of guanidine, methylguanidine, and creatinine as uremic toxins.

Because the three uremic guanidine compounds, guanidinosuccinic acid, methylguanidine and guanidinovaleric acid inhibited [^{14}C]TEA uptake by hOCT1 as well as hOCT2, the pharmacokinetics of the cationic drugs may be affected in the patients with renal failure. The [^{14}C]TEA uptake by hOCT1 was *trans*-stimulated by guanidinovaleric acid, suggesting the hOCT1-mediated transport of guanidinovaleric acid compensating the impaired renal function. It might relate to the fact that the serum level of guanidinovaleric acid in the patients with renal insufficiency was similar to normal values [10,13].

Among 14 guanidine compounds, aminoguanidine was found to be a selective substrate for hOCT2 compared to hOCT1 and hOCT3. A guanidine compound agmatine (1-amino-4-guanidobutane) was reported as a substrate for hOCT2 and hOCT3, but not for hOCT1 [28], while guanidine was transported by rOCT2, but not by rOCT1 and hOCT3 [6]. Therefore, aminoguanidine as well as agmatin and guanidine can be a good probe to examine the transport activity of hOCT2 in comparison with hOCT1 and hOCT3.

The apparent affinity of aminoguanidine for hOCT2 was similar to that of creatinine (K_m = 4.0 mM) [7] and lower than that of metformin (K_m = 1.4 mM) [19]. Aminoguanidine, which inhibits many diabetes-related complications, remains under therapeutic testing [16,17,29]. Because aminoguanidine was excreted into urine by tubular secretion as well as glomerular filtration and hOCT2 was the most abundant organic cation transporter in the basolateral membranes of human kidney [3,15], the secretion of aminoguanidine may be predominantly mediated by hOCT2.

In ACTION I trial (*A Clinical Trial In Overt Nephropathy of Type 1* Diabetics), which included patients with type 1 diabetes mellitus [17], aminoguanidine reduced significantly secondary measures of outcome such as proteinuria and had additional effects on diabetic retinopathy and circulating lipid levels. However, the reduction in the primary end point of time to doubling of the serum creatinine concentration was not statistically significant. Although creatinine clearance is often used for the estimation of GFR, creatinine is also excreted via tubular secretion mediated by hOCT2 [7,30,31]. Aminoguanidine might inhibit the transport of creatinine by hOCT2 and increase the serum concentration of creatinine without inducing renal impairment. Therefore, the other parameters whose elimination was unaffected by aminoguanidine should have been used.

Although creatinine and metformin were also excreted into urine through transport by hOCT2 [7,19], their IC_{50} values for aminoguanidine uptake by hOCT2 (creatinine, 42.4 mM; metformin, 2.37 mM) were much higher than the physiological concentrations of creatinine (about 45–85 μM for male and 30–60 μM for female) and metformin (about 15–25 μM) (Table 2, Fig. 7) [24,32–34]. Therefore, the transport of aminoguanidine mediated by hOCT2 is not likely to be affected by creatinine and metformin, and diabetic patients whose plasma creatinine concentrations are increased or who use metformin may be able to use aminoguanidine safely. It was reported that the maximum aminoguanidine concentration was only 40 μM, during the interdialytic period [15]. It is also probable that aminoguanidine has little effect on the transport of metformin mediated by hOCT2, at the physiological concentrations (Fig. 8).

In this study, we demonstrated that many guanidine compounds examined had relatively equal affinity to hOCT1 and hOCT2 and could not found the selectivity for hOCT2 simply by guanidino group. Among guanidine compounds, we newly discovered that aminoguanidine had greater affinity for hOCT2 than hOCT1, in addition to guanidine and creatinine. Therefore hOCT2 could function as a transporter for aminoguanidine at the basolateral membranes of renal proximal tubules. These findings will be helpful to elucidate the specificity of hOCT2, and clarify the pharmacokinetics of aminoguanidine.

Acknowledgements

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ORIGINAL ARTICLE

Identification of multidrug and toxin extrusion (MATE1 and MATE2-K) variants with complete loss of transport activity

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H⁺/organic cation antiporters (multidrug and toxin extrusion: MATE1 and MATE2-K) play important roles in the renal tubular secretion of cationic drugs. We have recently identified a regulatory single nucleotide polymorphism (SNP) of the *MATE1* gene (−32G>A). There is no other information about SNPs of the *MATE* gene. In this study, we evaluated the functional significance of genetic polymorphisms in *MATE1* and *MATE2-K*. We sequenced all exons of *MATE1* and *MATE2-K* genes in 89 Japanese subjects and identified coding SNPs (cSNPs) encoding MATE1 (V10L, G64D, A310V, D328A and N474S) and MATE2-K (K64N and G211V). All the variants except for MATE1 V10L showed significant decrease in transport activity. In particular, MATE1 G64D and MATE2-K G211V variants completely lost transport activities. When membrane expression level was evaluated by cell surface biotinylation, those of MATE1 (G64D and D328A) and MATE2-K (K64N and G211V) were significantly decreased compared with that of wild type. These findings suggested that the loss of transport activities of the MATE1 G64D and MATE2-K G211V variants were due to the alteration of protein expression in cell surface membranes. This is the first demonstration of functional impairment of the MATE family induced by cSNPs.
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Keywords: cSNP; H⁺/organic cation antiporter; MATE1; MATE2-K; pharmacogenomics; polymorphism; transporter

INTRODUCTION

In the proximal tubules of the 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.[1–4] Among human organic ion transporters located at the basolateral membranes, organic cation transporter 2 (OCT2), organic anion transporter 1 (OAT1) and OAT3 were isolated a decade ago, and have been well characterized as key transporters to regulate the renal handling of ionic drugs.[4,5] In contrast, the molecular functions of apical transporters have been only recently characterized. For example, multidrug resistance-associated protein 4 (MRP4) was demonstrated to be responsible for the renal elimination of antiviral drugs,[6] diuretics[7] and cephalosporin antibiotics.[8] Human orthologs of the multidrug and toxin extrusion (MATE) family, members of which confer multidrug resistance on bacteria, were identified most recently,[9,10] and named MATE1 (SLC47A1) and MATE2-K (SLC47A2). Both transporters are expressed mainly in the renal brush border membranes, and are able to transport tetraethylammonium (TEA) utilizing an oppositely directed H^+ gradient as a driving force,[11] indicating that MATE1 and MATE2-K are H⁺/organic cation antiporters. These findings have improved the molecular understanding of the transcellular transport of ionic drugs in the renal tubules.

