Urinary Vanin-1 As a Novel Biomarker for Early Detection of Drug-Induced Acute Kidney Injury

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Received February 2, 2012; accepted March 6, 2012

ABSTRACT

Drug-induced nephrotoxicity is a serious problem in patients with hospital-acquired acute kidney injury (AKI). A new renal biomarker is needed because traditional markers are not sensitive for early detection of drug-induced AKI. In a recent study, we demonstrated that vanin-1 is a novel candidate biomarker of nephrotoxicant-induced kidney injury. The objective of the present study is to determine whether the increase in urinary vanin-1 is detected before the elevations of serum creatinine or urinary N-acetyl-β-D-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 administration of a higher dose of cisplatin (10 mg/kg, a single intraperitoneal dose) or gentamicin (120 mg/kg per day, once daily intraperitoneal dose for 9 days), urinary vanin-1 was detected earlier than the other biomarkers. In rats treated with a lower dose of cisplatin (5 mg/kg, a single intraperitoneal dose) or gentamicin (40 mg/kg per day, once daily intraperitoneal dose 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 dose 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) has gradually increased (Xue et al., 2006; Hsu et al., 2007). Of note, 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 obvious increases in the traditional markers of kidney injury such as serum creatinine and blood urea nitrogen are seen 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 noninvasive indicators of kidney injury (Han and Bonventre, 2004) including N-acetyl-β-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, 2005; McIvor 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, 2004; Han et al., 2002; Vaidya et al., 2006). 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 it 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.

In a recent study, we reported that urinary concentration of vanin-1 elevated before the conventional markers changed in rats with nephrotoxicant-induced renal tubular injury (Hosohata et al., 2011). Vanin-1, an epithelial glycosylphospho-

ABBREVIATIONS: AKI, acute kidney injury; NAG, N-acetyl-β-D-glucosaminidase; Kim-1, kidney injury molecule-1; NGAL, neutrophil gelatinase-associated lipocalin; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
formed in accordance with the guidelines for animal research. 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-induced and gentamicin-induced kidney injury.

**Materials and 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 (7:00 AM–7:00 PM) and dark (7:00 PM–7:00 AM) cycle. Animals were fed regular chow (CE-2; CLEA Japan, Tokyo, Japan) and water ad libitum and were 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 injected intraperitoneally with either vehicle (0.9% sodium chloride) or 5 or 10 mg/kg 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 six 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 a daily intraperitoneal injection of either vehicle (0.9% sodium chloride) or 40 or 120 mg/kg 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 1000g 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 urine supernatant were used for the measurement of NAG by the colorimetric method (Wako Pure Chemical Industries, 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 a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA), 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 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) and frozen rapidly using dry ice. After the sections (4 μm) were cut, they were washed with PBS, fixed in 4% paraformaldehyde, and blocked in 1% donor equine serum (Thermo Fisher Scientific) in PBS for 30 min. The sections were then incubated with monoclonal anti-rat vanin-1 antibody (1: 50; Santa Cruz Biotechnology, Inc., Santa Cruz, 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, Carlsbad, CA) for 60 min at room temperature. At the final step, sections were washed with PBS and mounted with Vector Shield mounting reagent (Vector Laboratories, Burlingame, CA).

**Cell Culture.** The human proximal tubular cell line HK-2 was purchased from American Type Culture Collection (Manassas, VA), and cells were cultured in keratinocyte serum-free medium (Invitrogen) supplemented with 5 mg/ml human recombinant epidermal growth factor and 0.05 mg/ml bovine pituitary extract (Invitrogen). Cells were seeded on a 12-well plate at a density of 1 × 10⁵ cells/ml and maintained at 37°C in a humidified atmosphere of 5% CO₂. Subconfluent cells were treated for 24 h with cisplatin (1–10 μM), gentamicin (1–10 μM), 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 instructions. The concentration of total RNA was measured by a NanoDrop ND-1000 UV-visible 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 (Invitrogen), with the Applied Biosystems StepOnePlus real-time PCR system (Invitrogen). 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 identification numbers, and target exons were NM_001025623.1, Rn01537206_m1*, and 6-7 for Vnn1; NM_017008.3, Rn09999916_s1, and 1-1 for Gapdh; NM_004666.1, Hs01090582_m1, and 1-2 for VNN1; and NM_002046.3, Hs00999905_m1, and 3-3 for GAPDH, respectively. Data were analyzed using the comparative threshold cycle method.

**Statistical Analysis.** Data are expressed as the means ± S.E. Variables were compared using one-way analysis of variance followed by the Bonferroni multiple comparison test and the unpaired t-test as appropriate. For time course studies, the dose effects of drugs were compared using two-way analysis of variance followed by a Tukey test. p < 0.05 was considered 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 dose (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. 1, A and B). As shown in Fig. 2A, urinary vanin-1 began 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 in urinary NAG, Kim-1, and NGAL during the first 2 days. Likewise, the higher gentamicin dose (120 mg/kg per day, 9 days) caused histologically distinct renal damage (Fig. 1, C and D) 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 significantly elevated on day 5 and peaked on day 9, and urinary NAG showed a 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 dose of cisplatin (5 mg/kg) or gentamicin (40 mg/kg per day, 9 days). After treatment with the lower cisplatin dose, histopathologically mild changes were detected on day 5 (Fig. 1, A and B), 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 dose, mild renal damage was observed on day 9 (Fig. 1, C and D), 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 on days 1 and 5. On the other hand, vanin-1 mRNA decreased in the renal cortices of rats with administration of a higher gentamicin dose, but the differences did not reach 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

Fig. 1. Influence of cisplatin and gentamicin on renal morphology. A and C, representative histology of kidney sections from respective treatment group. Scale bars, 100 μm. B and 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.
degenerations of tubular cells on day 5 (Fig. 4). Furthermore, the in vitro studies showed that mRNA expression of vanin-1 was dose-dependently decreased after a 24-h exposure to cisplatin (1–100 μM) and gentamicin (1–10 mM), whereas it was significantly increased after a 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 evidence indicating that urinary vanin-1 is a novel biomarker for earlier detection of drug-induced AKI.

Vanin-1 is an epithelial ectoenzyme with pantetheinase activity, which catalyzes the conversion of pantetheine into pantothanic 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 enhanced glutathione synthetase activity, leading to the elevation of endogenous glutathione (5-L-glutamyl-L-cysteinylglycine) stores in tissues (Berruyer et al., 2004). 5-L-Glutamyl-L-cysteinylglycine 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. (2002) found that the renal vanin-1 mRNA level increased in the rats with an ischemia-reperfusion injury. 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). Vanin-1 protein expression was 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 of renal origin. Further studies are needed to confirm this point.

In a previous study, we showed that the mRNA levels of vanin-1 were significantly elevated in rat renal cortices and a human renal tubular cell line (HK-2 cells) after exposure to ethylene glycol for 3 weeks and 24 h, respectively (Hosohata et al., 2011). Vanin-1 protein expression was 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 of renal origin. Further studies are needed to confirm this point.

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increased after a 24-h exposure to cisplatin and gentamicin, although the mRNA level was significantly increased after 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 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 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. Of interest, Kim-1 mRNA expression is highly detected in injured and dilated tubules, and its renal and urinary protein levels remain higher for a longer duration after renal injury (Vaidya et al., 2006). Likewise, 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 (Mishra et al., 2003). In addition, NGAL also stimulates the production 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 an 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.

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