

Original Article

## Comparison of changes in urinary and blood levels of biomarkers associated with proximal tubular injury in rat models

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**Abstract:** To investigate useful biomarkers associated with proximal tubular injury, we assessed changes in levels of a focused set of biomarkers in urine and blood. Male rats administered a single dose or four doses of gentamicin (GM, 240 mg/kg/day) or a single dose of cisplatin (CDDP, 5 mg/kg) were euthanized on days 2 (the day after initial dosing) 5, or 12. At each time point, histopathological examination of the kidney and immunohistochemistry for biomarkers, kidney injury molecule-1 (Kim-1), lipocalin (NGAL), clusterin (CLU), cystatin C (CysC) and  $\beta$ 2-microglobulin ( $\beta$ 2M) were performed. Biomarker levels were measured in urine and blood. In both treatment groups, degenerated/necrotic proximal tubules and regenerated tubules were mainly observed on days 5 and 12, respectively. At the same time as these tubular injuries, urinary Kim-1, CysC and  $\beta$ 2M levels were increased. Moreover, urinary levels of CysC and  $\beta$ 2M in GM-treated animals and Kim-1 in CDDP-treated animals increased (on day 2) prior to tubular injury on day 5. This was considered to reflect the characteristics of drug toxicity. Although almost all of the biomarkers in blood were not sufficiently sensitive to detect proximal tubular injury, urinary and plasma  $\beta$ 2M levels simultaneously increased. Therefore, in addition to urinary Kim-1, CysC and  $\beta$ 2M levels, plasma  $\beta$ 2M levels were also considered useful for detecting proximal tubular injury. (DOI: 10.1293/tox.2014-0039; *J Toxicol Pathol* 2015; 28: 151–164)

**Key words:** kidney, proximal tubular injury, biomarker, gentamicin, cisplatin, immunohistochemistry

### Introduction

Renal injury can be fatal, making the kidney a major target organ for drug research and development. Drug-induced renal injury represents one of the primary causes of drug failure or adverse drug reactions (approximately 21%) in late-stage development<sup>1, 2</sup>. Therefore, early detection of nephrotoxicity is important for determining the safety of drug candidates. Blood urea nitrogen (BUN) and creatinine (CRE) are the most commonly used markers for detecting nephrotoxicity in traditional clinical pathology. However, these markers are not very specific or sensitive, as there is usually a significant loss of renal function before BUN and

CRE levels increase<sup>3</sup>.

Several renal injury biomarkers that are more specific and sensitive than BUN or CRE have already been reported. Regulatory authorities have judged 8 biomarkers— $\beta$ 2-microglobulin ( $\beta$ 2M), clusterin (CLU), cystatin C (CysC), kidney injury molecule-1 (Kim-1), albumin (ALB), urinary total protein (TP), trefoil factor 3 and renal papillary antigen-1—as acceptable for the detection of acute drug-induced renal toxicity and for providing additional and complementary information not available with the currently available standard parameters in specified contexts of non-clinical development<sup>4-7</sup>. Moreover, urinary alpha-glutathione S-transferase, mu-glutathione S-transferase, lipocalin (NGAL), and osteopontin have also been recognized as candidate biomarkers for nephrotoxicity<sup>8-10</sup>.

Proximal tubular injury is the most frequent drug-induced renal injury because a high number of xenobiotics are reabsorbed and excreted by proximal tubules<sup>11</sup>. Glomerular injury also leads to proximal tubular injury because persistent albuminuria following glomerular injury is harmful to renal tubular cells<sup>12</sup>. Therefore, detection of proximal tubular injury may provide a sensitive way to monitor most, but

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not all, renal toxicities. Gentamicin (GM) and/or cisplatin (CDDP) are often used to experimentally induce nephrotoxicity when investigating the usefulness and characteristics of biomarkers that detect proximal tubular injury. GM damages proximal convoluted tubules [S1 and S2 segments]<sup>13</sup>; CDDP primarily damages proximal straight tubules [S3 segments]<sup>14</sup>.

Biomarkers are usually examined in the urine because urine is readily accessible and repeated measurements can be obtained for individual animals/humans. This allows the investigator to track the onset and progression of a renal injury and recovery from it. In addition to examination in urine, biomarkers are also examined in blood (serum/plasma)<sup>15–17</sup>. Most studies that have evaluated blood biomarkers have been performed in humans, few studies have evaluated blood biomarkers in animals<sup>18</sup>. Nonetheless, evaluation of blood biomarkers in animals is convenient because blood collection can be routinely performed in preclinical studies. However, evaluation of either urinary or blood biomarkers provides limited information regarding the part of the nephron that is injured and involved with changes in biomarker levels. Tissue-based assays, such as immunohistochemistry, are important to confirm the origin of a biomarker and its relationship with a histopathological change.

Recently, there have been various reports that have compared urinary biomarkers with immunohistochemistry for biomarkers in rat models of proximal tubular injury<sup>19–21</sup>. However, there are a few reports that have evaluated blood biomarkers in correlation with proximal tubular injury in rat models. Moreover, there are no detailed reports integrating the changes in the biomarker levels using urine-based, blood-based and tissue-based approaches. Therefore, to investigate useful biomarkers in urine and blood, which could detect renal injury, we determined the time course changes in levels of a focused set of five biomarkers (Kim-1, NGAL, CLU, CysC and  $\beta$ 2M) that can reflect proximal tubular injury<sup>3</sup> in urine, blood and kidney tissues from GM- or CDDP-treated rats.

## Material and Methods

### *Animals and experimental design*

All procedures were performed after approval of the study by the Institutional Animal Care and Use Committee of Mitsubishi Tanabe Research Laboratories. Male Crl:CD(SD) rats were purchased from Charles River Laboratories (Kanagawa, Japan). The animals were maintained under controlled conditions (12-h light/dark cycle, temperature of  $23 \pm 3^\circ\text{C}$ , relative humidity of  $50 \pm 20\%$ ) and were given free access to food (autoclaved CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water. Seventy-two animals were allocated to following three groups of 24 animals each: a saline-treated group (control), a GM-treated group (GM group) and a CDDP-treated group (CDDP group). The animals were 6 weeks old at the time of GM or CDDP administration.

GM (240 mg/kg body weight; 5 mL/kg), CDDP (5 mg/

kg body weight; 2 mL/kg) or saline was administered intraperitoneally. Dosages were determined based on previous reports<sup>22, 23</sup> and a preliminary examination; we evaluated the kidney after slight injury, severe injury and recovery from injury at each time point. Twelve animals in each group were euthanized on day 2 after administration of a single dose; the day of initiation of administration was designated as day 1. Six animals after administration of GM for 4 days or administration of a single dose of CDDP were euthanized on days 5 or 12, respectively. Two animals in the CDDP group euthanized on day 12 were excluded from this study because of a dosing error.

### *Urinalysis and blood chemistry*

The animals in all groups were housed individually in stainless steel metabolic cages for approximately 24 h, and urine was sampled using the following schedule: days 1–2, animals euthanized on day 2; days 4–5, animals euthanized on days 5 and 12; days 11–12, animals euthanized on day 12.

At necropsy, blood was collected from the postcava under anesthesia with an intraperitoneal injection of thio-pental sodium (Ravonal<sup>®</sup>; Mitsubishi Tanabe Pharma Corporation). Serum samples were obtained by centrifugation at  $4^\circ\text{C}$ . Plasma samples were obtained after centrifugation at  $4^\circ\text{C}$  using a test tube containing dipotassium EDTA. These samples were stored in a freezer at  $-90$  to  $-70^\circ\text{C}$  until analysis.

Measurements of serum items, including CRE (Se-rotec, Tokyo, Japan) and BUN (Shino-Test, Tokyo, Japan), were performed using an automatic analyzer (Hitachi High-Technologies Corporation., Tokyo, Japan). Measurements of urinary and serum CysC (Mitsubishi Chemical Medicine, Tokyo, Japan) were also performed using this analyzer. Measurements of urinary and plasma Kim-1 (R&D Systems, Minneapolis, MN, USA), NGAL (BioPorto Diagnostics, Gentofte, Denmark), CLU (Life Diagnostics, Inc., West Chester, PA, USA) and  $\beta$ 2M (Mitsubishi Chemical Medicine) were performed using commercial ELISA kits.

