Letter

Neutrophil gelatinase-associated lipocalin, a sensitive urinary biomarker of acute kidney injury in dogs receiving gentamicin

Kiyonori Kai, Takashi Yamaguchi, Yu Yoshimatsu, Junzo Kinoshita,
Munehiro Teranishi and Wataru Takasaki

Medical Safety Research Laboratories, Daiichi Sankyo Co., Ltd., 717 Horikoshi, Fukuroi, Shizuoka 437-0065, Japan

(Received November 7, 2012; Accepted January 4, 2013)

ABSTRACT — A sensitive urinary biomarker for acute kidney injury (AKI) was investigated in beagle dogs with nephrotoxicity induced by gentamicin. Gentamicin sulphate at 25 or 50 mg/kg was injected (s.c.) for 9 days, and conventional urinalysis, ELISA assay of neutrophil gelatinase-associated lipocalin (NGAL) in urine, blood chemistry, and pathological examinations were performed. The dog given gentamicin at 25 mg/kg only showed slight deposition of lysosomal granules in the proximal tubular epithelium of the kidneys without any other significant changes even though urinary NGAL was elevated on Day 10 (day of necropsy). In the dog receiving gentamicin at 50 mg/kg, increases in urinary NGAL were observed on Days 3 and 5, and absence of urination, marked increases in serum urea nitrogen and creatinine, enlargement and discoloration of the kidneys with marked necrosis, and swelling of proximal epithelium were observed. In conclusion, urinary NGAL is considered to be a candidate as a sensitive predictable biomarker of AKI in the gentamicin-induced nephrotoxicity model in dogs.

Key words: NGAL, Dogs, Acute kidney injury, Urinary biomarker, Gentamicin

---

INTRODUCTION

Many therapeutic and diagnostic agents used in humans have the potential to cause renal damage. Hospital-based studies have suggested that the incidence of life-threatening nephrotoxicity causing admission or readmission to hospital is quite high, particularly in older people or those with impaired renal function; acute tubular necrosis is one type of pathology linked to drug-induced renal failure in patients (Greaves, 2012). Conceptually, acute renal failure (ARF) is defined as a rapid decrease of glomerular filtration rate (GFR), which can occur over hours to weeks, and it is usually reversible in humans. The clinical spectrum of decline in GFR is broad and any minor deterioration in GFR and kidney dysfunction should be diagnosed as the presence of kidney damage. Therefore the term ARF is replaced by that of acute kidney injury (AKI) and the term ARF preferably should be restricted to patients who have AKI and need renal replacement therapy. AKI is diagnosed from a progressive rise in serum creatinine (CRE) over several days, which may or may not be accompanied with oliguria. Serum CRE changes

can differ from actual GFR changes. Clinical diagnosis is often delayed due to lacking signs of renal function deteriorations. Delayed diagnosis is a problem because lacking signs of renal function deteriorations (Adiyanti and Loho, 2012). For a long time, blood urea nitrogen (UN) and CRE have been relied upon as the primary diagnostic biomarkers of drug-induced acute kidney tubular alterations. However recent studies reported that routine renal function tests based on serum CRE, UN and urine production were outdated because they failed to identify early stages of renal dysfunction and structural injury. Therefore 7 urinary biomarkers (Dieterle *et al.*, 2010); i.e., Kim-1, albumin, total protein, β_2 -microglobulin, cystatin C, clusterin, and trefoil factor-3, were subsequently proposed to and approved by the Food and Drug Administration (FDA), the European Medicines Agency (EMA), and the Pharmaceuticals and Medical Devices Agency (PMDA) as biomarkers to sensitively identify AKI in preclinical toxicity studies. Commercial tools to measure these urinary biomarkers were mainly validated in rats (Dieterle *et al.*, 2010; Gautier *et al.*, 2010; Hoffmann *et al.*, 2010; Tonomura *et al.*, 2010; Fuchs *et al.*, 2012). In

---

Correspondence: Kiyonori Kai (E-mail: kai.kiyonori.xb@daiichisankyo.co.jp)

addition to these biomarkers, neutrophil gelatinase-associated lipocal (NGAL), a member of the lipocalin superfamily, has been proposed as a candidate for a sensitive urinary biomarker to detect AKI (Bonventre *et al.*, 2010; Mori, 2005; Mori and Nakao, 2007). Unfortunately, in part due to the lack of commercially available assays for non-rodents, limited data comparing the performance of the exploratory and approved markers are publicly available so far.

In this study, we selected gentamicin, a well-known nephrotoxic agent that induces AKI in experimental animals and humans, and injected it subcutaneously to beagle dogs for 9 days and evaluated urinary NGAL using a commercial ELISA kit as a sensitive urinary biomarker in comparison with conventional blood and urinary toxicity makers and pathological examinations.

MATERIALS AND METHODS

Test article

ELTACIN injection, an injectable product of gentamicin sulfate was purchased from FujiPharma (Shizuoka, Japan).

