Renal tubular injury exacerbated by Vasohibin-1 deficiency in a murine cisplatin-induced acute kidney injury model

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Abstract

Acute kidney injury (AKI) is frequently encountered in clinical practice, particularly secondary to cardiovascular surgery and administration of nephrotoxic agents, and is increasingly recognized for initiating a transition to chronic kidney disease. Clarifying the pathogenesis of AKI could facilitate the development of novel preventive strategies because the occurrence of hospital-acquired AKI is often anticipated. Vasohibin-1 (VASH1) was initially identified as an antiangiogenic factor derived from endothelial cells. VASH1 expression in endothelial cells has subsequently been reported to enhance cellular stress tolerance. Considering the importance of maintaining peritubular capillaries in preventing the progression of AKI, this study aimed to examine whether VASH1 deletion is involved in the pathogenesis of cisplatin-induced AKI. For this, we injected male C57BL/6J wild-type (WT) and VASH1 heterozygous knockout ($VASH1^{+/-}$) mice with either 20 mg/kg of cisplatin or a vehicle solution intraperitoneally. Seventy-two hours after cisplatin injection, increased serum creatinine concentrations and renal tubular injury accompanied by apoptosis and oxidative stress were more prominent in the $VASH1^{+/-}$ mice than in the WT mice. Cisplatin-induced peritubular capillary loss was also accelerated by VASH1 deficiency. Moreover, the increased expression of intercellular adhesion molecule-1 in the peritubular capillaries of cisplatin-treated $VASH1^{+/-}$ mice was associated with a more marked infiltration of macrophages into the kidney. Taken together, VASH1 expression could have protective effects on cisplatin-induced AKI probably through maintaining the number and function of peritubular capillaries.

Keywords: Vasohibin-1, Acute kidney injury, Cisplatin, Peritubular capillary, Endothelial cells

Introduction

Acute kidney injury (AKI) is one of the current major problems in the field of nephrology. It has been estimated to affect approximately 20–200 individuals per million people (14), and 57% of patients with AKI have been admitted to an intensive care unit (13). AKI is known to be associated with increased risk for mortality and morbidity. Moreover, even the patients who survive AKI have a higher risk of developing chronic kidney disease. Importantly, in-hospital AKI frequently occurs secondary to medical treatments such as cardiac surgery or cancer chemotherapy. Because patients who undergo planned surgery or chemotherapy can be screened for risk of AKI in advance, specific strategies could potentially prevent AKI effectively in high-risk patients. Therefore, clarifying the pathogenesis of AKI is crucial for identifying novel targets.

Cisplatin nephropathy is a representative chemotherapeutic agent-related AKI. The molecular mechanisms underlying cisplatin-induced AKI have long been the subjects of research, and a number of studies have demonstrated the involvement of mitochondrial dysfunction, oxidative stress, inflammation, and apoptosis in renal tubular cells (25). Renal ischemia is involved in the pathogenesis of not only ischemic–reperfusion injury but also chemotherapeutic agent-related AKI. Loss of peritubular capillaries observed in the murine cisplatin nephropathy model can accelerate tubular cell injuries and renal dysfunction (15). Additionally, similar to the case of ischemic–reperfusion AKI, the activation of hypoxia-inducible factor (HIF)-1 reportedly protects the renal tubules from gentamicin-induced AKI (1). These findings suggest that renal vascular protection is a potential preventive strategy against chemotherapeutic agent-related AKI.

Angiogenesis is a physiological and pathological process of forming new blood vessels using existing vasculature. It is well-known to be controlled by the balance between

pro-angiogenic and anti-angiogenic factors. The vascular endothelial growth factor (VEGF)-A is the qualitatively and quantitatively predominant pro-angiogenic factor in the kidney and other organs. Although the major source of VEGF-A in the kidney is glomerular podocytes, renal tubular cells, particularly in those in the thick ascending limbs of Henle's loops, also express VEGF-A at a substantially lower level (35). A recent study showed that renal tubule-specific deletion of VEGF-A resulted in a marked reduction in interstitial capillary density (4), indicating that tubular VEGF-A expression is essential for the maintenance of peritubular capillaries in the kidney. Indeed, HIF-1 activation followed by VEGF-A upregulation ameliorated anti-cancer drug-induced AKI in rodents (1). Therefore, anti-angiogenic factors competing with VEGF-A may theologically exacerbate AKI. However, some anti-angiogenic factors may have renoprotective effects under certain circumstances (36).