Urinary data are shown as total urinary excretion measured over 24 h of collection. Urinary biomarker levels were not normalized to the contemporaneous urine creatinine level because urinary creatinine excretion in the setting of acute kidney injury is dynamic and its inclusion in the assessment of urinary biomarkers has been challenged<sup>24</sup>.

### *Histopathology*

Animals were exsanguinated by severing the abdominal aorta and postcava after blood collection, and then the kidneys were harvested. After fixing in 10% neutral buffered formalin, cross sections of the kidney showing papilla were dehydrated and embedded in paraffin. Paraffin sections (4  $\mu\text{m}$  thick) were stained with hematoxylin and eosin. Then, unblinded evaluation of the sections was performed.

### *Immunohistochemistry*

Deparaffinized sections were incubated with 3%  $\text{H}_2\text{O}_2$  in distilled water for 10 min and with Block Ace (Dainippon

**Table 1.** Primary Antibodies Used in this Study

Antigen	Host species	Clonality	Dilution	Antigen retrieval	Supplier
Kidney injury molecule-1 (Kim-1)	Goat	Polyclonal	1:300	MW <sup>a</sup>	R&D Systems, Minneapolis, MN, USA
Lipocalin (NGAL)	Goat	Polyclonal	1:300	AC <sup>b</sup> and TRY <sup>c</sup>	R&D Systems
Clusterin (CLU)	Goat	Polyclonal	1:50	None	R&D Systems
Cystatin C (CysC)	Rabbit	Polyclonal	1:1000	MW <sup>a</sup>	Abcam, Cambridge, UK
$\beta$ 2-microglobulin ( $\beta$ 2M)	Rabbit	Polyclonal	1:200	MW <sup>a</sup>	Lifespan BioSciences, Seattle, WA, USA

<sup>a</sup> Microwaved at 90°C for 10 min with citrate bufer (pH 6.0). <sup>b</sup> Autoclaved at 121°C for 4 min with citrate buffer (pH 6.0). <sup>c</sup> Treated with 0.1% trypsin for 2 min at 37°C.

**Table 2.** Histopathological Findings in the Kidney after Receiving GM or CDDP

Group	Findings	Grade	Day 2	Day 5	Day 12
GM			(12)	(6)	(6)
	Eosinophilic globules in the proximal convolved tubule in the cortex	+	0	2	0
		++	0	2	0
	Degeneration/necrosis of the proximal convolved tubular epithelium in the cortex	+	0	3	0
	Regeneration of the proximal convolved tubular epithelium in the cortex	+	0	0	2
		++	0	0	4
	Dilatation of the proximal tubule over the cortex and OSOM	++	0	0	1
	Infiltration of macrophage/lymphocyte in the cortex	+	0	4	4
		++	0	0	1
	CDDP			(12)	(6)
Nuclear chromatin margination of the proximal straight tubular epithelium in the OSOM		+	11	0	0
Degeneration/necrosis of the proximal straight tubular epithelium in the OSOM		+	0	0	3
		++	0	6	0
Regeneration of the proximal straight tubular epithelium in the OSOM		+	0	5	0
		++	0	0	4 <sup>a</sup>
Regeneration of the proximal convolved tubular epithelium in the cortex		+	0	0	4
Karyomegaly of the proximal straight tubular epithelium in the OSOM		+	0	6	0
		++	0	0	4
Infiltration of macrophage/lymphocyte in the OSOM		+	0	2	0

Results are expressed as number of animals with a given finding and grade. Numbers in parentheses are the numbers of animals examined. Grade: +, slight; ++, moderate. <sup>a</sup> Observed with slight dilatation.

Pharmaceutical Co., Ltd., Osaka, Japan) for 30 min at room temperature. Then, sections were incubated at 4°C for 16 h with primary antibodies. Details of the primary antibodies used are summarized in Table 1. Following incubation with Histofine Simple Stain MAX PO (Nichirei, Tokyo, Japan) for 30 min at room temperature, sections were developed using 0.05% 3,3-diaminobenzidine/H<sub>2</sub>O<sub>2</sub> as the chromogen. All immunostained slides were counterstained with hematoxylin.

Grading of immunoreactivity was performed as follows: -, negative; +, positive for a small area of tubular epithelium in the renal cortex or medulla; ++, positive for a large area of tubular epithelium in the renal cortex or medulla; +++, positive for a large area of tubular epithelium in both the renal cortex and medulla.

### Statistical analysis

A two-tailed Dunnett's multiple comparison test was employed for statistical evaluation of differences between control and treatment groups at a significance level of 5%. Blood chemistry and urinalysis values tended to show a log-

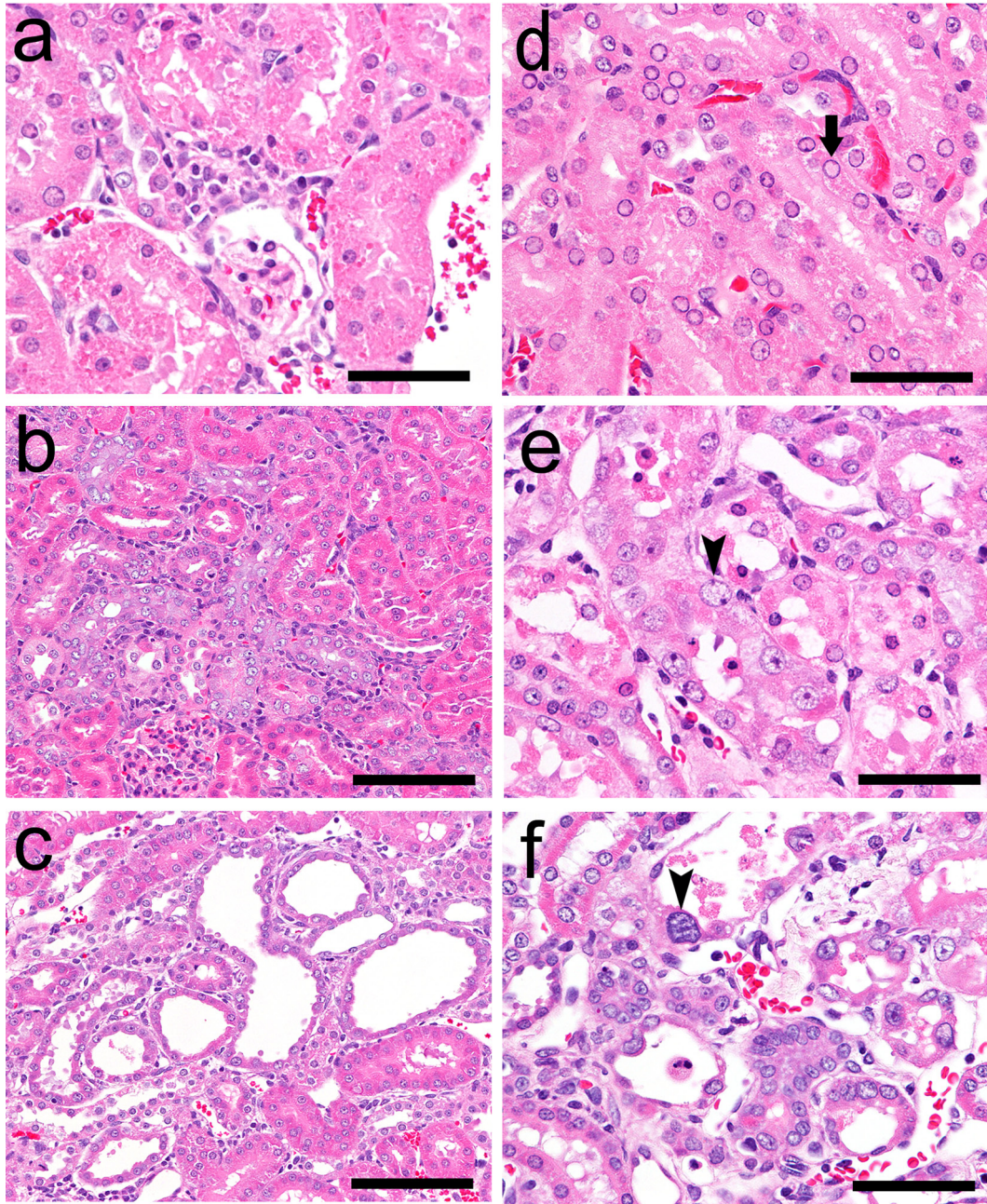
normal distribution; therefore, a logarithmic transformation was conducted before employing Dunnett's multiple comparison test. Values below the lower limit of quantification were regarded as half the lower limit of quantification for conducting logarithmic transformations.

