Urinary vanin-1 as a novel biomarker for early detection of drug-induced acute kidney injury

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Running title: Early detection of drug-induced kidney injury by vanin-1

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Abbreviations: AKI, acute kidney injury; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Kim-1, Kidney injury molecule-1; NAG,
N-acetyl-beta-D-glucosaminidase; NGAL, neutrophil gelatinase-associated lipocalin.

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Abstract

Drug-induced nephrotoxicity is a serious problem in cases with hospital-acquired acute kidney injury (AKI). New renal biomarker is needed because traditional markers are not sensitive for early detection of drug-induced AKI. Recently, we have demonstrated that vanin-1 is a novel candidate biomarker of nephrotoxicant-induced kidney injury. Objective of the present study is to determine whether the increase of urinary vanin-1 is detected before the elevations of serum creatinine, or urinary N-acetyl-beta-glucosaminidase (NAG), Kidney injury molecule-1 (Kim-1) and neutrophil gelatinase-associated lipocalin (NGAL) in the two well-established animal models of drug-induced AKI. After the dosing of a higher cisplatin (10 mg/kg, a single i.p. dosing) or gentamicin (120 mg/kg/day, once daily i.p. dosing for 9 days), urinary vanin-1 was detected earlier than the other biomarkers. In rats treated with a lower cisplatin (5 mg/kg, a single i.p. dosing) or gentamicin (40 mg/kg/day, once daily i.p. dosing for 9 days), serum creatinine and urinary NAG were not changed throughout the study period, whereas urinary vanin-1, Kim-1 and NGAL were significantly increased. The renal vanin-1 protein levels were significantly decreased in rats treated with the higher dose of cisplatin on day 5 and gentamicin on day 9, and the immunofluorescence analyses confirmed that vanin-1 immunoreactivity in tubular cells was reduced with the time after
the dosing of cisplatin, indicating that urinary vanin-1 was leaked from tubular cells.

These results suggest that compared with urinary Kim-1 and NGAL, urinary vanin-1 is an earlier and equally sensitive biomarker for drug-induced AKI.
Introduction

Despite technical improvements in clinical care and the development of preventive strategies, the incidence of acute kidney injury (AKI) is gradually increased (Xue et al., 2006; Hsu et al., 2007). Notably, drug-induced nephrotoxicity contributes to up to approximately 20% of cases of hospital-acquired AKI (Luyckx and Naicker, 2008). AKI is associated with a high rate (up to 50%) of morbidity and mortality in hospitalized patients (Thadhani et al., 1996; Chertow et al., 2005), mainly because the traditional markers of kidney injury such as serum creatinine and blood urea nitrogen obviously increase only at the advanced stage of renal damage. Therefore, new biomarkers for earlier and more accurate detection of drug-induced AKI are needed to promote early therapeutic intervention.

Several urinary biomarkers are used as the non-invasive indicators of kidney injury (Han and Bonventre, 2004) including N-acetyl-beta-D-glucosaminidase (NAG) (Tsutsumi and Neckers, 2007), Kidney injury molecule-1 (Kim-1) (Ichimura et al., 1998; Han et al., 2002; Ichimura et al., 2004; Vaidya et al., 2006) and neutrophil gelatinase-associated lipocalin (NGAL) (Mishra et al., 2003; Mishra et al., 2005; McIlroy et al., 2010). Kim-1 is a type 1 transmembrane protein that is not detected in normal tissue or urine, but is highly expressed in dedifferentiated proximal tubule epithelial cells after toxic injury (Ichimura et al., 1998; Han et al., 2002; Ichimura et al., 2004; Vaidya et
However, urinary Kim-1 is reported to increase after the peaks of urinary NAG and NGAL in patients with AKI after cardiac surgery (Han et al., 2009). The appearance of NGAL in the urine precedes that of other biomarkers such as NAG and β2-microglobulin after kidney injury (Mishra et al., 2003), but is also elevated under various pathological conditions such as pneumonia (Chan et al., 2009) and inflammatory bowel disease (Oikonomou et al., 2011). Therefore, these biomarkers might not be enough to make a definitive diagnosis of AKI at an early stage.