Vasohibin-1 (VASH1) was initially identified as a novel anti-angiogenic factor derived from endothelial cells (39). It has the unique characteristic of angiogenic regulation in a negative feedback manner; i.e., it is secreted from endothelial cells in response to pro-angiogenic stimuli, and it acts on newly formed vessels to terminate angiogenesis. The anti-angiogenic effects of VASH1 have been demonstrated in tumor transplant animal models(30, 39). Additionally, the adenoviral overexpression of VASH1 in murine diabetic models inhibited excess VEGF-A signaling in the glomeruli, leading to the improvement of diabetic glomerular alterations (24, 26). However, the endogenous expression of VASH1 in endothelial cells seems to be important in maintaining endothelial survival. The downregulation of VASH1 in cultured endothelial cells leads to cellular senescence and vulnerability to cellular stress (23). Therefore, normal VASH1 and VEGF-A expression in the kidney may be essential for maintaining the intact structure and function of peritubular

capillaries.

In this study, we examined the pathophysiological significance of endogenous VASH1 expression in cisplatin nephropathy using heterozygous VASH1 knockout mice. VASH1 deficiency leads to more severe renal tubular injury and peritubular capillary loss in conjugation with increased oxidative stress and macrophage infiltrations. Our results suggest that VASH1 expression play a protective role in the development of cisplatin-induced AKI.

Materials and Methods

Animals and experimental protocols

VASH1 heterozygous knockout ($VASH1^{+/-}$) mice were obtained from the Institute of Development, Aging, and Cancer at Tohoku University (Sendai, Japan) and bred at the Department of Animal Resources in the Advanced Science Research Center at Okayama University (Okayama, Japan). The experimental protocol was approved by the Animal Care and Use Committee at Okayama University (approval number OKU-2014509 and OKU-2017345). Male C57BL/6J wild-type (WT) and $VASH1^{+/-}$ mice were fed a standard pellet laboratory chow and were provided with water *ad libitum*. Experimental AKI was induced by a single intraperitoneal injection of 20 mg/kg of cisplatin (*cis*-diamineplatinum [II] dichloride; Sigma-Aldrich, St Louis, MO, USA). Control mice received a vehicle solution (normal saline). After 72 hours, and under anesthesia, blood samples were collected via the inferior vena cava, followed by the harvesting the kidneys. The mice were divided into the following four subgroups: 1) vehicle-treated WT (n = 5), 2) vehicle-treated $VASH1^{+/-}$ (n = 5), 3) cisplatin-treated WT (n = 6), and 4) cisplatin-treated $VASH1^{+/-}$ mice (n = 6). Serum creatinine and blood urea nitrogen (BUN) concentrations was measured by FUJIFILM Monolith Co., Ltd. (Tokyo, Japan).

Histological analysis and apoptosis detection

Formalin-fixed, paraffin-embedded kidney sections (4-μm thickness) were stained with periodic acid−Schiff (PAS) and observed under a light microscopy. Renal tubular injuries were quantified by calculating the percentage of injured tubules defined by epithelial cell detachment from the basement membrane, loss of brush border, or proteinaceous cast formation at 200× magnification (37, 38). The kidney sections were evaluated by two

investigators (S.T and K.T) in a blinded manner.

To detect apoptotic cells in the kidneys, the TACS 2 TdT-Blue Label *In Situ* Apoptosis Detection Kit (Trevigen, Gaithersburg, MD, USA) was used on frozen sections (8-μm thickness) according to the manufacturer's protocol (37). The nuclei were counterstained with Nuclear Fast Red. Positively stained cells were counted at 200× magnification in ten fields of view per section.

Immunohistochemistry

Formalin-fixed, paraffin-embedded sections (4-μm thickness) were used for the immunohistochemistry of malondialdehyde accumulation, as described previously (33, 34). Briefly, after deparaffinization, the sections were treated with 3% H_2O_2 for 10 min to inactivate endogenous peroxidase activity. Subsequently, the sections were incubated with primary antibodies overnight at 4 °C. Anti-malondialdehyde (MDA; JaICA, Fukuroi, Japan), anti-CD34 (Abcam, Cambridge, UK), and anti-mouse intercellular adhesion molecule-1 (ICAM-1; R&D Systems, Minneapolis, MN, USA) were used as the primary antibodies. After the sections were washed with phosphate-buffered saline, they were incubated with MACH2 Mouse HRP-Polymer (Biocare Medical, Pacheco, CA) for MAD, HRP anti-rat IgG (Vector laboratories, Burlingame, CA) for CD34, or Simple Stain MAX-PO(G) (Nichirei Bioscience Inc., Tokyo, Japan) for ICAM-1. ImmPACT DAB (Vector laboratories) was used as a chromogen. The nuclei were counterstained with hematoxylin. The semiquantitative staining score for MAD was determined at 200× magnification in ten fields of view per section as follows: area (0, none; 1, < 25%; 2, 26–50%; 3, > 50%) × intensity (1, weak; 2, moderate; 3, heavy) (3). The number of CD34-positive peritubular capillaries was counted at 200× magnification in ten fields of view per section.