Pearson's product-moment correlation coefficient was used to evaluate the linear association between the urinary and blood levels of each biomarker.

## Results

### GM group

The results of histopathological examination following GM treatment are presented in Table 2. There were no histopathological findings on day 2 (the day after administration of a single dose). Eosinophilic globules and degeneration/necrosis were noted in the proximal convoluted tubules in the cortex on day 5 (Fig. 1a), and regeneration of the tubules subsequently occurred on day 12 (7 days after administration for 4 days; Fig. 1b). On day 12, dilatation of proximal tubules over the cortex and the outer stripe of the outer



**Fig. 1.** Histopathological findings in the kidneys of rats following GM or CDDP administration. The following findings were obtained in the GM group: eosinophilic globules in and degeneration/necrosis of the proximal convoluted tubular epithelium in the cortex on day 5 (a), regeneration of the proximal convoluted tubular epithelium in the cortex on day 12 (b) and dilatation of the proximal tubule over the cortex and OSOM on day 12 (c). The following findings were observed in the CDDP group: nuclear chromatin margination of the proximal straight tubular epithelium (arrow) in the OSOM on day 2 (d) and degeneration/necrosis, regeneration, karyomegaly (arrow head) of the proximal straight tubular epithelium in the OSOM on day 5 (e) and day 12 (f). Hematoxylin and eosin staining. Bar = 50  $\mu\text{m}$  (a, d, e, f), 100  $\mu\text{m}$  (b, c).

medulla (OSOM) was observed in one animal (Fig. 1c). Although histopathological changes were observed from day 5, BUN and CRE were significantly increased only on day 12 (Table 3). Urinary TP and ALB levels were significantly increased from days 2 to 5 (Table 3).

On day 12, it was found that the TP and ALB levels had recovered to within the control range, except for the animal

with dilatation of the proximal tubules observed in histopathology; this animal showed extremely high TP and ALB levels (17.2 mg and 107.0  $\mu\text{g}$ , respectively). It also showed extremely high levels of the following biomarkers: urinary and plasma Kim-1 (723.9 ng and 1675.1 pg/mL, respectively) and urinary NGAL (28998.6 ng), CLU (13182.4 ng) and CysC (19.68  $\mu\text{g}$ ).

**Table 3.** Blood Chemistry and Urinalysis Parameters Except for Focused Biomarkers

	Serum			Urine		
	<i>n</i>	BUN mg/dL	CRE mg/dL	<i>n</i>	TP excretion mg	ALB excretion μg
Control						
Day 2	12	15.7 ± 3.0	0.19 ± 0.02	12	3.0 ± 1.2	0.0 ± 0.0
Day 5	6	13.1 ± 1.7	0.17 ± 0.01	12	2.9 ± 0.5	1.7 ± 0.8
Day 12	6	14.5 ± 2.5	0.21 ± 0.03	6	8.2 ± 1.0	2.7 ± 1.1
GM						
Day 2	12	15.4 ± 3.2	0.20 ± 0.02	12	6.5 ± 2.3**	2.9 ± 3.1**
Day 5	6	13.1 ± 3.1	0.23 ± 0.02	12	7.2 ± 3.4**	7.6 ± 6.5**
Day 12	6	20.7 ± 3.0**	0.28 ± 0.07**	6	10.1 ± 4.2	21.9 ± 41.8
CDDP						
Day 2	12	15.2 ± 1.5	0.20 ± 0.02	12	2.2 ± 0.5	0.0 ± 0.0
Day 5	6	36.3 ± 16.0**	0.74 ± 0.37**	10	16.8 ± 4.5**	98.1 ± 29.8**
Day 12	4	15.6 ± 1.4	0.25 ± 0.04*	4	7.6 ± 2.7	2.9 ± 0.7

Values are shown as the group mean ± SD. *n*: Number of animals examined. \* $P < 0.05$  and \*\* $P < 0.01$  vs. time-matched control group (two-tailed Dunnett's multiple comparison test).

(1) Kim-1: The urinary Kim-1 level was significantly increased on days 5 and 12, whereas the plasma Kim-1 level was increased only on day 12 (Fig. 2a, b). Although the plasma Kim-1 levels increased after the urinary Kim-1 levels, the two were strongly correlated [coefficient of correlation ( $R$ ) = 0.96,  $P < 0.01$ ; Fig. 3a], and both the levels of urinary and plasma Kim-1 increased from day 5 to 12.

The results of immunohistochemistry following GM treatment are presented in Table 4. Kim-1 immunoreactivity was absent in control animals at every time point. In GM-treated animals, Kim-1 immunoreactivity was detected in a part of the proximal convoluted tubules in a nephron that displayed eosinophilic globules or degeneration/necrosis on day 5 (Fig. 4a). Kim-1 immunoreactivity was also detected in both the regenerated tubules (Fig. 4b) and dilated tubules on day 12 (Fig. 4c).

(2) NGAL: The urinary NGAL levels on days 5 and 12 were increased and showed equal values, although the increase on day 5 was not statistically significant (Fig. 2c). Although the urinary NGAL levels were showed a strong correlation with the plasma levels ( $R = 0.89$ ,  $P < 0.01$ ; Fig. 3b), the plasma NGAL were not increased in majority of the animals at any time point (Fig. 2d).

NGAL immunoreactivity was absent in control animals at every time point. NGAL immunoreactivity in GM-treated animal was similar to that of Kim-1. However, the immunoreactivity was observed sporadically with granular staining in a part of the proximal convoluted tubules that displayed eosinophilic globules or degeneration/necrosis on day 5 (Fig. 5a). NGAL immunoreactivity was also detected in a few regenerated tubules (Fig. 5b) and a part of the dilated tubules on day 12 (Fig. 5c).

(3) CLU: The urinary CLU levels were significantly increased only on day 12, but the plasma CLU levels showed no increase at any time point (Fig. 2e, f). In addition, there was no significant correlation between urinary and plasma CLU levels ( $R = 0.10$ ,  $P = 0.65$ ; Fig. 3c).

CLU immunoreactivity was absent in control animals

at every time point. CLU immunoreactivity in the GM-treated group was also absent on days 2 and 5, but it was detected in a few normal distal tubules (Fig. 6a) and a part of the dilated tubules on day 12 (Fig. 6b).

(4) CysC: The urinary CysC levels significantly increased on days 2 and 5 (Fig. 2g); however, the serum CysC levels showed no increase at any time point (Fig. 2g, h) and no significant correlation with the urinary levels ( $R = 0.25$ ,  $P = 0.26$ ; Fig. 3d). The urinary CysC levels increased from days 2 to 5. The increased mean urinary CysC level on day 12 was considered to reflect the data from the animal with proximal tubule dilatation because the CysC levels of the other animals were similar to the levels of the control animals.