It is widely recognized that there is a large variation in the responses to drugs among individuals. Many enzymes involved in drug metabolism, such as cytochrome P450 and uridine diphosphate-glucuronosyltransferase are known to be polymorphic and have been associated with variations in blood concentrations of drugs.[12] In addition to drug-metabolizing enzymes, the clinical significance of genetic variation of drug transporters has been demonstrated.[13] For example, polymorphisms of *SLCO1B1*, which encodes the organic anion transporting polypeptide 1B1 to mediate the hepatic uptake of pravastatin, contribute to the interindividual variability in the disposition of pravastatin.[14] Recent studies of OCT have demonstrated that polymorphisms of the *OCT1* gene in Caucasians and the renal *OCT2* gene in Koreans are responsible for the interindividual differences in the therapeutic efficacy and pharmacokinetics of metformin, an anti-diabetic agent.[15–17]

Metformin showed large interindividual variation in renal clearance, and a potential genetic contribution by the renal transporter was speculated.[18] Because metformin is also a superior substrate for

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MATE1 and MATE2-K,[10,19] polymorphisms of *MATE1* and *MATE2-K* genes may be involved in the interindividual difference in the renal clearance. We have recently identified a single nucleotide polymorphism (SNP) in the promoter region of *MATE1* (−32G>A), which causes a decrease in Sp1 binding and promoter activity of approximately 50%.[20] However, other genetic information for these transporters, especially the polymorphisms in the coding region, and their effect on functional properties, have not been well evaluated. In this study, therefore, we screened for polymorphisms in all exons of *MATE1* and *MATE2-K* genes, and examined their transport activities by *in vitro* transient expression system.

MATERIALS AND METHODS

Materials

[^{14}C]TEA bromide (2.035 GBq mmol^{-1}) and [^{14}C]metformin (1.998 GBq mmol^{-1}) were obtained from American Radiolabeled Chemicals Inc. (St Louis, MO, USA) and Moravek Biochemicals Inc. (Brea, CA, USA), respectively. All other chemicals used were of the highest purity available.

Identification of SNPs of *MATE1* and *MATE2-K* genes

Genomic DNA was isolated from peripheral blood from 89 Japanese subjects with renal diseases using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). Genotyping was investigated by direct sequencing. PCR primers were designed to span all 17 exons of *MATE1* and *MATE2-K* (GenBank accession number NT_010718) (Table 1). The PCR conditions were 94 °C for 3 min, followed by 40 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, and then a final extension at 72 °C for 10 min, except for MATE1 exon 1. The condition for MATE1 exon 1 was 94 °C for 1 min, followed by 35 cycles of 94 °C for 30 s and 68 °C for 3 min, and then a final extension at 68 °C for 3 min. The PCR products were sequenced using a multicapillary DNA sequencer RISA384 system (Shimadzu, Kyoto, Japan). This study was conducted in accordance with the Declaration of Helsinki and its amendments and was approved by the Ethics Committee of Kyoto University Graduate School and Faculty of Medicine. All subjects gave their written informed consents.

Construction of non-synonymous variants of MATE1 and MATE2-K

MATE1 and MATE2-K cDNA were excised from MATE1/pcDNA3.1 and MATE2-K/pcDNA3.1,[10] and were subcloned into pcDNA3.1/nV5-DEST (Invitrogen, Carsbad, CA, USA) to yield nV5-MATE1 and nV5-MATE2-K. Non-synonymous variants were constructed by the site-directed mutagenesis of nV5-MATE1 and nV5-MATE2-K, using a QuikChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) with the primers listed in Table 2. The nucleotide sequences of these constructs were confirmed using a multicapillary DNA sequencer RISA384 system (Shimadzu).

Transport studies

HEK293 cells (ATCC CRL-1573; American Type Culture Collection) were cultured in complete medium consisting of Dulbecco's modified Eagle's medium (Sigma Chemical Co., St Louis, MO, USA) with 10% fetal bovine serum (Invitrogen) in an atmosphere of 5% CO_2 and 95% air at 37 °C. cDNA plasmid transfection (Figure 2: 25 ng; Figure 3: 100 ng; Figure 6: 100 ng for MATE1 and 200 ng for MATE2-K) and cellular uptake of [^{14}C]TEA and [^{14}C]metformin were reported earlier.[11,19,21,22]

Table 1 Primers used for direct sequencing

Gene	*Location*	*Forward primer (5′ to 3′)*	*Reverse primer (5′ to 3′)*	*Amplified length (bp)*
MATE1	Exon 1	CGCAGTGGTGCAGAGAGAGGTGCAA	AGTCACCCGCGGAGGCAGAAATCAC	451
	Exon 2	AAGGTGGCAGAGGCTCACTGAAGTT	TCTGTGTAGGTTTCAGCCACTACAT	339
	Exon 3	TGAAGGAGGAGCTTTGCAGGCTCTT	CCTGCCAGTGGAGCTCTTCCATCTA	248
	Exon 4	CTTTGTGTGGCACAATTGAAGGCTT	CACCCAGACAGGATAATCTTTCCGT	303
	Exon 5	CTTCTGCCTAACTTTCCCTGGAAAC	CTGAGCTCACAGATATGGTGGCTAC	192
	Exon 6	CTGCCGTGTGACCTCACTTCTGTGT	GGTCCCTGGTCCTGGAGTATCTTCA	208
	Exon 7	GCCTGTGTGTGCTTGGGTAGCAGAA	CGCATGGACACAAGAACCAGCTGAA	279
	Exon 8, 9, 10	ATGAGTCTCCCCTCCTCACTGAGTT	TGCCTGTGCTCATCCATAGACTCTT	633
	Exon 11, 12	ATGAGGCTGCTTCTCTGCACGTGTT	CAGCAATGTTTCTGAACAGCCTGAT	481
	Exon 13	CCACTGCGCCTAGCCAGAAAGCTAT	CCCTCCTCTCAGCTGAAATTTACCA	224
	Exon 14	CTCGGGAGATGGGAGTGTTTCAAGA	AAGACCCGTGTGCTCCGACGGTCAT	276
	Exon 15	CTCCACCTCAGCCATGAAAGCAGAT	AGGGAGAGCCAGATCAGATCCTGTT	289
	Exon 16	TGGCTTGGCTCTTCCTAAACTAGGT	TAGCAGCAAATCTAGCTGTGTCTCA	258
	Exon 17	CTCTCCACTATTAGCACATATTCCTT	ATCCATGGGCACACCTGAATGACAT	436
MATE2-K	Exon 1	CTCATCCCACAAGTTGCCATGGTAG	GCACATTTCTGGATCCTGCCTGCAA	369
	Exon 2	CCTCAAAGCTGGAGAGGCCTGTCTT	GGCTGTGTCTTCCCATCCCTGACCA	297
	Exon 3	GGCACACAGCACATGAGGCTGCTGA	TGCCATCTCCATGGCACCTGTGGAA	292
	Exon 4	TCAGGAAGGCCGCTGTGCCATTACA	TGAGGGCTGGGCATCTTCAGGGTTT	400
	Exon 5	GAGGTTTCACAGTCCTGGCTGAGAC	AGGGATCTTCCGCAGCAGATAGAGT	262
	Exon 6	CAATCTGGGGTACTATGTCCTGGAA	GCTGGTTCACAGATGGTGGAGAGAA	252
	Exon 7	CCTTCTCTCCACCATCTGTGAACCA	CAGGATGGTGACTGATCTGTCTCCA	422
	Exon 8	CCCTGGTTGAGTCTGATCCCAGGAT	TCCAACAGGCTCTACTGCACCCTCT	351
	Exon 9	AATGCCCAGTGCCTGAGCCTGCTAA	TGAGGGCCTGGCCAGTGAAGCTGGAA	403
	Exon 10	TCCCCAAAGCAAAGCAGCGTCCTGT	GGGAGACAGAGATAGCTTCAGGTGA	254
	Exon 11	CTCTTACACTGCATGCCTGAGATCT	TCACAGCAGCAGGGAAGGAGTGAGT	488
	Exon 12	GGCTGGGCTGACTTGCACTGACATA	CCCAGCACTGAGCCAGGAATGTGAT	275
	Exon 13	CTCTGGGCTAGCAGTGCCAGTTACA	CAAGTTCATCCTCACAGCCCTGCGA	317
	Exon 14, 15	TGCCATGCGAATGGCTTAGCACAGT	CTGGGCATTTCTGGCTGAGTAGTCA	483
	Exon 16	CAGTGAAGGGGTGAACTGTTGAGCT	CACAGAGGGCAGACAAGAGCAACAT	225
	Exon 17	CACAGCCAGGTGGTTAACCTAGGTT	ACCTGCACTAGACCCCATTGGTGTT	416