Frozen kidney sections (4-μm thickness) were used for the immunohistochemistry of macrophage infiltration, as described previously (26). After treatment with 3% H_2O_2 for 10 min, the sections were incubated with rat anti-mouse F4/80 antibody (Serotec, Oxford, UK) overnight at 4 °C. A biotinylated anti-rat IgG antibody (Vector laboratories) and Vectastain ABC Elite reagent kit (Vector laboratories) were used for immunoperoxidase staining. ImmPACT DAB was used as a chromogen, and sections were counterstained with hematoxylin. F4/80-positive cells were counted at 200× magnification in ten fields of view per section.

Immunoblotting

Total protein was extracted from whole kidney tissues using the RIPA Lysis Buffer (Santa Cruz Biotechnology, Dallas, TX, USA). Immunoblotting was performed as described previously (37, 38). 40 μg of the extracted protein samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto the nitrocellulose membranes. Anti-VASH1 antibody (Novus Biologicals, Centennial, CO, USA), anti-4-hydroxy-2-nonenal (4-HNE) antibody (JaICA), and anti-β-actin antibody (Sigma-Aldrich) were used as the primary antibodies. Peroxidase-conjugated anti-rabbit or mouse antibody (Cell Signaling, Danvers, MA, USA) was applied as a secondary antibody. Enzyme activity was detected using the ECL Western Blotting Detection Kit (GE Healthcare, Buckinghamshire, UK), and images were obtained using the ImageQuant LAS 4000 (GE Healthcare). The density of bands was determined using ImageJ software and expressed relative to the density of β-actin.

Real-time polymerase chain reaction

RNA extraction and cDNA preparation were performed using the RNeasy Mini Kit (Qiagen, Chatsworth, CA, USA) and SuperScript II Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, USA), as described previously (21). cDNA was added to the Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) with specific oligonucleotide primers as summarized in Table 1. Quantitative real-time PCR was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems). The amount of PCR products was normalized with *Actb* mRNA-encoding β-actin.

Cell culture

Primary human umbilical vein endothelial cells (HUVECs; Lonza, Basel, Switzerland) were cultured in endothelial basal medium containing 2% fetal bovine serum and growth supplements (EGM™-2 Bullet Kit™; Lonza) with penicillin (100 U/ml) and streptomycin (100 μg/ml). For the experiments, HUVECs at less than ten passage were seeded on collagen I-coated 6-well plates. At 70-80% confluency, the cells were incubated in the above-mentioned medium with 0, 10, 50, or 100 μM of cisplatin for 12 hours, or with 100 μM of cisplatin for 0, 3, 6, or 12 hours, and were then harvested.

Statistical analysis

All values were expressed as means ± standard deviation (SD). One-way ANOVA with a *post-hoc* Scheffe's test were used for inter-group comparisons of multiple variables. Statistical analyses were performed using the JMP 10 software (SAS Institute Inc, Cary, NC, USA). The level of $P < 0.05$ was considered statistically significant.

Results

Decreased VASH1 expression in cisplatin-induced kidney injury

Serum levels of BUN, a marker of renal dysfunction, in WT mice began to increase by 48 hours after cisplatin injection (Fig. 1A). Interestingly, renal *Vash1* mRNA expression tended to decrease before cisplatin-induced elevation of BUN levels and was significantly reduced after 72 hours (Fig. 1B). The same tendency of decreased renal VASH1 protein expression in cisplatin-treated WT mice was confirmed by immunoblotting (Fig. 1C and 1D).