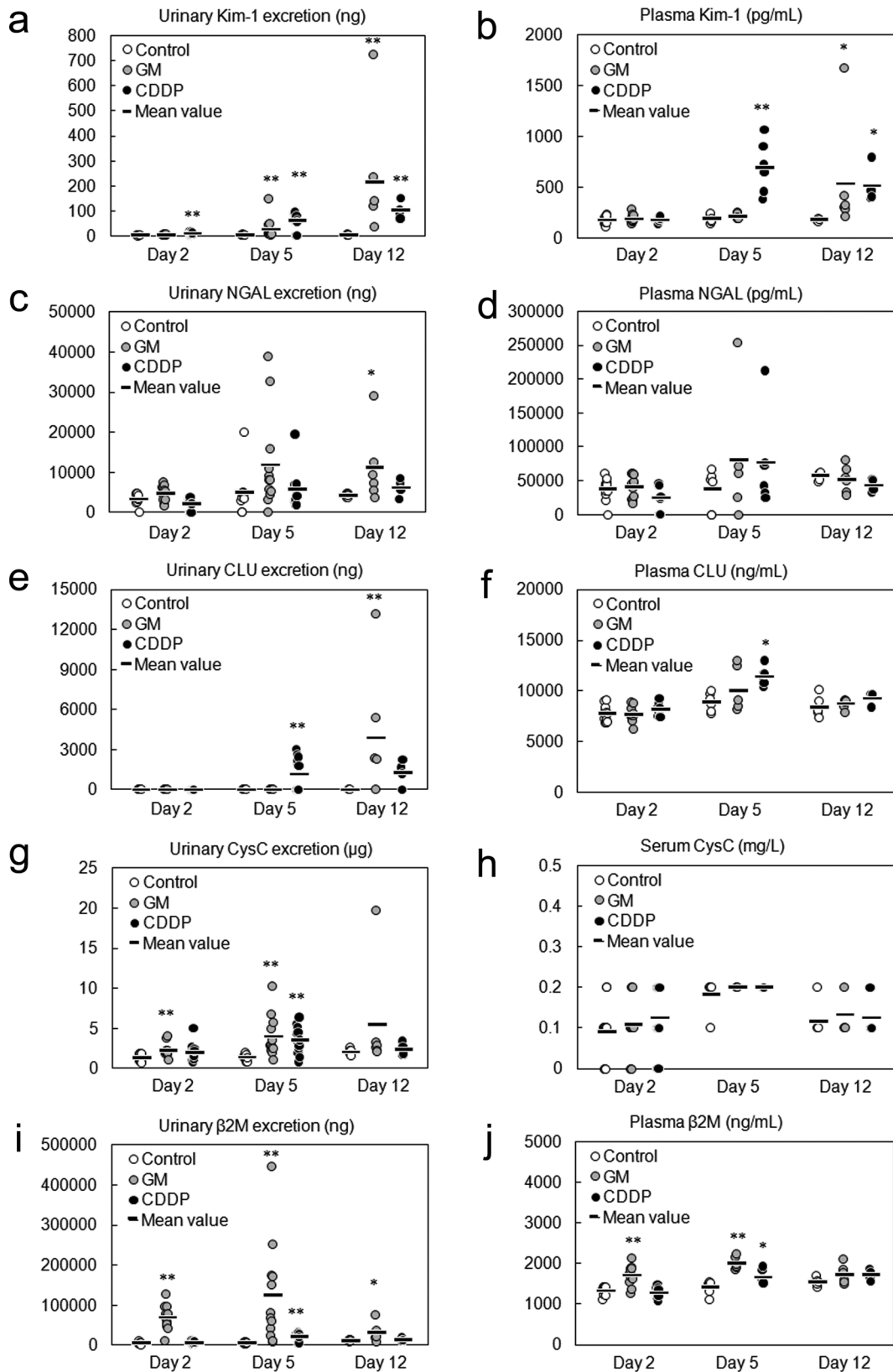
CysC immunoreactivity was present in the proximal convoluted tubules in control animals at every time point (Fig. 7a). In the kidney of the GM-treated animals on day 5, CysC immunoreactivity was more evident than that in the control animals, displaying the strongest intensity in the proximal convoluted tubules (Fig. 7b). In addition to this area, CysC immunoreactivity was detected in a part of the dilated tubules on day 12 (Fig. 7c).

(5)  $\beta$ 2M: The urinary  $\beta$ 2M levels were significantly increased at every time point, whereas the plasma  $\beta$ 2M levels were significantly increased on days 2 and 5 (Fig. 2i, j). Both the urinary and plasma  $\beta$ 2M levels increased from days 2 to 5 and decreased from days 5 to 12, although no significant correlation was observed ( $R = 0.33$ ,  $P = 0.13$ ; Fig. 3e).

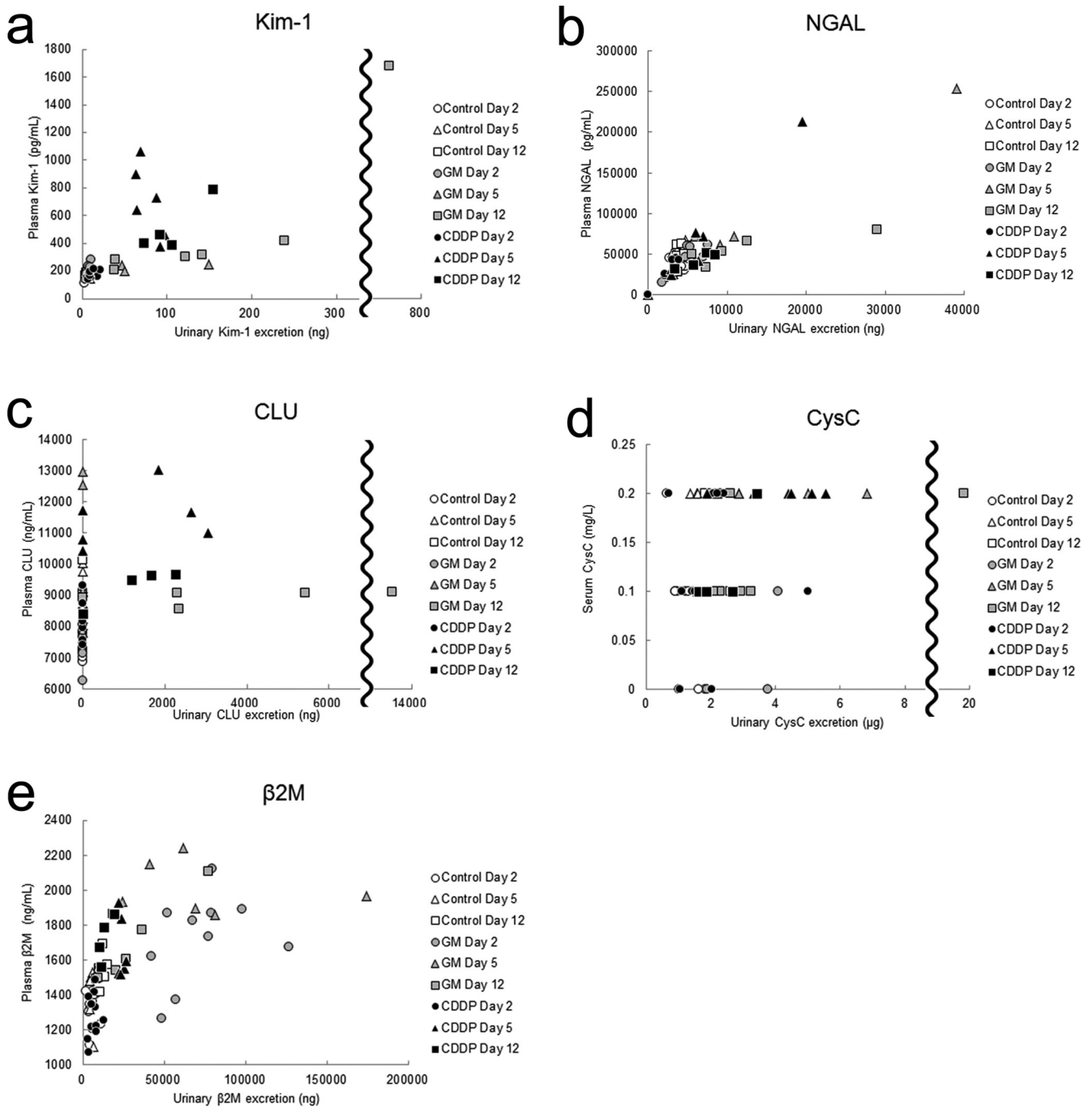
$\beta$ 2M immunoreactivity was observed in the proximal convoluted tubules in the control animals (Fig. 8), and the immunoreactivity was not affected by GM administration.