Recently, we reported that urinary concentration of vanin-1 elevated before the conventional markers changed in rats with the nephrotoxicant-induced renal tubular injury (Hosohata et al., 2011). Vanin-1, an epithelial glycosylphosphatidylinositol-anchored pantetheinase (Aurrand-Lions et al., 1996; Pitari et al., 2000), participates in the response to oxidative stress in vivo (Berruyer et al., 2004). A recent study showed that the protein levels of renal vanin-1 increased in rats with streptozotocin-induced diabetic nephropathy, and urinary vanin-1 was detected in patients with diabetic nephropathy (Fugmann et al., 2011). Therefore, it is anticipated that urinary vanin-1 is a potential biomarker for the early detection of AKI. However, the utility of urinary vanin-1 in drug-induced AKI has not been examined. To address this issue, we determined whether urinary vanin-1 was detected before the elevations of
serum creatinine, and urinary NAG, Kim-1 and NGAL in the two well-established animal models of drug-induced AKI, cisplatin- and gentamicin-induced kidney injury.
Methods

Animal experiments

Male Wistar rats were purchased at the age of 7 weeks from Charles River Japan (Yokohama, Japan). The rats were maintained under a specific pathogen-free condition, and controlled temperature and humidity with a 12-h light (0700–1900 h) - dark (1900–0700 h) cycle. Animals were fed a regular chow (CE-2; CLEA Japan, Tokyo, Japan) and water ad libitum, and housed in metabolic cages from 3 days before the treatment. All animal procedures were approved by the Animal Care and Use Committee of Jichi Medical University (Shimotsuke, Japan) and performed in accordance with the guidelines for animal research.

Cisplatin-induced nephrotoxicity model

Rats were intraperitoneally (i.p.) injected either vehicle (0.9% sodium chloride), or 5 or 10 mg/kg of cisplatin on day 0. Tail-vein blood was obtained on days 0, 1, 2, 3 and 5, and 24-h urine samples were collected for 6 consecutive days. Samples were stored at -80°C until analysis. The animals were anesthetized with pentobarbital sodium (50 mg/kg, i.p.) to obtain kidney samples on days 1 and 5 (n = 4 in each group).
Gentamicin-induced nephrotoxicity model

Rats received daily i.p. injection of either vehicle (0.9% sodium chloride), or 40 or 120 mg/kg of gentamicin sulfate (n = 4 in each group) for 9 days. The 24-h urine samples were collected on days 0, 1, 2, 3, 4, 5, and 9, and were stored at -80°C until analysis. The animals were anesthetized with pentobarbital sodium (50 mg/kg, i.p.) to obtain blood from vena cava and kidney samples on day 9.

Laboratory measurements

Urine and blood samples were centrifuged at 1,000 g for 10 min, and the supernatant and serum were used to measure creatinine by the Jaffe method with a commercial kit (Wako Pure Chemical Industries, Osaka, Japan) and vanin-1 using an enzyme-linked immunosorbent assay (ELISA) kit (Uscn Life Science Inc., Wuhan, China), respectively. The urinary supernatant were used for the measurement of NAG by the colorimetric method (Wako), Kim-1 using an ELISA kit (R&D Systems, Minneapolis, MN), and NGAL using an ELISA kit (BioPorto Diagnostics, Gentofte, Denmark).

Measurement of vanin-1 protein in the kidney
Sample lysates were prepared from the kidneys of rats on days 1 and 5 after dosing of cisplatin, and on day 9 after daily dosing of gentamicin. Twenty-five micrograms of total protein, which were measured using BCA protein assay kit (Thermo scientific, Rockford, IL), were loaded onto the microplates for ELISA of vanin-1 (Uscn Life Science Inc).

**Histopathology**

Rat kidneys were fixed in 10% formalin/phosphate-buffered saline (PBS) overnight, dehydrated by passing through an ascending ethanol series, and embedded in paraffin wax. The paraffin-embedded tissue sections (4 μm) were deparaffinized in xylene, rehydrated with a series of alcohol washes, and then stained with periodic acid-Schiff (PAS) reagent and hematoxylin. The morphologic evaluation was performed using well-established criteria (Kelleher et al., 1987; Yamamoto et al., 2007) in a blind manner.