VASH1 deficiency accelerated cisplatin-induced kidney injury

As reported previously (10), VASH1 heterozygous knockout mice had the same appearance as that of the WT mice. In the kidneys of VAHS1$^{+/-}$ mice, endogenous *Vash1* mRNA expression was significantly reduced compared with the WT mice (Fig. 2A). Renal *Vash1* mRNA expression in the WT mice significantly decreased after cisplatin injection, and the level in the VASH1$^{+/-}$ mice remained markedly low (Fig. 2A). Although there was no difference in body weight between the WT and VASH1$^{+/-}$ mice (21.2 ± 1.2 g vs. 21.9 ± 1.3 g) after cisplatin treatment, the increased kidney weight/body weight ratio tended to be greater in the VASH1$^{+/-}$ mice than in the WT mice (Fig. 2B), indicating renal swelling in the VASH1$^{+/-}$ mice. The renal dysfunction induced by cisplatin was accessed by measuring the serum blood urea nitrogen (BUN) and creatinine levels. Both blood markers were significantly increased in the cisplatin-treated WT group compared with the vehicle-treated group, whereas VASH1 deficiency resulted in more severe renal dysfunction after cisplatin injection (Fig. 2C and 2D). Histologically, there was no difference between the vehicle-treated WT and VASH1$^{+/-}$ mice. Cisplatin treatment induced acute tubular necrosis, indicated by epithelial cell detachment, brush border loss, and intratubular cast formation (Fig. 2E). The

percentage of injured tubules was significantly higher in the cisplatin-treated VASH1-deficient mice than in the WT mice (Fig. 2F). These results suggest that VASH1 deficiency accelerated cisplatin-induced renal tubular cell injuries, leading to more severe renal dysfunction.

VASH1 deficiency promoted renal apoptosis and oxidative stress in cisplatin-treated mice

Along with the abovementioned results, TUNEL staining showed that cisplatin increased significantly in a number of apoptotic cells in the kidneys of VASH1$^{+/-}$ mice compared with those of the WT mice (Fig. 3A and 3B). The accumulation of oxidative stress markers such as MDA and 4-HNE was also more prominent in the kidney tissues of the cisplatin-treated VASH1$^{+/-}$ mice, determined by immunohistochemistry (Fig. 3C and 3D) and immunoblot (Fig. 3E and 3F), than in the WT mice. Because VASH1 increases the expression of SOD2 and Sirt1 to improve stress tolerance in endothelial cells *in vitro* (23), the mRNA levels for these molecules were evaluated in the kidneys. The mRNA levels between the non-treated WT and VASH1$^{+/-}$ mice did not differ (Fig. 4A and 4B). The level of *Sod2* mRNA decreased after cisplatin injection, and a significantly greater decrease occurred in the VASH1$^{+/-}$ mice (Fig. 4A). Although the difference in renal Sirt1 mRNA levels between the vehicle-treated and cisplatin-treated WT mice was not statistically significant, *Sirt1* significantly decreased in the cisplatin-treated VASH1$^{+/-}$ mice (Fig. 4B).

VASH1 deficiency resulted in increased vascular permeability in cisplatin-treated mice

As reported previously, the number of peritubular capillaries in the kidney cortex tended to be lower in the cisplatin-treated WT mice. Cisplatin-induced peritubular capillary loss was more prominent In the VASH1$^{+/-}$ mice than in the WT mice (Fig. 5A and 5B).

Additionally, the renal mRNA level of the *Vegfa* encoding major pro-angiogenic factor VEGF-A was significantly reduced after cisplatin injection in the WT mice, whereas no statistically significant differences were found in the *Vegfa* mRNA levels between the cisplatin-treated WT and VASH1$^{+/-}$ mice (Fig. 5C). The renal expression of angiopoietin-1 (Ang-1), a crucial factor for maintaining vascular stability, was also decreased in the cisplatin-treated WT mice. Interestingly, the decreased expression of Ang-1 was significantly greater in the VASH1$^{+/-}$ mice than in the WT mice (Fig. 5D), suggesting that VASH1 deficiency promotes vascular instability in cisplatin-induced AKI. Furthermore, the upregulation of ICAM-1 caused by cisplatin treatment was markedly enhanced in the VASH1$^{+/-}$ mice compared with the WT mice (Fig. 5E). Immunohistochemistry showed that the ICAM-1 expression was mainly localized in the capillary endothelial cells and partially localized in the interstitial cells in both vehicle- and cisplatin-treated mice (Fig. 5F). Therefore, the increased ICAM-1 expression may be associated with endothelial damage after cisplatin treatment.

VASH1 deficiency exacerbated renal macrophage infiltration in cisplatin-treated mice

Along with the increased accumulation of oxidative stress, cisplatin treatment resulted in F4/80-positive macrophage infiltration in the renal interstitial area in the WT mice. The cisplatin-treated VASH1$^{+/-}$ mice showed significantly enhanced macrophage infiltration in the kidney compared with the WT mice (Fig. 6A and 6B). However, the increased renal expression of *Ccl2* mRNA, which encodes monocyte chemotactic protein-1 (MCP-1), that was observed in the cisplatin-treated WT mice was not found to be elevated in the VASH1$^{+/-}$ AKI mice (Fig. 6C). In light of these results, we concluded that the increased macrophage infiltration observed in the cisplatin-treated VASH1$^{+/-}$ mice was likely due to the upregulation

of adhesion molecules in the damaged endothelial cells rather than the increased expression of chemoattractants in the renal tubular cells.