Table 2 Primers used for site-directed mutagenesis

Gene	*Name*	*Direction*	*Sequence (5'-3')*	*Position*
			Primers for the site-directed mutagenesis	
MATE1	V10L	F	GGAGCCCGCGCCA**T**TGCGCGGAGGCC	15/40
		R	GGCCTCCGCGCA**A**TGGCGCGGGCTCC	40/15
	G64D	F	CCGTGTTCTGTG**A**CCACCTGGGCAAGCTGG	179/208
		R	CCAGCTTGCCCAGGTGG**T**CACAGAACACGG	208/179
	A310V	F	CATGGTCCCTG**T**AGGCTTCAGTGTGGCTGCC	918/948
		R	GGCAGCCACACTGAAGCCT**A**CAGGGACCATG	948/918
	D328A	F	CGCTCTGGGTGCTGGAG**C**CATGGAGCAGG	966/994
		R	CCTGCTCCATG**G**CTCCAGCACCCAGAGCG	994/966
	N474S	F	GGCTCAGGTACACGCCA**G**TTTGAAAGTAAACAACGTGCC	1404/1442
		R	GGCACGTTGTTTACTTTCAAA**C**TGGCGTGTACCTGAGCC	1442/1404
MATE2-K	K64N	F	GGCACCTGGGCAA**T**GTGGAGCTGGC	179/203
		R	GCCAGCTCCAC**A**TTGCCCAGGTGCC	203/179
	G211V	F	GGGGGTCAGGG**T**CTCCGCCTATGCC	621/645
		R	GGCATAGGCGGAG**A**CCCTGACCCCC	645/621

Abbreviations: F, forward; R, reverse.
Mutations introduced into the oligonucleotides are shown in bold.

Table 3 cSNPs of the *MATE1* and *MATE2-K* in 89 Japanese subjects

Location	*SNP*	*dbSNP (NCBI)*	*Effects*	*Allelic frequency (%)*	*Genotype (n)*
MATE1					
Exon1	28G>T	ss104806851	V10L	2.2	G/G 85, G/T 4, T/T 0
Exon1	33C>T	ss104806852	R11R	0.6	C/C 88, C/T 1, T/T 0
Exon1	126T>C	ss104806853	A42A	0.6	T/T 88, T/C 1, C/C 0
Exon2	191G>A	ss104806854	G64D	0.6	G/G 88, G/A 1, A/A 0
Exon8	708C>T	ss104806855	L236L	9.6	C/C 74, C/T 13, T/T 2
Exon11	929C>T	ss104806856	A310V	2.2	C/C 85, C/T 4, T/T 0
Exon11	983A>C	ss104806857	D328A	0.6	A/A 88, A/C 1, C/C 0
Exon16	1421A>G	ss104806858	N474S	0.6	A/A 88, A/G 1, G/G 0
MATE2-K					
Exon2	192G>T	ss104806859	K64N	0.6	G/G 88, G/T 1, T/T 0
Exon2	207G>A	ss104806860	S69S	5.6	G/G 79, G/A 10, A/A 0
Exon4	345C>A	ss104806861	G115G	36.5	C/C 37, C/A 39, A/A 13
Exon8	632_633GC>TT	ss104806862	G211V	1.7	GC/GC 86, GC/TT 3, TT/TT 0
Exon10	885C>T	ss104806863	Y295Y	48.9	C/C 25, C/T 41, T/T 23

Abbreviations: cSNP, coding single nucleotide polymorphism; MATE, multidrug and toxin extrusion.

Cell surface biotinylation

Cell surface biotinylation was performed according to our earlier methods[22] with some modifications. HEK293 cells were grown on poly-D-lysine-coated 12-well plates and transfected with MATE1 or MATE2-K cDNA plasmids (50 ng for MATE1 and 200 ng for MATE2-K). At 48 h after the transfection, cells were washed three times with 1 ml ice-cold phosphate-buffered saline with Ca and Mg (138 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 9.6 mM Na_2HPO_4, 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$, pH 7.3) and then treated with 400 µl of membrane-impermeable biotinylating agent, sulfo-NHS-SS-biotin (Pierce, Rockford, IL, USA) (1.5 mg ml^{-1}) at 4 °C for 1 h. Subsequently, the cells were washed three times with 1 ml ice-cold phosphate-buffered saline with Ca and Mg containing 100 mM glycine and then incubated for 20 min at 4 °C with the same buffer to remove the remaining labeling agent. After being washed with phosphate-buffered saline with Ca and Mg, cells were disrupted with 400 µl of lysis buffer (10 mM Tris-base, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100 and 1% protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan), pH 7.4) at 4 °C for 1 h with constant agitation. Following centrifugation, 50 µl of streptavidin agarose beads (Pierce) was added to 300 µl of cell lysate and incubated for 1 h at room temperature to isolate the biotinylated membrane proteins.