**Immunofluorescence analyses of vanin-1**

For immunohistochemistry, tissue sections (5 mm) were embedded in Tissue-Tek OCT compound (Sakura Finetek, Tokyo, Japan), frozen rapidly using dry ice. After the
sections (4 μm) were cut, these were washed with PBS and fixed in 4% paraformaldehyde and blocked in 1% donor equine serum (Thermo Fisher Scientific, Waltham, MA) in PBS for 30 min. The sections were then incubated with monoclonal anti-rat vanin-1 antibody (1:50; Santa Cruz Biotechnology, Avenue, CA) diluted in the blocking solution at 4°C overnight. The sections were washed with PBS and incubated with Cy3-labeled donkey anti-goat IgG (1:200; Abcam, Cambridge, UK) and Alexa Fluor 488-labeled phalloidin (Invitrogen) for 60 min at room temperature. At the final step, sections were washed with PBS and mounted with Vector Shield mounting reagent (Vector, Burlingame, CA).

Cell culture

Human proximal tubular cell line, HK-2 cells, were purchased from American Type Culture Collection (Manassas, VA) and were cultured in keratinocyte serum-free medium (Life Technologies, Carlsbad, CA) supplemented with 5 ng/ml human recombinant epidermal growth factor and 0.05 mg/ml bovine pituitary extract (Life Technologies). Cells were seeded on a 12-well plate at a density of 1 × 10^5 cells/ml and maintained at 37°C in a humidified atmosphere of 5% CO_2. Subconfluent cells were treated for 24 h with cisplatin (1-100 μM), gentamicin (1-10 mM), or ethylene glycol
(100 μM), which was used as a positive control (Hosohata et al., 2011).

Isolation of RNA

Total RNA was isolated from in vivo and in vitro samples using an RNeasy Mini Kit (QIAGEN, Valencia, CA) according to the manufacturer’s instruction. The concentration of total RNA was measured by a NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Real-time quantitative PCR

Isolated total RNA was reverse-transcribed with a PrimeScript RT reagent Kit (Takara Bio Inc., Otsu, Japan). Real-time quantitative PCR was performed in a total volume of 20 μl, containing 9 μl of reverse-transcribed cDNA, 1 μl of each primer and the probe (TaqMan gene expression assays) and 10 μl of TaqMan Fast Universal PCR Master Mix (Life Technologies), with the Applied Biosystems StepOnePlus Real-Time PCR system (Life Technologies). To control for variation in the amount of cDNA available for PCR in the different samples, mRNA expression levels of the target sequences were normalized to the expression of an internal control, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The GenBank accession
numbers, assay ID, and target exons were NM_001025623.1, Rn01537206_m1*, and 6-7 for Vnn1; and NM_017008.3, Rn99999916_s1, and 1-1 for Gapdh; NM_004666.1, Hs00190582_m1, and 1-2 for VNN1; and NM_002046.3, Hs99999905_m1, and 3-3 for GAPDH, respectively. Data were analyzed using the comparative threshold cycle method.

**Statistical analysis**

Data are expressed as the means ± SE. Variables were compared using the one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test and the unpaired t-test as appropriate. For time course studies, the dose effects of drugs were compared using the two-way ANOVA followed by Tukey's test. The p value less than 0.05 was considered to be significant. All statistical analyses were conducted with GraphPad PRISM, version 4 (GraphPad Software, San Diego, CA).
Results

Time course of serum creatinine, and urinary NAG, vanin-1, Kim-1 and NGAL in rats treated with cisplatin or gentamicin