Cisplatin decreased VASH1 expression and enhanced ICAM-1 expression in cultured endothelial cells

Because previous reports have demonstrated that cisplatin caused direct toxicity to endothelial cells (6, 25), we examined VASH1 expression in cisplatin-treated HUVECs *in vitro*. VASH1 expression in HUVECs was significantly decreased by high-dose cisplatin (Fig. 7A), and the decreased VASH1 expression was time-dependent (Fig. 7B). Furthermore, the result was in accordance with the decreased expression of vascular endothelial (VE)-cadherin encoded by *CDH5*, which blocks vascular permeability (Fig. 7C). Conversely, cisplatin increased the ICAM-1 expression in HUVECs in a dose-dependent manner (Fig. 7D), as observed in the peritubular capillaries of cisplatin-treated mice (Fig. 5E and 5F).

Discussion

In this study, we demonstrate that VASH1 deficiency results in the exacerbation of cisplatin-induced renal dysfunction and injury, as well as in oxidative stress and apoptosis, probably due to vascular vulnerability and accelerated macrophage infiltration.

VASH1 is mainly produced and secreted by vascular endothelial cells. A recent report demonstrated that VASH1 expression in renal cell carcinoma was sporadically found in tumor microvessels, and that VASH1 density was a significant predictor of poor prognosis and metastasis, suggesting that VASH1 expression might reflect the actual activity of tumor angiogenesis (22). In contrast, in the kidney of patients with chronic kidney disease, VASH1 immunoreactivity is observed in the glomerular and peritubular capillary endothelial cells (8), and it can be detected in human plasma and urine samples (9). Unfortunately, localization of VASH1 in the murine kidney could not be determined by immunohistochemistry in this study, because of the poor specificity of currently available antibodies for VASH1. To date, the anti-angiogenic effects of VASH1 have been well demonstrated in various animal models. For example, the adenoviral overexpression of VASH1 in mice inoculated with Lewis lung carcinoma cells inhibited tumor angiogenesis and growth (12). The increased glomerular endothelial area observed in animals with type 1 and type 2 diabetic nephropathy was largely prevented by VASH1-expressing adenoviral infection (24, 26). However, unlike most other anti-angiogenic agents, VASH1 does not induce endothelial apoptosis but rather seems to maintain vascular stability during active angiogenesis. In fact, VASH1 overexpression in non-diabetic mice did not decrease the number of glomerular capillaries (24). Moreover, endogenous VASH1 expression in the endothelial cells was shown to be necessary for cellular stress tolerance and survival (23). Therefore, VASH1 acts as an endothelial-protecting factor in stress conditions. Because cisplatin-induced AKI is

associated with the loss of peritubular capillaries, as observed in the present and previous studies (15), endogenous VASH1 expression should have protective effects on the capillary endothelium. Nevertheless, VASH1 deficiency does not result in any vascular phenotype. A recent study reported that homozygous VASH1 knockout mice exhibited mild insulin resistance with healthy longevity (31), suggesting that VASH1 does not play an essential role in maintaining vascular integrity in unstressed conditions. Although there have been no reports of mutations in human *VASH1*, VASH1 deficiency may enhance susceptibility to cancer progression (16) as well as various vascular diseases.

Cisplatin-induced AKI is considered to be caused by the direct effects of cisplatin on the renal tubular cells because these cells uptake cisplatin through organic cation transporters, and its intracellular concentration becomes five times higher than its serum level (18). Previous reports have shown that cisplatin induces the apoptosis of renal tubular cells (25). Such events result in mitochondrial dysfunction accompanied by the accumulation of lipid peroxidative products such as MDA and 4-HNE in the kidney, as observed in this study. Given that VASH1 deficiency results in the exacerbation of cisplatin-induced AKI with tubular cell apoptosis, oxidative stress, and inflammation, the endogenous expression of VASH1 may play a protective role against cisplatin-induced AKI. The abovementioned endothelial stress tolerance and survival caused by VASH1 are reportedly associated with the upregulation of SOD2 and Sirt1 (23). Kidney-specific Sirt1 overexpression reduces reactive oxygen species production and tubular apoptotic cells (7), whereas Sirt1 induced SOD2 expression prevents oxidative stress via FOXO activation (11). Therefore, a more prominent reduction in Sirt1 and SOD2 in the cisplatin-treated VASH1$^{+/-}$ mice than in the cisplatin-treated WT mice may contribute to increased oxidative stress and apoptotic cells, leading to severe renal dysfunction and tubular injury.