Western blot analysis and quantification of band density

Isolated biotinylated membrane proteins were subjected to western blot analysis according to NuPAGE manufacturer's instructions (Invitrogen). Monoclonal anti-V5 antibody (Invitrogen) (1:2500 dilution) or Na^+/K^+-ATPase antibody (1:10 000 dilution; Upstate Biotechnology, Lake Placid, NY, USA) was used as the primary antibody. A peroxidase-conjugated anti-mouse IgG antibody was used for the detection of bound antibodies, and the blots were visualized by chemiluminescence on X-ray film. Quantification of band density was performed on scanned images using ImageJ, a public domain image-processing program (W Rasband, National Institute of Mental Health, Bethesda, MD, USA). The optical density of each lane was plotted, and the area under the curve was measured.

Statistical analysis

Kinetic parameter data were statistically analyzed with unpaired *t*-test compared with the values for the wild type. The other experimental data were statistically analyzed with the one-way analysis of variance followed by Dunnett's test.

RESULTS

Identification of *MATE1* and *MATE2-K* SNPs

All 17 exons of the *MATE1* and *MATE2-K* genes were sequenced to find SNPs in 89 Japanese subjects. In this study, eight *MATE1* SNPs and five *MATE2-K* SNPs were identified in the coding region (Table 3). The allelic frequencies for the non-synonymous SNPs ranged from 0.6 to 2.2%. Figure 1 shows the position of mutated amino-acid residues in the predicted secondary structure of MATE1 (a) and MATE2-K (b), respectively. Only Ala310 was localized in the transmembrane domain (TMD), and other amino-acid residues are located at the intra- or extracellular domains.

Transport studies of the MATE1 and MATE2-K variants

To assess the functional alterations caused by the non-synonymous SNPs of both genes, [^{14}C]TEA transport activity by the variants was evaluated by *in vitro* transient expression system. As shown in Figure 2a, [^{14}C]TEA uptake by the MATE1 G64D variant was completely abolished. Other MATE1 variants except for the MATE1 V10L variant also showed a significant reduction in [^{14}C]TEA transport activity, and the order of the remaining transport activities were as follows: wild type=V10L>N474S>D328A=A310V. [^{14}C]Metformin uptake by various variants was similar to [^{14}C]TEA uptake (Figure 2b). Both the MATE2-K variants showed significant decrease in [^{14}C]TEA and [^{14}C]metformin uptake, and the transport activity of MATE2-K G211V was completely abolished (Figures 3a and b).

Cell surface expression levels of the MATE1 and MATE2-K variants

To determine whether the reduced transport activity of these variants was due to the decreased expression of transporter proteins in the plasma membranes, cell surface biotinylation followed by western blot analysis was carried out. Among the MATE1 variants, the cell surface expression level of MATE1 G64D and D328A showed a decrease to approximately 10 and 20% compared with that of the wild-type MATE1 (Figure 4), which are well correlated with the reduction ratios of the transport activity for these variants (Figures 2a and b). Other MATE1 variants exhibited similar cell surface expression level with wild-type MATE1. In the MATE2-K, both the MATE2-K K64N and MATE2-K G211V variants showed a decrease to approximately 50 and 1% compared with that of the wild-type MATE2-K, respectively (Figure 5). These reduction ratios were well correlated with those of transport activities of both the MATE2-K variants (Figures 3a and b). These findings suggested that the low transport activities of MATE1 G64D, D328A and two MATE2-K variants were due to the alteration of protein expression in cell surface membranes.

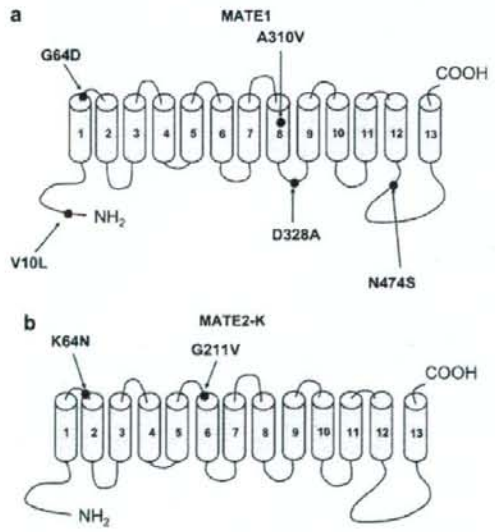

Figure 1 Locations of mutated amino-acid residues caused by non-synonymous single nucleotide polymorphisms (SNPs) in the secondary structure of multidrug and toxin extrusion 1 (MATE1) (**a**) or MATE2-K (**b**) protein. Amino-acid numbers are shown.

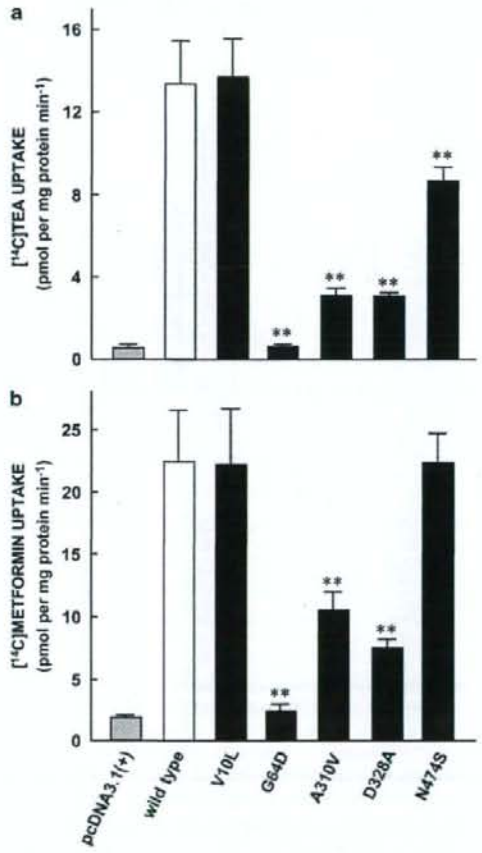

Figure 2 Uptake of [^{14}C]TEA (tetraethylammonium) (**a**) and [^{14}C]metformin (**b**) by HEK293 cells transiently expressing wild type or various multidrug and toxin extrusion 1 (MATE1) variants. The cells were preincubated with incubation medium (pH 7.4) in the presence of 30 mM ammonium chloride for 20 min. Then, the preincubation medium was removed, and the cells were incubated with 5 μM of [^{14}C]TEA or 10 μM of [^{14}C]metformin for 1 min at 37 °C. Each column represents the mean ± s.d. of six monolayers from two independent experiments. $^{**}P<0.01$, significantly different from the values for the wild type.