#### CDDP group

The results of histopathological examination following CDDP treatment are presented in Table 2. On day 2, nuclear chromatin margination was observed in the proximal straight tubule in the OSOM (Fig. 1d). On day 5, degeneration/necrosis and regeneration of the proximal straight tu-



**Fig. 2.** Time course of the appearance of urinary and blood biomarkers in rats given a single administration or 4 administrations of gentamicin (GM, 240 mg/kg/day), rats given a single administration of cisplatin (CDDP, 5 mg/kg) or rats used as the vehicle control. The urinary and serum/plasma levels of Kim-1(a, b), NGAL (c, d), CLU (e, f), CysC (g, h) and β2M (i, j) were assayed at the indicated time points (the day after initial dosing shown as day 2). Individual data and mean values are expressed. The sample numbers were as follows: n = 12 for urinalysis on days 2 and 5 and blood analysis on day 2, except for the CDDP group (n = 10 for urinalysis on day 5), and n = 6 for urinalysis on day 12 and blood analysis on days 5 and 12, except for the CDDP group (n = 4 for urinalysis and blood analysis on day 12). \* $P < 0.05$  and \*\* $P < 0.01$  vs. control group (two-tailed Dunnett's multiple comparison test).



**Fig. 3.** Relationship between the urinary and serum/plasma levels of biomarkers. Pearson’s product-moment correlation coefficient was used to evaluate the linear association between the urinary and blood levels of each biomarker. Kim-1 (GM group, coefficient of correlation (R) = 0.96,  $P < 0.01$ ; CDDP group,  $R = 0.71$ ,  $P < 0.01$ ) (a), NGAL (GM group,  $R = 0.89$ ,  $P < 0.01$ ; CDDP group,  $R = 0.95$ ,  $P < 0.01$ ) (b), CLU (GM group,  $R = 0.10$ ,  $P = 0.65$ ; CDDP group,  $R = 0.60$ ,  $P < 0.01$ ) (c), CysC (GM group,  $R = 0.25$ ,  $P = 0.26$ , CDDP group,  $R = 0.40$ ,  $P = 0.07$ ) (d),  $\beta 2M$  (GM group,  $R = 0.33$ ,  $P = 0.13$ , CDDP group,  $R = 0.68$ ,  $P < 0.01$ ) (e).

bule and karyomegaly in the proximal straight tubule were observed in the OSOM (Fig. 1e). Although the majority of these changes were still observed on day 12, the degenerated/necrotic areas began to be replaced by regenerated tubules (Fig. 1f) accompanied by slight dilatation. The BUN, CRE, ALB and TP levels showed significant increases mainly on day 5, and the levels on day 12 were similar to

those of the control levels (Table 3).

(1) Kim-1: The urinary Kim-1 levels significantly increased at every point (Fig. 2a). The plasma Kim-1 level was significantly increased on days 5 and 12 (Fig. 2b). The plasma Kim-1 levels showed a strong correlation with the urinary levels ( $R = 0.71$ ,  $P < 0.01$ ; Fig. 3a). The urinary and plasma Kim-1 levels increased from days 2 to 5 and re-

**Table 4.** Immunohistochemistry in the Kidney after Administration of GM or CDDP

Group	Biomarkers	Grade	Control			GM			CDDP		
			Day 2	Day 5	Day 12	Day 2	Day 5	Day 12	Day 2	Day 5	Day 12
			(12)	(6)	(6)	(12)	(6)	(6)	(12)	(6)	(4)
Kim-1	-		12	6	6	12	0	0	0	0	0
	+		0	0	0	0	1	0	12	0	0
	++		0	0	0	0	5	5	0	4	0
	+++		0	0	0	0	0	1 <sup>a</sup>	0	2	4
NGAL	-		12	6	6	12	1	0	0	0	0
	+		0	0	0	0	3	5	12	0	1
	++		0	0	0	0	2	1 <sup>a</sup>	0	4	3
	+++		0	0	0	0	0	0	0	2	0
CLU	-		12	6	6	12	6	1	12	0	0
	+		0	0	0	0	0	4	0	2	1
	++		0	0	0	0	0	1 <sup>a</sup>	0	2	3
	+++		0	0	0	0	0	0	0	2	0
CysC	++		12	6	6	12	6 <sup>b</sup>	5	12	2	2
	+++		0	0	0	0	0	1 <sup>a</sup>	0	4	2
β2M	++		12	6	6	12	6	6	12	6	4

Results are expressed as the number of animals with a grade for each biomarker. Numbers in parentheses are the numbers of animals examined. Grade: -, negative; +, positive for a small area of tubular epithelium in the renal cortex or medulla; ++, positive for a large area of tubular epithelium in the renal cortex or medulla; +++, positive for a large area of tubular epithelium in both the renal cortex and medulla. <sup>a</sup> Observed with dilated renal tubular epithelium. <sup>b</sup> Increased intensity compared with the immunohistochemical expression in the control group.

mained at high levels until day 12.

The results of immunohistochemistry following CDDP treatment are presented in Table 4. Kim-1 immunoreactivity was detected in a few proximal straight tubules on day 2 (Fig. 4d). This immunoreactivity corresponded poorly with that of the lesion with nuclear chromatin margination. Kim-1 immunoreactivity was also detected in the degenerated/necrotic and regenerated tubules on days 5 and 12, respectively (Fig. 4e, f).

(2) NGAL: There were no apparent increases in the urinary and plasma NGAL levels at any time point (Fig. 2c, d), although the urinary and plasma levels showed correlation ( $R=0.95$ ,  $P<0.01$ ; Fig. 3b).

NGAL immunoreactivity was detected in a few proximal straight tubules in the OSOM on day 2 (Fig. 5d). Similar to Kim-1, NGAL immunoreactivity corresponded poorly with that of the lesion with nuclear chromatin margination. NGAL immunoreactivity was also detected in degenerated/necrotic and regenerated tubules on days 5 and 12, respectively, and their lumens on day 5 (Fig. 5e, f). The intensity of immunoreactivity decreased from days 5 to 12.

(3) CLU: The urinary CLU levels increased on days 5 and 12, although the increase on day 12 was not significant (Fig. 2e, f). Plasma CLU levels were also increased on day 5 and then decreased from days 5 to 12. The urinary and plasma CLU levels showed moderate correlation ( $R=0.60$ ,  $P<0.01$ ; Fig. 3c).

CLU immunoreactivity was detected in a part of the degenerated/necrotic tubules in the OSOM on days 5 and 12 (Fig. 6c, e), and the intensity of immunoreactivity de-

creased from days 5 to 12. CLU immunoreactivity was also observed in the lumens of normal tubules in the medulla and in the normal distal tubules over the medulla and cortex on day 5 (Fig. 6c, d).

(4) CysC: Urinary CysC levels significantly increased on day 5; however, serum CysC levels did not change at any time point (Fig. 2g, h) and did not correlate with the urinary levels ( $R=0.40$ ,  $P=0.07$ ; Fig. 3d).

CysC immunoreactivity was present in the proximal convoluted tubules in the cortex of the kidneys of control animals (Fig. 7d). CDDP-treated animals showed additional CysC immunoreactivity in a part of the tubules including degenerated/necrotic and regenerated tubules and the tubular lumens of the medulla on days 5 (Fig. 7e) and 12.