Previous reports have also suggested the possible involvement of renal vascular injury in cisplatin nephropathy. In the rat cisplatin nephropathy model, renal blood flow was decreased at 24–72 hours after treatment (41), possibly leading to ischemic renal tubular injury. Cisplatin reportedly has a direct toxic effect on endothelial cells due to ATP depletion and caspase-3 activation (6). This study has shown that the cisplatin-induced loss of peritubular capillaries may be responsible for such ischemic tubular injury. Conversely, renal tubular cells produce VEGF-A and Ang-1, which are essential for maintaining an intact endothelium in the peritubular capillaries. In recent studies, tubule-specific VEGF-A knockout mice showed a marked decrease in peritubular capillary density (4). Tubule-specific Ang-1 overexpression promoted the growth of the peritubular capillary network (28), whereas Ang-1 knockout mice showed accelerated peritubular capillary rarefaction after ureteral obstruction (19). Therefore, cisplatin and the decreased renal expression of VEGF-A and Ang-1 may cause endothelial damage, subsequently leading to the upregulation of ICAM-1.

Inflammatory infiltration is also involved in the pathogenesis of cisplatin-induced AKI. Although macrophage infiltration is considered to be late-occurring, a recent animal experiment showed that F4/80-positive macrophages were infiltrated in the renal interstitium at 1 day after cisplatin injection (20). Macrophage infiltration could be driven by the upregulation of macrophage chemoattractant MCP-1. Indeed, cisplatin induces MCP-1 in renal tubular cells (29), and the urinary MCP-1 level is elevated in patients who receive cisplatin-containing chemotherapy (27). We have previously reported that VASH1 deficiency results in marked glomerular macrophage infiltration caused by diabetes, with increased MCP-1 expression (10). In this study, however, the increased expression of MCP-1 cannot account for the more prominent macrophage infiltration in the cisplatin-treated VASH1$^{+/-}$

mice compared with the WT mice, because there was no difference in *Ccl2* mRNA levels between these groups. Given that endothelial ICAM-1 is associated with the recruitment of macrophages (5), increased ICAM-1 expression in the peritubular capillaries may facilitate renal macrophage infiltration in cisplatin-treated VASH1$^{+/-}$ mice.

Furthermore, our *in vitro* study demonstrated that cisplatin directly acted on endothelial cells to reduce the VASH1 expression. However, some previous reports have showed that cisplatin upregulates ICAM-1 expression in cultured endothelial cells through an NF-κB-independent or dependent manner (32, 42). In the present study, cisplatin decreased VE-cadherin, an endothelium-specific tight junction protein, and increased ICAM-1 along with a decrease in VASH1 levels. Such alterations in the expression of endothelial proteins might provide a basis for the cisplatin-induced increase in vascular permeability and inflammatory infiltration.

This study had limitation that VASH1$^{+/-}$ animals used were conventional gene knockout mice, rather than conditional endothelium-specific knockout mice. Therefore, the VASH1 expressed outside the endothelial cells may be important in protecting the renal tubular cells from cisplatin-induced AKI. A recent report has underscored the physiological roles of VASH1 in other cell types such as neurons (2) and fibroblasts (40). However, given the evidence that VASH1 expression is almost restricted to the endothelial cells (17), we believe that the endothelial expression of VASH1 plays a protective role against cisplatin-induced loss of peritubular capillaries and subsequent renal tubular injury. Indeed, our *in vitro* study revealed that cisplatin directly reduced VASH1 expression in endothelial cells. Another limitation was that not all C57BL/6J WT mice used in this study were littermate controls. As VASH1$^{+/-}$ mice had been backcrossed with the same strain as the WT mice, it was unlikely that strain variance might have affected the entire results. However, the

possibility that strain variance had affected the sensitivity to cisplatin cannot be excluded in this study.

In conclusion, VASH1 deficiency results in the exacerbation of cisplatin-induced AKI. It is essential in maintaining the integrity of peritubular capillaries under endothelial stress condition caused by cisplatin. The preservation of VASH1 expression in the peritubular capillaries may be a promising strategy against chemotherapeutic drug-induced AKI given the anti-cancer effect of VASH1.

Acknowledgements

We would like to thank Ms. Yoshiko Hata for providing technical assistance in the cell culture experiments.

Grants

This study was supported by funds from JSPS KAKENHI Grant Number JP15K09263 (2015-2017 to K.T) and the Cooperative Research Project Program of Joint Usage/Research Center at the Institute of Development, Aging and Cancer, Tohoku University (2017-2018 to K.T).