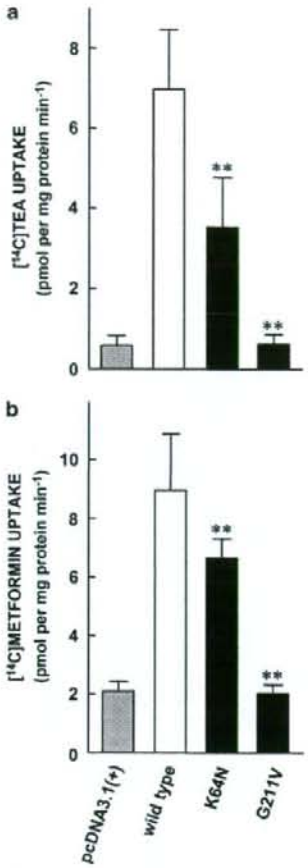

Figure 3 Uptake of [^{14}C]TEA (tetraethylammonium) (**a**) and [^{14}C]metformin (**b**) by HEK293 cells transiently expressing wild type or variants of multidrug and toxin extrusion 2-K (MATE2-K). The procedures are identical to those described in the legend of Figure 2. Each column represents the mean ± s.d. of six monolayers from two independent experiments. $^{**}P<0.01$, significantly different from the values for the wild type.

Comparison of functional characteristics between wild type and the variants of MATE1 and MATE2-K

To estimate kinetic parameters for [^{14}C]TEA uptake by several MATE1 and MATE2-K variants, concentration-dependent uptake was carried out (Figures 6a and b). The [^{14}C]TEA uptake by the MATE1 and MATE2-K variants exhibited saturable kinetics, following the Michaelis–Menten equation. The apparent maximal uptake velocity (V_{max}), Michaelis–Menten constant (K_m) and V_{max}/K_m values are summarized in Table 4. V_{max} values of MATE1 A310V, D328A and MATE2-K K64N were significantly decreased. K_m values of MATE1 A310V and N474S were significantly increased.

DISCUSSION

MATE1 and MATE2-K function as H^+/organic cation antiporters at the renal brush border membranes and play crucial roles in the renal handling of cationic drugs, such as cimetidine, metformin and oxaliplatin.[23] In this study, we identified five and two non-synonymous SNPs in their genes, all of which induced a reduction of transport activity except for MATE1 V10L. In MATE1, both the cell surface expression and transport activity of the G64D and D328A variants were significantly reduced to approximately 10 and 20% compared with that of the wild type. These findings suggest that reduced protein expression levels in the plasma membrane can account for the decrease in transport activity of MATE1 with G64D and D328A. Previously, we indicated that Cys63 of MATE1 plays an important role in substrate binding,[22] and Cys63 is the neighboring amino-acid residue of Gly64. Thus, regarding G64D, the change from the small side chain (Gly) to the bulkier and polar side chain (Asp) may inhibit the substrate binding and decrease transport activities, in addition to reduced protein expression levels in the plasma membrane.

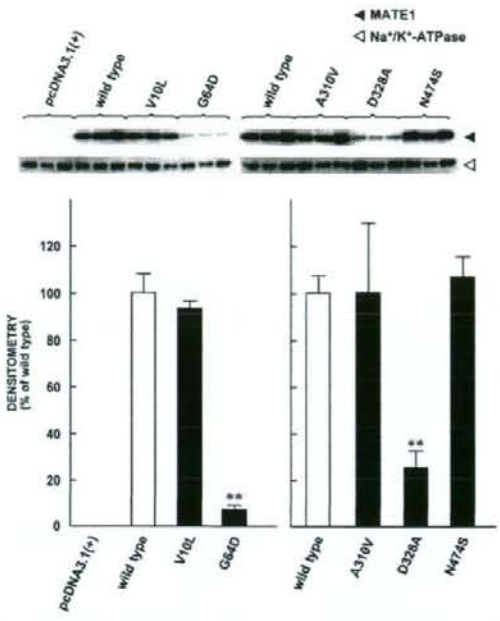

Figure 4 Western blot analysis of cell surface biotinylated proteins obtained from HEK293 cells transiently expressing wild type or variants of multidrug and toxin extrusion 1 (MATE1). Cell surface membrane fractions prepared by cell surface biotinylation were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (4–12%) and blotted onto polyvinylidene fluoride (PVDF) membranes. Each column represents the mean ± s.d. of three monolayers. $^{**}P<0.01$, significantly different from the values for the wild type.

On the other hand, N474S sustained a modest level of transport activity. The small impact of Asn474 on MATE1 function may be due to its position, in the intracellular loop between TMD12 and TMD13. Three-dimensional models of MATE1 will clarify the importance of these amino-acid residues.

In the MATE1 protein, Gly64 and Asn474 are conserved in the rat (AB248823), mouse (AAH31436) and rabbit (EF120627) orthologs, suggesting that these two amino-acid residues are essential. Ala310 of MATE1 is conserved in the rat ortholog only. The transport activity of A310V showed a decrease to approximately 20% compared with that of the wild type, though its membrane expression level was same as the wild type. Kinetic analysis indicated that K_m value for MATE1

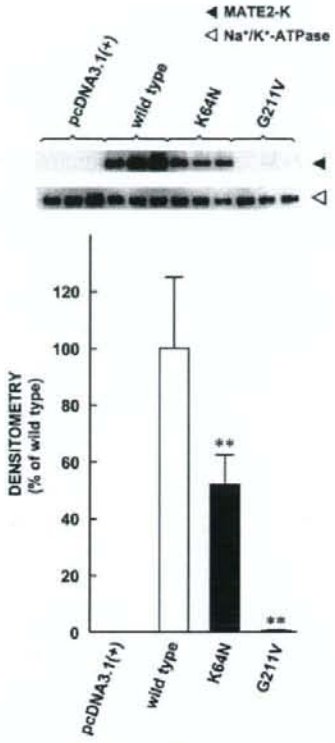

Figure 5 Western blot analysis of cell surface biotinylated proteins obtained from HEK293 cells transiently expressing wild type or variants of multidrug and toxin extrusion 2-K (MATE2-K). The procedures are identical to those described in the legend of Figure 4. Each column represents the mean ± s.d. of three monolayers. $^{**}P<0.01$, significantly different from the values for the wild type.