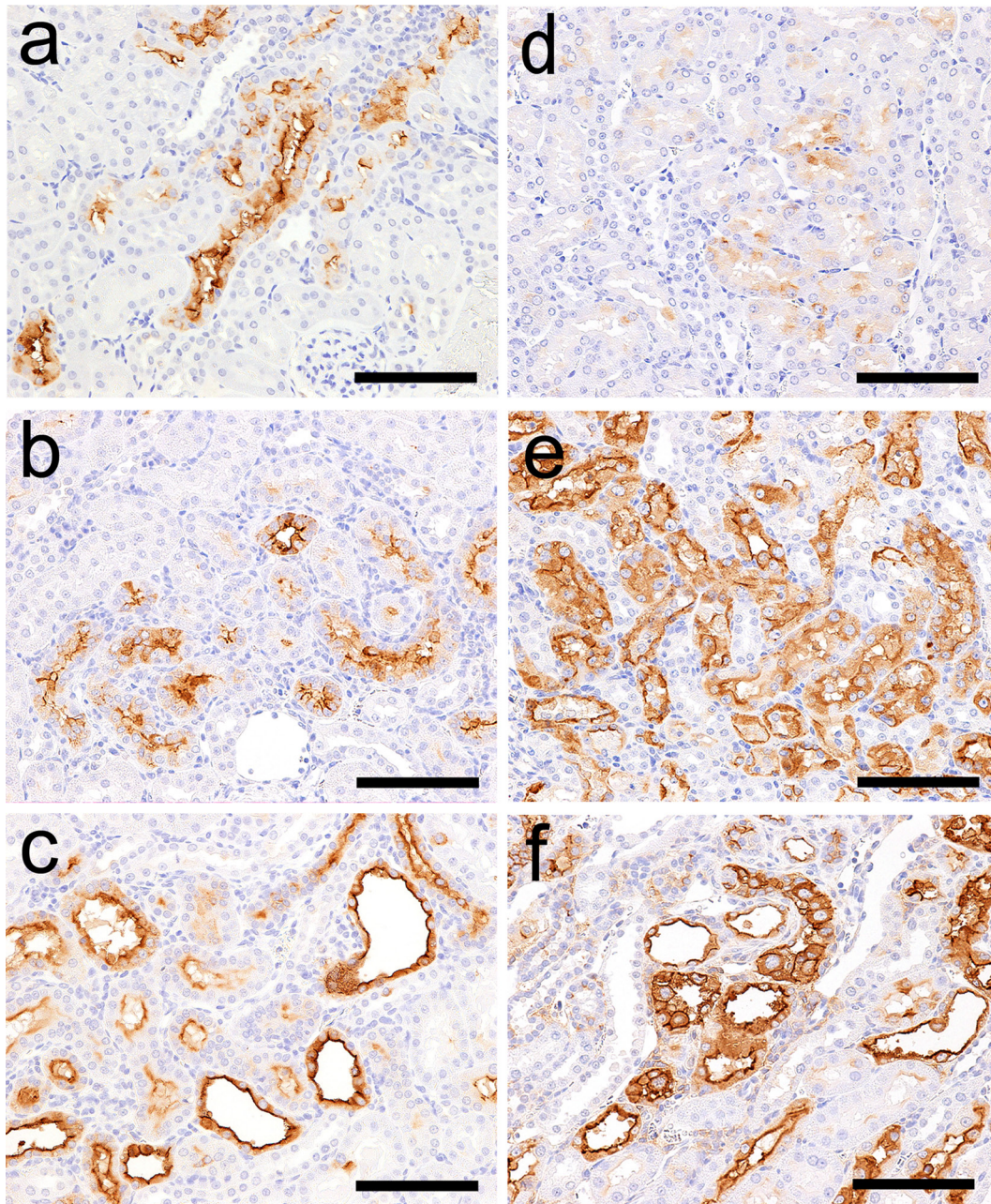
(5) β2M: Urinary and plasma levels of β2M significantly increased on day 5 (Fig. 2f, j), and they were moderately correlated ( $R=0.68$ ,  $P<0.01$ ; Fig. 3e).

In immunohistochemistry, there was no significant difference between control and CDDP-treated animals.

## Discussion

To investigate useful biomarkers that can detect proximal tubular injury, we determined the time course changes in levels of five biomarkers (Kim-1, NGAL, CLU, CysC and β2M) in urine and blood. Immunohistochemical analysis for these biomarkers was also performed to confirm the association between histopathological injury and biomarker behaviors in the kidney. Overall results are summarized in Table 5.

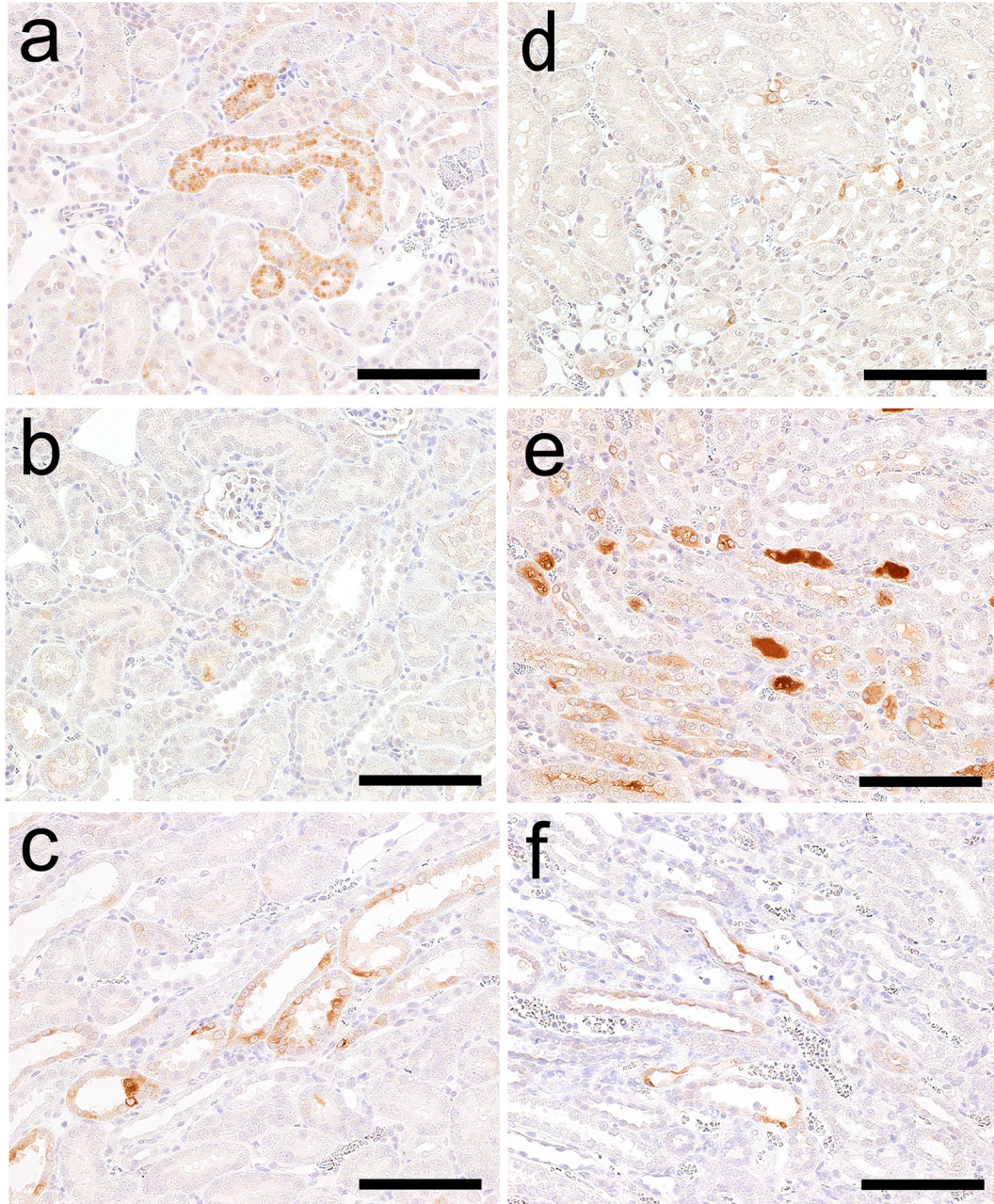




**Fig. 4.** Kim-1 immunohistochemistry in the kidneys of rats following administration of GM or CDDP. Kim-1 immunoreactivity in a part of the proximal convoluted tubules in a nephron on day 5 (a), regenerated tubules on day 12 (b) and dilated tubules on day 12 (c) in the GM group. Kim-1 immunoreactivity in a few proximal straight tubules in the OSOM on day 2 (d) and the immunoreactivity in degenerate/necrotic and regenerated tubules on day 5 (e) and day 12 (f) in the CDDP group. Bar = 100  $\mu$ m (a–f).