After the treatment with the higher cisplatin (10 mg/kg, i.p.), histological examination revealed slight changes (degeneration of tubular cells, tubular dilatation, and brush-border loss) on day 1 and severe changes on day 5 (Fig. 1A and 1B). As shown in Fig. 2A, urinary vanin-1 initiated to elevate on day 1 and significantly increased on day 2, which lasted up to day 5. On the other hand, there were no significant increases in serum creatinine, or urinary NAG, Kim-1 and NGAL during the first 2 days. Similarly, the higher gentamicin (120 mg/kg/day, 9 days) caused histologically distinct renal damages (Fig. 1C and 1D) and earlier elevation of urinary vanin-1 as follows (Fig. 2B); Urinary vanin-1 significantly increased on day 4 and peaked on day 5, whereas urinary Kim-1 and NGAL significant elevated on day 5 and peaked on day 9, and urinary NAG showed significant increase only on day 9. Contrary to urinary vanin-1, serum vanin-1 concentration was almost undetectable both in the cisplatin- and gentamicin-treated rats, as well as in the controls (data not shown).

Next, we examined whether urinary vanin-1 was more sensitive than other traditional markers using the lower cisplatin (5 mg/kg) or gentamicin (40 mg/kg/day, 9
After the treatment with the lower cisplatin, histopathologically mild changes were detected on day 5 (Fig. 1A and 1B), but serum creatinine, and urinary NAG and NGAL did not increase throughout the study period (Fig. 2A). On the other hand, both urinary vanin-1 and Kim-1 gradually increased up to day 5 (Fig. 2A). As for the lower gentamicin, mild renal damages were observed on day 9 (Fig. 1C and 1D), but serum creatinine and urinary NAG did not elevate during the study period (Fig. 2B). However, urinary vanin-1 as well as urinary Kim-1 and NGAL significantly increased on day 9.

Vanin-1 mRNA expression and protein levels in the kidney

Because the profile of the time-course of urinary vanin-1 was different from those of urinary Kim-1 and NGAL, we examined the changes in vanin-1 expression level in the renal cortices. As shown in Fig. 3A, vanin-1 mRNA expression levels in the renal cortices did not significantly differ among the control and cisplatin-treated groups both on days 1 and 5. On the other hand, vanin-1 mRNA decreased in the renal cortices of rats with dosing of a higher gentamicin, but the differences did not reach to a statistical significance. In addition, vanin-1 protein levels in the renal cortices dose-dependently decreased in the cisplatin-treated rats on day 5 and in the gentamicin-treated animals on day 9 (Fig. 3B). Consistent with these findings, the immunofluorescent analysis
showed that vanin-1 protein was abundantly expressed in tubules of renal cortices in control rats (Fig. 4), but its expression was sparsely detected with the degenerations of tubular cells on day 5 (Fig. 4). Furthermore, the in vitro studies showed that mRNA expressions of vanin-1 were dose-dependently decreased after 24-h exposure to cisplatin (1-100 μM) and gentamicin (1-10 mM), whereas it was significantly increased after 24-h exposure to ethylene glycol in human proximal tubular cell line, HK-2 cells (Fig. 5).
Discussion

In this study, we demonstrated for the first time that the elevation of urinary vanin-1 preceded those of serum creatinine, and urinary NAG, Kim-1 and NGAL in the drug-induced AKI models. Moreover, urinary vanin-1 was as sensitive as urinary Kim-1 and NGAL in the mild nephrotoxicity models. Thus, this study provides the evidence indicating that urinary vanin-1 is a novel biomarker for an earlier detection of drug-induced AKI.

Vanin-1 is an epithelial ectoenzyme with pantetheinase activity, which catalyzes the conversion of pantetheine into pantothenic acid (vitamin B5) and cysteamine (Aurrand-Lions et al., 1996; Pitari et al., 2000). In vanin-1⁻/⁻ mice, the lack of cysteamine is associated with an enhanced γ-glutamylcysteine synthetase activity leading to the elevation of endogenous glutathione (5-L-glutamyl-L-cysteinylglycine, GSH) stores in tissues (Berruyer et al., 2004). GSH exerts an important function in protecting tissues against the degenerating effects of oxidative damage by scavenging free radicals from endogenous or exogenous compounds (Meister and Anderson, 1983). As a result, vanin-1⁻/⁻ mice are resistant to 2,4,6-trinitrobenzene sulfonic acid-induced colitis and mortality (Berruyer et al., 2006). As for renal vanin-1, Yoshida et al. found that renal vanin-1 mRNA level increased in the rats with an ischemia-reperfusion injury
(Yoshida et al., 2002). In addition, a recent study showed that the protein levels of renal vanin-1 increased in rats with streptozotocin-induced diabetic nephropathy, and urinary vanin-1 was detected in patients with diabetic nephropathy (Fugmann et al., 2011). The vanin-1 protein expression were detected in the pancreas of patients with pancreatic cancer-associated diabetes mellitus, but the western blotting results for vanin-1 in serum were not available (Huang et al., 2010). Therefore, it is speculated that urinary vanin-1 might be renal origin. Further studies are needed to confirm this point.