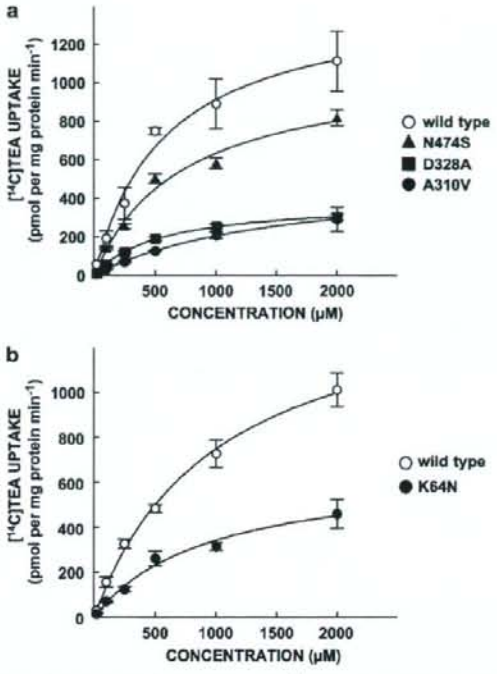

Figure 6 Concentration dependence of [^{14}C]TEA (tetraethylammonium) uptake by HEK293 cells transiently expressing wild type or variants of multidrug and toxin extrusion 1 (MATE1) (**a**) and MATE2-K (**b**). The figures show a specific uptake of [^{14}C]TEA obtained by subtracting the non-saturable components, which were estimated in the presence of 5 mM unlabeled compound. Each point represents the mean ± s.d. of three monolayers from a typical experiment.

A310V was significantly increased. These findings suggested that substitution of Ala for Val may inhibit the substrate binding or translocation because Ala310 was localized in the TMD.

In MATE2-K protein, Lys64 and Gly211 are conserved in the rabbit ortholog (EF121852). As for two MATE2-K SNPs, the alterations in transport activity were in accordance with the alterations in the cell surface expression of MATE2-K protein, indicating that the reduced function of MATE2-K K64N and MATE2-K G211V is mainly due to the decreased expression at the plasma membrane. Actually, it was demonstrated that V_{max} value of MATE2-K K64N was significantly decreased. These findings may provide important information to elucidate molecular mechanisms of membrane trafficking and stability of the MATE2-K protein in the plasma membrane.

There are several reports that investigated the cell surface expression level caused by coding SNPs (cSNPs) between HEK293 cells and tissues. For example, the human organic anion-transporting polypeptide 1B1 protein expression level was not changed by substitution of Leu643 to Phe in both transfected HEK293 cells and liver samples.[24] In this study, we examined the effect of cSNPs on cell surface expression level only *in vitro*. Our *in vitro* data are difficult to extrapolate to the case *in vivo* in renal proximal tubules; however, these data suggest that altered cell surface expression level may occur in individuals with MATE1 G64D, D328A and both the MATE2-K variants. Future studies on cell surface expression level of MATE1 and MATE2-K protein in human proximal tubules with these variants will elucidate whether *in vitro* data in this study are consistent with the case *in vivo*.

Metformin, a biguanide agent, is mainly excreted into the urine mostly through tubular secretion and shows large interindividual variation of renal clearance.[18] Recently, it has been demonstrated that SNPs of the hepatic *OCT1* gene in Caucasians and renal *OCT2* gene in Koreans are responsible for the interindividual differences in the therapeutic efficacy and pharmacokinetics of metformin.[15–17] On the other hand, Shikata *et al.*[25] reported that *OCT1* and *OCT2* polymorphisms contribute little to the clinical efficacy of metformin in Japanese. Previously, we demonstrated that metformin is a good substrate not only for OCT2 but also for MATE1 and MATE2-K.[19,26] Therefore, the SNPs of *MATE1* and *MATE2-K* genes identified in this study may be involved in the interindividual difference in the renal clearance of metformin in Japanese. However, as the allelic frequencies of *MATE1* and *MATE2-K* SNPs are not very high, these SNPs cannot fully account for the large interindividual variation in the renal clearance of metformin.

We reported that the kidney-specific expression of OCT2 is involved in the renal distribution and accumulation of the anticancer agent

Table 4 Kinetic parameters of [^{14}C]TEA uptake determined by HEK293 cells transiently expressing wild type or variants of MATE1 and MATE2-K

	$V_{max} \pm$ s.e. (*nmol per mg protein min^{-1}*)	$K_m \pm$ s.e. (*mM*)	$V_{max}/K_m \pm$ s.e. (*μl per mg protein min^{-1}*)
MATE1			
Wild type	1.94 ± 0.37	0.49 ± 0.05	4.10 ± 0.88
A310V	0.74 ± 0.17*	1.84 ± 0.38*	0.40 ± 0.03**
D328A	0.53 ± 0.06**	0.63 ± 0.04	0.84 ± 0.09*
N474S	1.36 ± 0.25	0.70 ± 0.06*	1.92 ± 0.20
MATE2-K			
Wild type	1.99 ± 0.44	1.39 ± 0.47	1.56 ± 0.14
K64N	0.73 ± 0.03*	0.79 ± 0.05	0.93 ± 0.08**

Abbreviations: MATE, multidrug and toxin extrusion; TEA, tetraethylammonium.
The values were calculated from four separate experiments performed in three monolayers.
*P<0.05, **P<0.01, significantly different from the values for the wild type.

cisplatin.[27,28] As there is little transport of cisplatin by MATE1 and MATE2-K, cisplatin is accumulated in the proximal tubular cells causing nephrotoxicity. A low-nephrotoxic platinum anticancer agent, oxaliplatin, was transported by OCT2 and MATE2-K,[27,28] suggesting that oxaliplatin does not accumulate in the renal proximal tubular cells. Therefore, loss of function of MATE2-K caused by cSNPs may lead to the accumulation of oxaliplatin in the kidney and the subsequent nephrotoxicity. Future study will be needed to clarify the clinical implications of the SNPs of both genes identified in this study.

In conclusion, five non-synonymous SNPs in the *MATE1* and two non-synonymous SNPs in the *MATE2-K* genes were identified in Japanese subjects for the first time. All of the mutated proteins except for MATE1 V10L showed a significant decrease in transport activity, and especially those of MATE1 G64D and MATE2-K G211V were completely abolished by the impairment of cell surface expression. These polymorphisms may affect the renal handling of various cationic drugs and cause drug-induced nephrotoxicity.