This study suggested that urinary Kim-1, CysC and  $\beta$ 2M levels are especially useful for detecting proximal tubular injury. Although urinary levels of all five biomarkers are known to reflect a proximal tubular injury<sup>3</sup>, NGAL and CLU did not increase in association with degeneration/necrosis of the proximal tubule (on day 5) in GM and/or CDDP groups. Moreover, the changes in the urinary CysC and  $\beta$ 2M levels were considered to successfully represent the time course changes in histopathology; the increased levels of these biomarkers accompanying histological in-

jury (on day 5) returned to close to the control range in the recovery phase (on day 12). Immunohistochemical analysis was useful in confirming the association of proximal tubular injury with urinary levels of Kim-1 but not with urinary levels of CysC and  $\beta$ 2M. The immunoreactivity patterns of CysC changed in both GM- and CDDP-treated animals and resembled those in the past reports<sup>25, 26</sup>; however, the changes in CysC immunoreactivity did not correlate with the time of increase of CysC levels in urine, and no change in  $\beta$ 2M immunoreactivity was observed in any of the treated

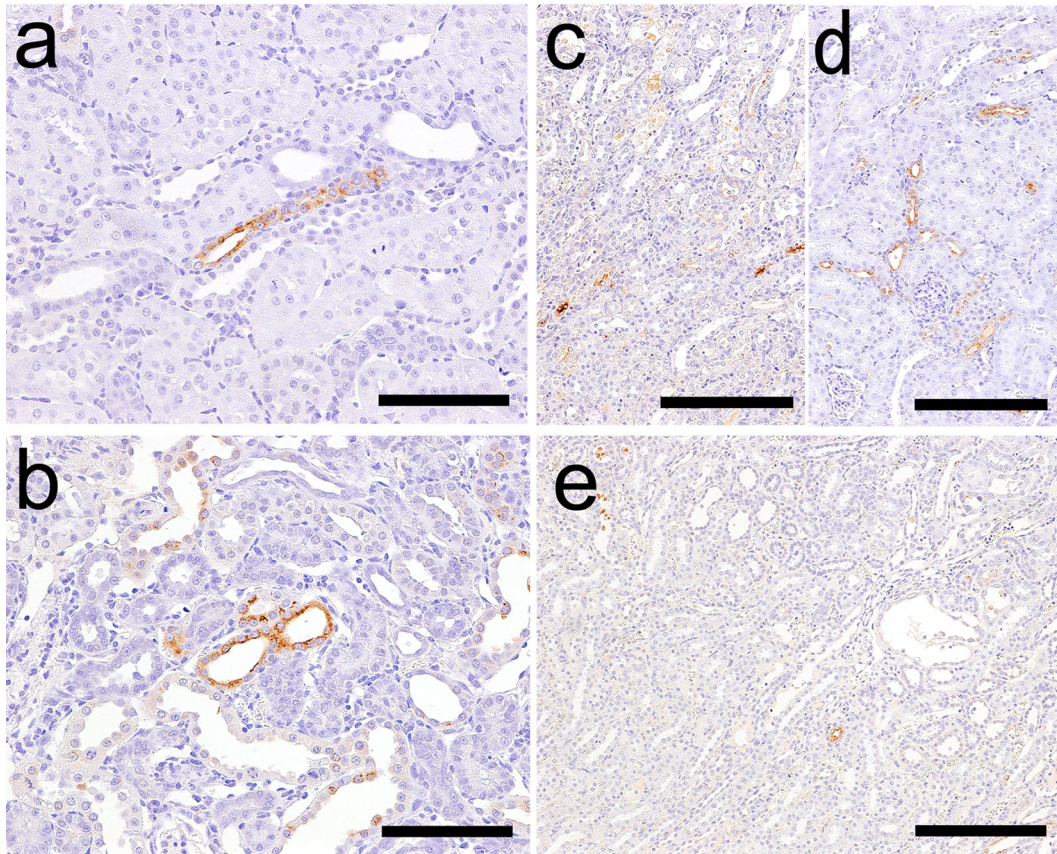


**Fig. 5.** NGAL immunohistochemistry in the kidneys of rats following administration of GM or CDDP. NGAL immunoreactivity in a part of the proximal convoluted tubules sporadically on day 5 (a) and in a few regenerated tubules (b) and a part of dilated tubules on day 12 (c) in the GM group. NGAL immunoreactivity in a few proximal straight tubules in the OSOM on day 2 (d) and in degenerate/necrotic and regenerated tubules and their lumens on day 5 (e) and in a few degenerate/necrotic and regenerated tubules on day 12 (f) in the CDDP group. Bar = 100  $\mu\text{m}$  (a–f).

animals in this study.

We also revealed that urinary Kim-1, CysC and  $\beta$ 2M levels increased prior to degeneration/necrosis of the proximal tubule, depending on the toxicity characteristics of the treatment. In the GM group, the CysC and  $\beta$ 2M levels markedly increased on day 2 without any histopathological findings. CysC and  $\beta$ 2M are low-molecular-weight proteins that are almost completely filtered from the blood in the glomerulus, and the filtered CysC and  $\beta$ 2M are reabsorbed

and metabolized by the proximal convoluted tubules<sup>3,11</sup>. An increase in the urinary levels of these biomarkers indicates impaired tubular reabsorption, reflecting renal tubular injury or protein overload, such as that from glomerular injury<sup>11,27</sup>. In addition, polycationic drugs such as GM increase the glomerular filtration of large anionic proteins, such as albumin, through a change in electrical charge<sup>11,28</sup>. Coupled with the increase of TP and ALB levels observed at the same time point, the increase in urinary CysC and  $\beta$ 2M levels



**Fig. 6.** CLU immunohistochemistry in the kidneys of rats following administration of GM or CDDP. CLU immunoreactivity in a few normal distal tubules in the cortex (a) and in a part of the dilated tubules on day 12 (b) in the GM group. CLU immunoreactivity in a part of degenerate/necrotic tubules and lumens of normal tubules in the medulla (c) and the distal tubules over the cortex to medulla on day 5 (d) and in a few degenerate/necrotic tubules in the medulla on day 12 (e) in the CDDP group. Bar = 100  $\mu$ m (a, b), 200  $\mu$ m (c–e).

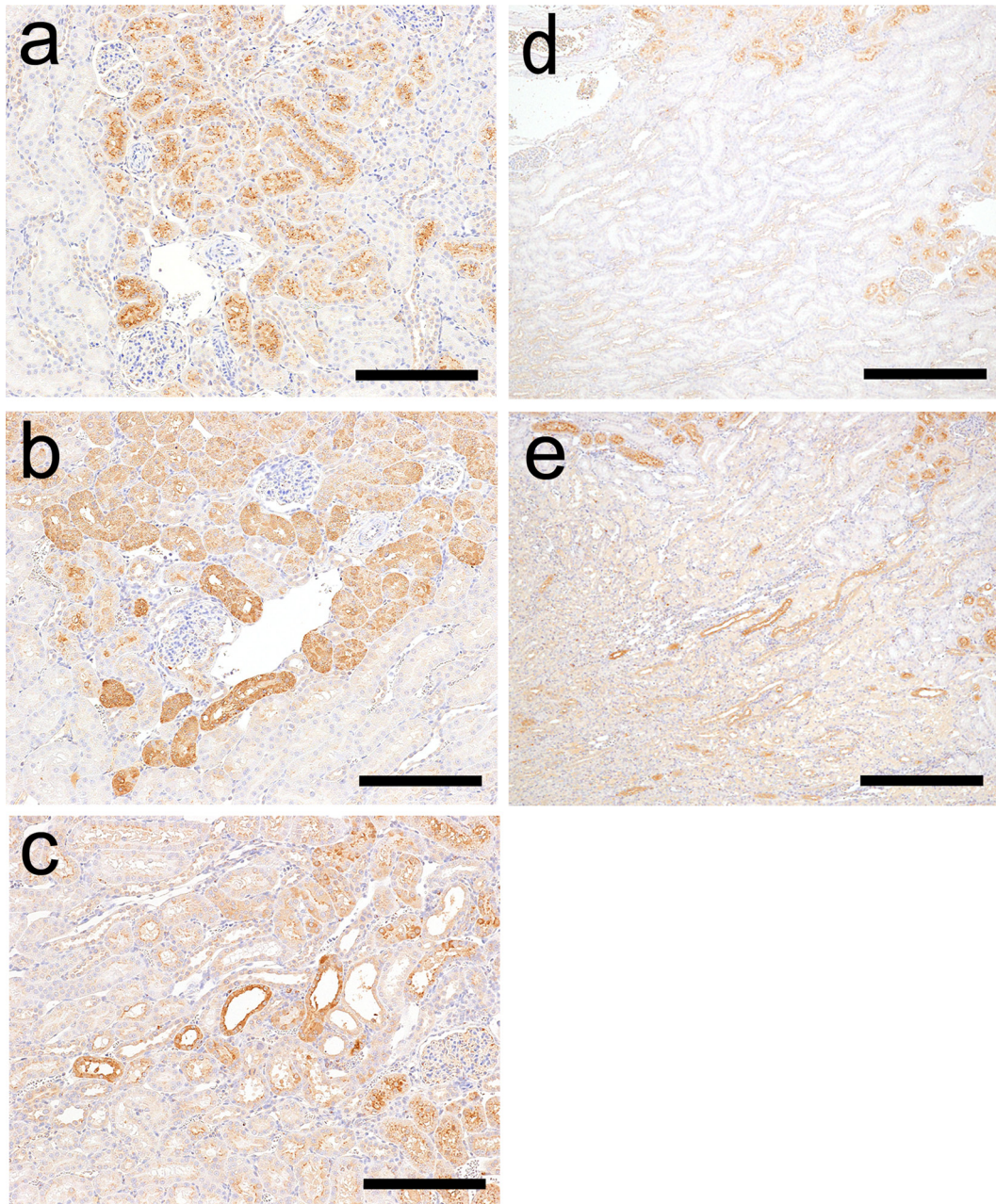
without histopathological findings suggests the impairment of tubular reabsorption resulting from protein overload, not proximal tubular injury. On the other hand, urinary Kim-1 levels significantly increased on day 2 before degeneration/necrosis of the proximal tubule in the CDDP group. In the case of CDDP, proximal tubular injury begins with oxidative damage of cellular components<sup>14</sup>; thus, the levels of Kim-1, which is expressed in the injured renal tubular epithelial membrane<sup>29, 30</sup>, changed first, reflecting cytotoxicity. As for the nuclear margination observed at the same time point, its involvement in the changes in urinary Kim-1 levels was not completely understood because of the inconsistency between the lesion and immunoreactivity. However, considering that both of them were observed in the same area, nuclear margination may be the histopathological event that leads to degeneration/necrosis of the proximal tubule.