Previously, we showed that the mRNA levels of vanin-1 significantly elevated in rat renal cortices and human renal tubular cell line (HK-2 cells) after the exposure to ethylene glycol for 3 weeks and 24 h, respectively (Hosohata et al., 2011). In the present study, renal vanin-1 protein was significantly decreased in rats treated with a higher dose of cisplatin and gentamicin. In addition, in vitro studies showed that mRNA expressions of vanin-1 were dose-dependently decreased after the 24-h exposure to cisplatin and gentamicin, although the mRNA level was significantly increased after the exposure to ethylene glycol as shown previously (Hosohata et al., 2011). Cisplatin (Lieberthal et al., 1998) and gentamicin (Li et al., 2009; Pessoa et al., 2009) are known to directly cause renal cell death whereas the toxicity of ethylene glycol is per se low.
(Poldelski et al., 2001). Ethylene glycol-induced renal toxicity is caused by the accumulation of its metabolites (Poldelski et al., 2001) and/or obstruction of renal tubules by the metabolite-related crystals (Hackett et al., 1990). Thus, the differences in the mechanism underlying their toxicities and that progression of the injury may lead to the distinct patterns of vanin-1 expression in the models investigated. Because AKI has a wide variety of causes, such as sepsis and ischemia, future studies are needed to determine whether urinary vanin-1 is detectable and useful in all kinds of AKI.

In this study, we found that the time-course of urinary vanin-1 was different from those of Kim-1 and NGAL in the drug-induced AKI models. Interestingly, Kim-1 mRNA expression is highly detected in the injured and dilated tubules, and its renal and urinary protein levels are kept to be higher for a longer duration after renal injury (Vaidya et al., 2006). Similarly, NGAL mRNA is highly expressed in the damaged tubules (Mishra et al., 2003). In addition, NGAL is also synthesized in the loop of Henle and collecting ducts in an AKI model (Schmidt-Ott et al., 2007). On the other hand, we showed in this study that the mRNA expression of vanin-1 was not increased by cisplatin and gentamicin, and its renal protein was decreased with the progression of the tubular degeneration. In addition, vanin-1 is highly expressed in normal tissues in the kidney of human (Jansen et al., 2009) and rodent (Pitari et al., 2000), whereas Kim-1 is not
Therefore, it is speculated that urinary vanin-1 was leaked from renal tissues at least in the drug-induced AKI models examined. As shown in Figure 2, the increase in urinary vanin-1 on day 1 after higher dose of cisplatin was not significant, whereas those on days 2-5 were significant, suggesting that renal vanin-1 was starting to obviously leak into urine on day 2 and continued to leak at least until day 5. As a result, the decrease in vanin-1 protein level might become apparent on day 5.

Considering the distinct features of vanin-1 from other biomarkers, the combined use of vanin-1 with Kim-1 or NGAL will be more useful for the diagnosis of drug-induced AKI and the prediction of damaged renal sites.

In summary, the present data suggest that, compared with urinary Kim-1 and NGAL, urinary vanin-1, which is leaked from tubular cells, is the earlier and equally sensitive biomarker for drug-induced AKI.
Authorship Contributions

Participated in research design: Hosohata, Ando, and Fujimura.

Conducted experiments: Hosohata and Ando.

Performed data analysis: Hosohata, Ando, and Fujimura.