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≪AKI の診断についてのトピック≫

AKI 診断の新たな指標，今後の展開

市村隆治　　増田智先*

要旨

- 既存の腎機能マーカーを AKI の指標として用いるには限界があり，より迅速性，正確性および特異性を兼ね備えた新しいバイオマーカーの開発と臨床応用が望まれている．
- 新規 AKI 尿中バイオマーカーの開発，評価が現在進められており，有望な候補分子の同定もなされている．
- 既存の腎機能マーカーにこれらの新規尿中バイオマーカーを併用することによって，バイオマーカーそれぞれの特異性と，感度や精度の強化に加え，さらに新薬開発領域における前臨床試験への応用も期待される．

はじめに

acute kidney injury(AKI)は，48 時間以内の急激な血清クレアチニン値の上昇と定義される臨床状態で，外傷や腎臓の構造的・機能的な損傷に起因する．一方，血清クレアチニン濃度や blood urea nitrogen(BUN)値など既存の腎機能マーカーは，感度と特異性が低く，AKI の早期発見と迅速な対応の妨げとなる場合がある．これらの問題を克服するために，新しいバイオマーカーの同定と，その臨床応用に関する研究が進められている．

本稿では，近年新しい急性腎不全のカテゴリーとして位置づけられつつある AKI の定義と，新規尿中バイオマーカーの関係について，測定技術も含めて述べる．また，AKI に関するいくつかの優れた総説が発表され，尿中バイオマーカーの詳細な記述がされていることから[1]，ここでは AKI バイオマーカーの現状と将来について簡潔な記述を行うこととする．

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AKI の定義と尿中バイオマーカー

1．新しい AKI の定義

AKI に関する詳細な記述は他稿に譲るとして，ここでは尿中バイオマーカーとの関係という側面から述べる．最近，Acute Kidney Injury Network(AKIN)[2]による AKI の定義が新しく提唱された．それを次に示す．

次のいずれかの指標によって決定される急激(48 時間以内)な腎機能低下を AKI と呼ぶ．

① 絶対値血清クレアチニン濃度の上昇が 0.3 mg/dl(≧26.4 μmol/dl)以上．
② 相対値血清クレアチニン濃度の上昇が 50%(1.5 倍)以上．
③ 尿量の低下が 0.5 ml/kg/hr 以下で 6 時間以上継続．

2．血清クレアチニンと BUN

臨床において AKI と診断する際にもっともよく使用される指標は，血清クレアチニン濃度の上昇，BUN 値の上昇である．AKI に伴う糸球体濾過速度(GFR)低下によって，正常な腎では尿中へと

排泄される血中の不要老廃物であるクレアチニンや BUN が，体内に貯留することに起因する．したがって，血清クレアチニン濃度および BUN 値の上昇は，AKI によって低下した腎機能ともっとも密接に関連すると考えられている．しかしながら，血清クレアチニン濃度，BUN は感度と特異性が低いこと，早期の検出が困難ということが指摘されている[3]．また AKI では重篤な場合を除いて，尿量は正常か，あるいは増加することもある[4]．現在 AKI の診断において，これら既存マーカーの弱点が問題となっている．

3．AKI に対する新規尿中バイオマーカー

これまで，AKI に関するさまざまな疫学的研究がなされており，その重篤性や生存率の低さなどが注目されている[4]．当然，AKI の診断やそれに対する治療方針の決定も，血清クレアチニン濃度や BUN などのマーカーに依存しているのが現状であろう．前述したとおり，血清クレアチニン濃度や BUN は，AKI に対する感度と特異性が低いことに加えて，レスポンスが遅いことやばらつきが大きいことも問題とされている．したがって，これらに代わる新しいバイオマーカーが必要であり，速やかで正確に腎機能の変化を診断しうる技術の開発が望まれている．

また，臨床使用可能なバイオマーカーは，新薬開発過程における前臨床試験にも非常に有効であることが期待される．新薬の重篤な副作用の問題など，新薬開発における安全性試験に有用な腎毒性尿中バイオマーカーの利用が期待されている．

kidney injury molecule (KIM)-1 は，筆者らによって発見されたものであり，これまでラットの腎毒性惹起による AKI の尿中バイオマーカー候補として検討されてきた[5,6]．さらに，国際生命科学協会 ILSI コンソーシアム（多くの製薬会社共同による腎毒性 AKI バイオマーカーの探査と評価）によって，KIM-1 が前臨床試験用 AKI 尿中バイオマーカーの有力な候補分子として同定されている[7]．

AKI の病理と尿中バイオマーカーの関係 ——臓器レベルから細胞レベルのバイオマーカーへ○

虚血や毒性物質による組織損傷が AKI の一般的な原因であることは，他稿にくわしく述べられている（<Special Article>AKI の疫学と病態生理）．これらの原因による尿細管上皮細胞の損傷と壊死が基本であり，この損傷のメカニズムは，臓器と細胞の二つのレベルで理解することができる[8]．

臓器内における灌流異常と血流低下，尿細管機能障害と細胞死，尿管閉塞，炎症反応と白血球の浸潤，GFR を含む腎機能の低下といったさまざまな障害が引き起こされ，続いて血清クレアチニン濃度や BUN が上昇する．

この段階で AKI マーカー（クレアチニン，BUN）が検出されるが，ほぼすべての障害が進行しており，当然この段階よりも早期に AKI をみつけることが重要であろう．

直接と間接の障害が尿細管上皮細胞の損傷を引き起こし，それが尿細管機能障害，上皮細胞の極性喪失，細胞膜，細胞死と細胞自体の脱落，尿管閉塞，炎症反応に繋がり，臓器レベルの AKI へと進行していく．この段階で AKI による細胞レベルの変化が尿中バイオマーカーで検出できれば，より早期の発見となる．

AKI の特徴は，尿細管上皮細胞の損傷の後に修復機構が働いて，再生可能な点にある．細胞レベルの修復に引き続き，組織，臓器レベルでの修復が行われる．

AKI における細胞レベルの病理と修復機構を理解することは，AKI の臓器，組織レベルにおける病理の理解と，新しい尿中バイオマーカーの発見・評価に重要である．なぜなら，この機構においてさまざまな蛋白質が発現し，放出・分泌されるが，これら蛋白質は何らかの形で尿細管の損傷と修復の機構に関連すると考えられる．さらに，それらの蛋白質が新規尿中バイオマーカーとしても有望視されている．

AKI 新規尿中バイオマーカー——なぜ新しいバイオマーカーが必要なのか？

1．既存の AKI マーカーの弱点を克服できるか？

すでに一部述べたように，もっとも一般的な既存マーカーである血清クレアチニン濃度，BUN は低感度かつ特異性が低いため，AKI の診断基準を上回るまでの時間が浪費され，その間の AKI 進行に対処することができない．また特異性の低さは，確実な診断と治療のための決断に影響を及ぼす．

当然，これらの弱点を克服するために既存マーカーの技術的な改善か，新しい代替の尿中バイオマーカーの開発と評価が必要とされている．したがって，理想的なバイオマーカーの特性としては迅速性，正確性，特異性，高感度ならびに簡便性といったものが求められる．いかに微小な損傷も，早期に高感度で検出できるマーカーが理想とされる．