We revealed that both urinary and plasma  $\beta$ 2M are sufficiently sensitive in detecting proximal tubular injury. Notably, plasma  $\beta$ 2M levels increased in a manner similar to urinary  $\beta$ 2M level, although the magnitude of the changes was not perfectly correlated in terms of individual values. According to several reports from human studies, increased

$\beta$ 2M levels in blood correlate with a decreased glomerular filtration rate<sup>31, 32</sup>, whereas increased  $\beta$ 2M levels in urine correlate with the impairment of tubular reabsorption<sup>11, 27</sup>. Therefore, the plasma  $\beta$ 2M level is considered to reflect a decreased glomerular filtration rate in parallel with a proximal tubular injury, although the relationship between the urinary and plasma  $\beta$ 2M levels is still not completely understood.

In contrast, serum CysC, which has characteristics similar to those of  $\beta$ 2M, did not change at any time point. This may be because of the difference in the measuring method: measurement with an autoanalyzer could not detect the changes in serum CysC levels because the sensitivity of the autoanalyzer was lower than that of commercial ELISA kits.

It was also noteworthy that plasma Kim-1 levels revealed good associations with urinary Kim-1 levels, although the sensitivity of plasma Kim-1 was inferior to that of urinary Kim-1. A recent study reported that plasma Kim-1 levels increased after GM treatment in rats<sup>18</sup>. Although the exact mechanism of the increase in blood Kim-1 levels remains unclear, it may be due to its direct leakage into



**Fig. 7.** CysC immunohistochemistry in the kidneys of rats following administration of GM, CDDP or a vehicle control. CysC immunoreactivity in the proximal convoluted tubules in control animals on day 5 (a, d). CysC immunoreactivity with a strong intensity in the proximal convoluted tubules of rats on day 5 (b) and immunoreactivity in a part of dilated tubules in addition to the proximal convoluted tubules on day 12 (c) in the GM group. CysC immunoreactivity in a part of the tubules including degenerated/necrotic and regenerated tubules and the tubular lumens of the medulla on day 5 (e). Bar = 200  $\mu\text{m}$  (a, b, c), 400  $\mu\text{m}$  (d, e).

the blood circulation because disturbance of cell polarity or permeability could occur after proximal tubular injury.

In conclusion, this study suggested that urinary Kim-1, CysC and  $\beta 2\text{M}$  levels are useful biomarkers for detecting proximal tubular injury because an increase in them is accompanied by degeneration/necrosis of the proximal tubule, and an increase in them was also observed prior to degeneration/necrosis depending on the toxicity characteristics. Moreover, we revealed that not only urinary biomarker levels but also plasma  $\beta 2\text{M}$  levels are useful for detecting proximal tubular injury because the latter increased in a manner

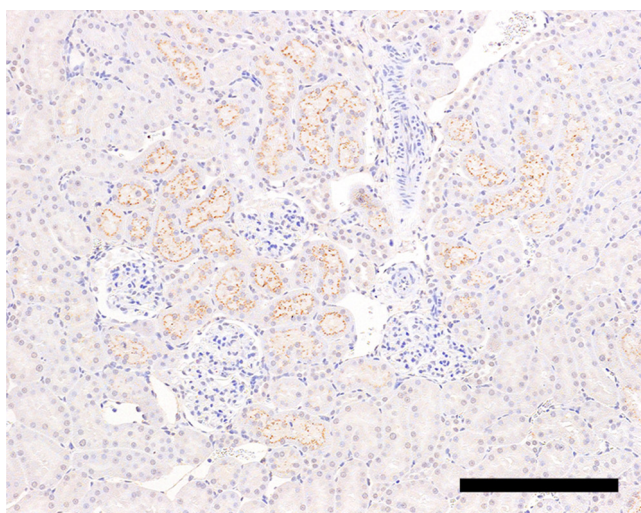
similar to that observed for urinary  $\beta 2\text{M}$  levels. Although the increase in plasma  $\beta 2\text{M}$  levels was considered to reflect the decrease in glomerular filtration rate rather than the direct effect of a proximal tubular injury, plasma  $\beta 2\text{M}$  levels could be an alternative biomarker in preclinical studies in cases where urine samples are unavailable.

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**Table 5.** Summary of the Changes in Histopathology and Biomarkers

		GM			CDDP		
		Day 2	Day 5	Day 12	Day 2	Day 5	Day 12
<b>Histopathology (HE)</b>							
	Eosinophilic granule	-	+-+++	-	-	-	-
	Chromatin margination	-	-	-	+	-	-
	Degeneration/necrosis	-	+	-	-	++	+
	Regeneration	-	-	+-+++	-	+	++
<b>BUN/CRE (sera)</b>		-	-	↑	-	↑↑	-
<b>TP/ALB (urine)</b>		↑	↑↑	-	-	↑	-
<b>Kim-1</b>	Urine	-	↑	↑↑	↑	↑↑	↑↑
	Plasma/sera	-	-	↑	-	↑	↑
	IHC	-	+-+++	++-+++	+	++-+++	+++
<b>NGAL</b>	Urine	-	(↑)	↑	-	-	-
	Plasma/sera	-	-	-	-	-	-
	IHC	-	--+++	+-+++	+	++-+++	+-+++
<b>CLU</b>	Urine	-	-	↑	-	↑	(↑)
	Plasma/sera	-	-	-	-	↑	-
	IHC	-	-	--+++	-	+-+++	+-+++
<b>CysC</b>	Urine	↑	↑↑	-	-	↑↑	-
	Plasma/sera	-	-	-	-	-	-
	IHC	-	++ <sup>a</sup>	++-+++ <sup>b</sup>	-	++-+++	++-+++
<b>β2M</b>	Urine	↑	↑↑	↑	-	↑	-
	Plasma/sera	↑	↑	-	-	↑	-
	IHC	-	-	-	-	-	-

-, no noteworthy changes; -, +, ++, +++, the grade of histopathology/immunohistochemistry. (↑), increase tendency; ↑, significant increase; ↑↑, more apparent increase than ↑. <sup>a</sup> Increased intensity compared with the immunohistochemical expression in the control group. <sup>b</sup> Increased only in one animal.



**Fig. 8.** β2M immunoreactivity in the proximal convoluted tubules of a control animal on day 5. Bar = 200 μm.

**Disclosure of Potential Conflicts of Interest:** The authors declare that they have no competing interests.

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