Wrote or contributed to the writing of the manuscript: Hosohata, Ando, and Fujimura.
References


Footnotes

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Figure legends

Figure 1. Influence of cisplatin and gentamicin on renal morphology. A, C: Representative histologies of kidney sections from respective treatment group. Scale bars, 100 μm. B, D: Acute tubular necrosis (ATN) score was calculated as a total of each index: brush border loss, casts, tubular dilatation and necrosis, and interstitial edema, which were graded on a 0 to 2 scale (n = 4; *p < 0.05, **p < 0.01).

Figure 2. Time course of urinary vanin-1 and traditional biomarkers in rats treated with cisplatin or gentamicin. Twenty-four h urine was collected in rats treated with cisplatin (A; 5 or 10 mg/kg, i.p.) or gentamicin (B; 40 or 120 mg/kg/day for 9 days, i.p.). Concentrations of urinary N-acetyl-beta-D-glucosaminidase (NAG), vanin-1, Kidney injury molecule-1 (Kim-1), and neutrophil gelatinase-associated lipocalin (NGAL) were normalized with urinary creatinine concentration. Data are the means ± SE, n = 4 at each time point. *p < 0.05, **p < 0.01 vs. controls.

Figure 3. The mRNA and protein expression level of vanin-1 in the kidney of rats treated with cisplatin or gentamicin. Sample lysates were prepared from rat kidneys on days 1 and 5 after dosing of cisplatin (5 or 10 mg/kg, i.p.), and on day 9 after
dosing of gentamicin (40 or 120 mg/kg for 9 days, i.p.), respectively. The mRNA expression level was normalized to that of GAPDH. Data are the means ± SE, n = 4 in each group. *p < 0.05, **p < 0.01.

Figure 4. Immunohistochemical analyses of vanin-1 expression in the renal cortex. Vanin-1 and F-actin were stained with a vanin-1 antibody (red) and phalloidin (green), respectively. Scale bars, 50 μm.

Figure 5. The mRNA expression level of vanin-1 in the human proximal tubular cell lines, HK-2 cells. Cells were exposed to cisplatin (A; 1, 10, and 100 μM), gentamicin (B; 1, 5, and 10 mM), or ethylene glycol (C; 100 μM) for 24 h. Data are the means ± SE, n = 6 in each group. N.D., Not Detected. *p < 0.05, **p < 0.01.
Figure 1.

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Figure 1.

B

Day 1

Day 5

ATN score

Control 5 mg/kg 10 mg/kg Cisplatin

Control 5 mg/kg 10 mg/kg Cisplatin

**
Figure 1.

C

<table>
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**Figure 1.**

D

![Bar chart showing ATN score for different doses of Gentamicin](chart.png)
Figure 2.

A  Cisplatin

- **10 mg/kg cisplatin**
- ▲ 5 mg/kg cisplatin
- ○ Control

B  Gentamicin

- ○ Control
- ▲ 40 mg/kg/day gentamicin
- ▲ 120 mg/kg/day gentamicin

Serum Creatinine (mg/dl)

Urinary NAG (U/g Creatinine)

Urinary Vanin-1 (ng/mg Creatinine)

Urinary Kim-1 (ng/mg Creatinine)

Urinary NGAL (ng/mg Creatinine)

Days
Figure 3.

A

Cisplatin

Day 1

Day 5

Gentamicin

Relative mRNA expression

Control 5 mg/kg 10 mg/kg

Control 5 mg/kg 10 mg/kg

Control 40 mg/kg 120 mg/kg

B

Cisplatin

Day 1

Day 5

Gentamicin

Vanin-1 protein (ng/mg protein)

Control 5 mg/kg 10 mg/kg

Control 5 mg/kg 10 mg/kg

Control 40 mg/kg 120 mg/kg
Figure 4.
Figure 5.

(A) Relative Vanin-1 mRNA expression in response to different concentrations of Cisplatin:
- Control
- 1 µM
- 10 µM
- 100 µM

(B) Relative Vanin-1 mRNA expression in response to various concentrations of Gentamicin:
- Control
- 1 mM
- 5 mM
- 10 mM

(C) Relative Vanin-1 mRNA expression in response to Ethylene glycol:
- Control
- Ethylene glycol