Disclosures

Jun Wada receives speaker honoraria from Astellas, Boehringer Ingelheim, Daiichi Sankyo, Novartis, and Tanabe Mitsubishi, and receives grant support from Astellas, Bayer, Baxter, Chugai, Daiichi-Sankyo, Kissei, Kyowa Hakko Kirin, MSD, Novartis, Novo Nordisk, Ono, Otsuka, Pfizer, Teijin, Torii, and Takeda.

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Figure legends

Fig. 1. Renal VASH1 expression in cisplatin-induced acute kidney injury.

(A) Increased blood urea nitrogen was observed at 48 hours after cisplatin injection in wild-type (WT) mice. (B) Temporal changes in renal *Vash1* mRNA expression determined by real-time PCR after cisplatin treatment. (C) Immunoblot for VASH1 and β-actin. Arrow indicates VASH1 immunoreactive bands, and additional bands are observed around 50 kDa. Each lane was loaded with 40 μg of protein. (D) Densitometry of the immunoblot normalized to β-actin. n = 4 at each time point. $^{*}P < 0.01$ vs. cisplatin-untreated (0 hour) mice. Each column shows the mean ± SD.

Fig. 2. VASH1 deficiency accelerated cisplatin-induced renal dysfunction and renal tubular injury.

(A) *Vash1* mRNA expression determined by real-time PCR in the renal cortex from WT and VASH1 heterozygous knockout (VASH1$^{+/-}$) mice with or without cisplatin injection. The increase in kidney weight to body weight ratio (B), blood urea nitrogen (C), and serum creatinine levels (D) induced by cisplatin treatment was greater in VASH1$^{+/-}$ than in WT mice. (E) Representative light microscopic images of the renal cortex from vehicle-treated WT (WT-veh), vehicle-treated VASH1$^{+/-}$ (VASH1$^{+/-}$-veh), cisplatin-treated WT (WT-cis), and cisplatin-treated VASH1$^{+/-}$ (VASH1$^{+/-}$-cis) mice (periodic acid–Schiff staining, original magnification, ×200). (F) Percentage of injured tubules including epithelial cell detachment, loss of brush border, or proteinaceous cast formation in these kidney sections. $^{*}P < 0.01$ and $^{\S}P < 0.05$ vs. vehicle-treated WT mice, $^{\#}P < 0.05$ vs. cisplatin-treated WT mice. Each column shows the mean ± SD.

Fig. 3. VASH1 deficiency enhanced oxidative stress and apoptosis in cisplatin-induced renal tubular injury.

(A) Representative kidney images following TUNEL staining for vehicle-treated WT (WT-veh), vehicle-treated VASH1$^{+/-}$ (VASH1$^{+/-}$-veh), cisplatin-treated WT (WT-cis), and cisplatin-treated VASH1$^{+/-}$ (VASH1$^{+/-}$-cis) mice (original magnification, ×200). Arrows indicate TUNEL positive nuclei. (B) The increased number of TUNEL-positive nuclei was significantly greater in VASH1$^{+/-}$ mice than in WT mice. (C) Representative images of immunohistochemistry for malondialdehyde (MDA) in the kidney from each group (original magnification, ×200). (D) Semi-quantitative analysis of MDA immunohistochemistry. (E) Immunoblot for 4-hidroxynolenal (4-HNE) and β-actin. Each lane was loaded with 40 μg of protein. (F) Densitometry of the immunoblot for 4-HNE normalized to β-actin. $^*P < 0.01$ vs. vehicle-treated WT mice, $^{\#}P < 0.05$ vs. cisplatin-treated WT mice. Each column shows the mean ± SD.

Fig. 4. VASH1 deficiency decreased expression of SOD2 and Sirt1 in cisplatin-induced AKI. *Sod2* (A) and *Sirt1* (B) mRNA expressions determined by real-time PCR in the renal cortex from vehicle- and cisplatin-treated WT and VASH1$^{+/-}$ mice. Data were normalized with expression of *Actb* mRNA-encoding β-actin. $^*P < 0.01$ and $^{\S}P < 0.05$ vs. vehicle-treated WT mice, $^{\#}P < 0.05$ vs. cisplatin-treated WT mice. Each column shows the mean ± SD.

Fig. 5. VASH1 deficiency promoted peritubular capillary loss and increased expression of ICAM-1 in cisplatin-induced renal injury.