2．新しい尿中バイオマーカー——特異性と多重性

この新しい尿中バイオマーカーについて，次の 2 点を最初に強調しておかなければならない．すなわち，① 特異性の高い，高感度な新しい尿中バイオマーカー（特異性），② 複数のバイオマーカーを同時に測定することが可能な技術的改善（多重性）．一つのバイオマーカーに依存しなければならない理由はなく，複数の新しいバイオマーカーを同時に開発することによって相補的効果が期待できる（多重 ELISA の応用など）．

3．最近の技術的進歩——ジェノミクス，プロテオミクスの応用

ジェノミクス，プロテオミクスの応用が新しいバイオマーカーの発見に大きく寄与してきた．とくに後述する NGAL や KIM-1 の発見は，端的な例としてあげられる．

また，他領域の例として心筋障害のバイオマーカーであるトロポニンなどの開発も重要な例である．AKI は全身的な循環状態の影響も大いに受けることから，心筋梗塞の早期発見は，AKI の早期発見においても重要である．

4．尿中バイオマーカーの検出方法と問題点

1）**酵素活性**：酵素活性を比色定量法にて測定する．比較的簡単で安価であるが，標本の酵素活性を維持する必要がある．

2）**ELISA**：特異的抗体を使用する方法であるが，比較的長時間を要すること，抗体の品質管理が重要である．

3）**多重 ELISA**：ELISA のより発展した方法で，同時多項目測定を可能とし，すでに実用段階にある．専用の機器（Luminex™など）が必要である．

4）**新技術**：抗 KIM-1 抗体を使ったトランジスター・バイオセンサーが開発されつつある[9]．今後，スクリーンテスト用の簡単な方法として，蛋白尿検査のようなディップスティックの開発が求められるであろう．

代表的新規 AKI 尿中バイオマーカー

1．AKI 尿中バイオマーカーの種類とその生物学的役割

AKI 尿中バイオマーカーの発現，分泌はそれら蛋白質の生物学的役割と密接に関係している．すなわち，それら蛋白質は腎障害とそれによる修復過程における放出，発現，分泌を受けることに着目され，バイオマーカーとして利用されるであろう．たとえば，損傷によって尿中へ放出される尿細管上皮細胞の酵素，尿中へ分泌されるストレス応答性蛋白質，炎症性蛋白質，および修復機能蛋白質などが，尿中バイオマーカーに利用されることになる．

以下に，代表的新規尿中 AKI バイオマーカーを例として，いくつかあげる（Table 1）．すべてではないが，これらは一定の研究（基礎と臨床）と開発が進んだ有望なバイオマーカー候補と考えられる．

2．kidney injury molecule-1（KIM-1）

KIM-1 は筆者らがラット腎より単離した後に[5]，T 細胞から異なる機能を有する蛋白質としても単離され，T-cell immunoglobulin- and mucin-

Table 1. 代表的な新規尿中 AKI バイオマーカー

バイオマーカー	特　徴	臨床研究	問題点	検出法
KIM-1	クレアチニンよりも早く検出 さまざまな原因による AKI で検出されている	尿中 KIM-1 は AKI で尿細管円柱より早く検出，AKI 後の生存率や透析との関連性が示唆されている 間質性線維化症や移植腎生着率との関連性が期待されている	有用なバイオマーカーと期待されることから，他のバイオマーカーとの共用の可能性も含めて，今後さらに検討・評価されるべきであろう	ELISA 多重 ELISA
NGAL	クレアチニンよりも早く検出	小児科領域での心肺バイパス手術時，また成人の心臓手術においても早期の有効なバイオマーカーであることが証明されている 入院早期の患者において，尿中 NGAL 値と透析の必要性との関連性について示唆されている	炎症や感染症において血中の NGAL が上昇することが知られている したがって，炎症や感染症と AKI が同時に引き起こされる敗血症のような病態では，NGAL モニターによる診断の信頼性に問題がある	ELISA 多重 ELISA
NAG	酵素活性の測定による検出法であるため，簡便かつ感度が高い	糸球体腎炎，糖尿病性腎症，心血管バイパス手術，ICU 患者の AKI，不良予後との関係などで評価されている	尿素，重金属による活性阻害が知られている 他の疾患における尿中の NAG が検出されていることから，今後 AKI の尿中バイオマーカーとしての特異性が評価されるべきであろう 一方，ばらつきが大きく，治療判断に影響を及ぼしている	酵素活性の比色定量
L-FABP	AKI において損傷を受けた近位尿細管から尿中へ放出	非糖尿病性の慢性腎不全進行や，IgA 腎症診断用のバイオマーカーとして検討された 造影剤による腎障害や，人工心肺装置使用後の AKI において，有用なバイオマーカーであるという報告がなされている	AKI 尿中バイオマーカーとしての特性，どのようなタイプの AKI でバイオマーカーとして有用であるかの臨床研究が必要と思われる AKI によって発現誘導を受けるのはヒトに限られ，マウスやラットにおいてそのメカニズムは保存されていないことから，新薬開発段階の安全性試験における腎毒性のバイオマーカーとしての有用性には，疑問が残る	ELISA

domain-containing molecule-1（TIM-1）とも呼ばれているが，これらのコードするアミノ酸配列は同じである．また，Hepatitis A virus cellular receptor 1（HAVCR1）としても知られている．

KIM-1 は一つの膜貫通領域をもつ膜蛋白質であり，その細胞外ドメインは，免疫グロブリン（Ig）ドメインと，多数の糖鎖が付加したムチン（mucin）ドメインからなる．KIM-1 は，正常な腎臓ではまったくみられないが，尿細管上皮細胞の損傷によって発現が強く誘導される[5,6]．プロテアーゼ依存的に細胞外ドメインが切断され，尿中へと分泌される．また，KIM-1 は尿細管管腔側の刷子縁膜に局在するため，切断されずに膜と同時に放出される場合もある．さまざまな原因による AKI において，KIM-1 蛋白質はその尿中に検出されることから，高感度で早期の AKI バイオマーカーであることが知られている[10,11]．

KIM-1 の生物学的機能は長らく不明であったが，最近リン脂質であるホスファチジルセリンの受容体であり，アポトーシス細胞の貪食・処理を担うことが，筆者らを含めて，いくつかの研究グループから報告されている[12~14]．

3．neutrophil gelatinase-associated lipocalin（NGAL）

低分子量蛋白質であり，好中球で産生・分泌される．ペプチドを介して鉄イオンを結合する．AKI において近位尿細管で産生され，尿中へも分泌される．クレアチニンの変化よりも早く検出す