(A) Representative images of immunohistochemistry for CD34 in the kidneys from vehicle-treated WT (WT-veh), vehicle-treated VASH1$^{+/-}$ (VASH1$^{+/-}$-veh), cisplatin-treated

WT (WT-cis), and cisplatin-treated VASH1$^{+/-}$ (VASH1$^{+/-}$-cis) mice (original magnification, ×200). (B) The number of CD34-positive peritubular capillaries was significantly decreased after cisplatin treatment in VASH1$^{+/-}$ compared with WT mice. Expressions of *Vegfa* (C), *Angpt1* (D), and *Icam1* mRNA in the renal cortex from vehicle- and cisplatin-treated WT and VASH1$^{+/-}$ mice. Data were normalized with expression of *Actb* mRNA. (F) Representative images of immunohistochemistry for ICAM-1 in the kidneys from each group. $^{*}P < 0.01$ and $^{\S}P < 0.05$ vs. vehicle-treated WT mice, $^{\#}P < 0.05$ vs. cisplatin-treated WT mice. Each column shows the mean ± SD.

Fig. 6. VASH1 deficiency accelerated renal macrophage infiltration in cisplatin-induced renal injury.

(A) Representative images of immunohistochemistry for F4/80, a marker of macrophage, in the kidneys from vehicle-treated WT (WT-veh), vehicle-treated VASH1$^{+/-}$ (VASH1$^{+/-}$-veh), cisplatin-treated WT (WT-cis), and cisplatin-treated VASH1$^{+/-}$ (VASH1$^{+/-}$-cis) mice (original magnification, ×200). Arrows indicate F4/80-positive cells. (B) The number of F4/80-positive macrophages induced by cisplatin treatment was significantly larger in VASH1$^{+/-}$ than in WT mice. (C) *Ccl2* mRNA expression in the renal cortex from each group. Data were normalized with expression of *Actb* mRNA. $^{*}P < 0.01$ vs. vehicle-treated WT mice, $^{\#}P < 0.05$ vs. cisplatin-treated WT mice. Each column shows the mean ± SD.

Fig. 7. Cisplatin directly decreased VASH1 expression in cultured endothelial cells.

VASH1 mRNA expression determined by real-time PCR in HUVECs treated with 0, 10, 50 or 100 μM of cisplatin for 12 hours (A) and with 100 μM of cisplatin for 0, 3, 6 or 12 hours (B). Expression of *CDH5* (C) and *ICAM1* (D) mRNA in cisplatin-treated HUVECs. n = 4 for each

concentration and each time point. Data were normalized with expression of *Actb* mRNA. $^{*}P$ < 0.01 vs. 0 μM of cisplatin, $^{\S}P$ < 0.01 vs. cisplatin treatment for 0 hour. Each column shows the mean ± SD.

Table 1 Primer sequences for real-time PCR

Genes	Proteins		Sequences
Mouse			
Vash1	Vasohibin-1	Forward	AGCACAGAGAGATGAGGAAC
		Reverse	CGTCGTCGGCTGGAAAGTAG
Sod2	SOD2	Forward	GAGAATCTCAGTGCTCACTCGTGTC
		Reverse	GGAACCCTAAATGCTGCCAGTC
Sirt1	Sirtuin-1	Forward	CAGACCCTCAAGCCATGTTTGATA
		Reverse	TTGGATTCCTGCAACCTGCTC
Vegfa	VEGF-A	Forward	ACATTGGCTCACTTCCAGAAACAC
		Reverse	TGGTTGGAACCGGCATCTTTA
Angpt1	Angiopoietin-1	Forward	CCGAGCCTACTCACAGTACGACAG
		Reverse	TGAAATCGGCACCGTGTAAGA
Icam1	ICAM-1	Forward	AACTGTGGCACCGTGCAGTC
		Reverse	AGGGTGAGGTCCTTGCCTACTTG
Ccl2	MCP-1	Forward	AGGTCCCTGTCATGCTTCT
		Reverse	CTGCTGGTGATCCTCTTGT
Actb	β-actin	Forward	CATCCGTAAAGACCTCTATGCCAAC
		Reverse	ATGGAGCCACCGATCCACA
Human			
VASH1	Vasohibin-1	Forward	AAGCCCTTACCTGCTTGCTGTC
		Reverse	ACCACTATGTCCTGTGCTGGTCTC
CDH5	VE-cadherin	Forward	TTTCACTGCAAACACACCTTGGA
		Reverse	GCTTTGCCCAGAGTGGGAAC
ICAM1	ICAM-1	Forward	AACTGACACCTTTGTTAGCCACCTC
		Reverse	TGTCCAGACATGACCGCTGA
ACTB	β-actin	Forward	TGGCACCCAGCACAATGAA
		Reverse	CTAAGTCATAGTCCGCCTAGAAGCA