Animals and housing conditions

Two male beagle dogs were purchased from Narc Corporation (Chiba, Japan) for gentamicin treatment, and 6 males and 6 females were additionally obtained from Marshall BioResources, Japan (Ibaraki, Japan) for collection background data of urinalysis. The dogs were individually housed in stainless steel cages (W93.5 cm × W80 cm × H78.5 cm) in an air-conditioned room (temperature, 18 to 28°C; relative humidity, 30 to 70%). A light/dark cycle of 12 hr and ventilation rate of 10 to 20 air changes/hr were used in the animal rooms. Two hundred and twenty grams of basal diet (Certified Canine Diet 5007, PMI Nutrition International, Inc., St. Louis, MO, USA) was given to each dog in the morning and tap water was available *ad libitum*. All experimental procedures were performed in accordance with the Guidelines for Animal Experimentation issued by the Japanese Association for Laboratory Animal Science (Japanese Association for Laboratory Animal Science, 1987).

Experimental design

Two male dogs (Narc beagles, approximately 23 months old, 9.6–10.1 kg) were used for gentamicin treatment; no control group was used because the main objective this study was to induce renal toxicity in dogs by gentamicin and in accordance with the 3Rs (Replacement, Reduction, and Refinement) policy. In the reports of

repeated intramuscular doses of gentamicin in dogs, gentamicin (i.m., 15 mg/kg twice daily) administration for 4 and 7 days induced necrosis of proximal tubules (Dobyan *et al.*, 1982) and intramuscular injection (20 mg/kg) once daily for 9 days induced nephrotoxicity characterized only with significant elevation of serum biochemical markers (Varzi *et al.*, 2007). To reduce local irritation induced by gentamicin, subcutaneous (s.c.) injection was selected and 2 dose levels (25 and 50 mg/kg, once daily) for 9 days were determined for the treatment in this study. ELTACIN injection (40 mg/ml) was administered subcutaneously to the dorsal neck region at 0.625 (25 mg/kg) and 1.25 ml/kg (50 mg/kg) to 1 dog each for 9 days, respectively. The day of the first dosing was regarded as Day 1. The 2 dogs were systemically anesthetized intravenously with pentobarbital sodium (Somnopentyl Injection, Kyoritsu Seiyaku Corporation, Tokyo, Japan) and euthanized by exsanguination from the carotid arteries on Day 10 (the day after the last dosing).

Clinical observation, urinalysis and blood chemistry

Clinical signs were observed immediately after administration and food consumption was calculated from the difference between the supplied and residual amounts daily. The body weights were measured on Days 1, 5 and 10. For urinalysis, the water supply was suspended to avoid contamination of spilled water into the urine during urine collection. The fresh urine samples were collected from 7 to 9 am on Days -6 (6 days before the first dosing), 3, 5, and 10 and the volume of urine accumulated for 2 hr was measured using a graduated cylinder. The fresh urine samples were centrifuged at 1,500 rpm for 5 min (GS-6K, Beckman Instruments Inc., Tokyo, Japan), and the supernatants were analyzed. Urinary creatinine (CRE), γ -glutamyl-transferase (GGT), N-acetyl- β -D-glucosaminidase (NAG), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), protein (PRO), glucose (GLU), inorganic phosphorus (IP), sodium (Na), and potassium (K) were measured using an autoanalyzer (TBA-200FR, Toshiba Medical Systems Co., Ltd., Tokyo, Japan). Urinary NGAL was measured using a NGAL ELISA kit (BIOPORTO® Diagnostics, Gentofte, Denmark) for dogs according to the manufacturer's instructions. The detection range of urinary NGAL was 4 to 400 pg/ml. The values of the aforementioned parameters except for urinary volume and CRE were normalized based on the corresponding urinary CRE (unit /g · CRE) as Waikar *et al.* (2010) suggested. Blood samples were obtained from the jugular vein on Days -5, 1, 3, 5 (in the morning, before the injection of gentamicin) and

10 (before the necropsy), and placed in separating agent-containing spitz tubes (Separapid tube S, Sekisui Medical Co., Ltd., Tokyo, Japan). Thereafter, the samples were allowed to stand at room temperature for about 30 min and centrifuged at 3,000 rpm and 4°C for 10 min with a refrigerated centrifuge (CF9RX, Hitachi Koki Co., Ltd., Tokyo, Japan). Serum urea nitrogen (UN) and CRE were measured using an autoanalyzer (TBA-200FR). For background data for urinalysis, fresh urine (2 hr; 7 to 9 am) and pooled urine (15 hr; 4 pm to 7 am) were collected from 6 males (14 to 17 months old, 8.30 to 10.65 kg) and 6 females (14 to 17 months old, 4.75 to 8.90 kg) using naive Marshall beagle dogs and analyzed as described above.

Pathological examinations

Immediately after euthanasia on Day 10, the two dogs receiving gentamicin were necropsied and examined macroscopically. The kidneys were fixed in 10 vol% neutral buffered formalin and embedded in paraffin. Thereafter the specimens were cut at approximately 5 µm in thickness and stained with hematoxylin and eosin (H&E) for light microscopic examination. Histopathological findings of the lesions were classified and scored for several areas in each dog as follows: mild degree - sporadic distribution and difficult to be detected at a low magnification; moderate degree - easily found at a low magnification; marked degree - extensive distribution and easily found at a low magnification. In order to identify the eosinophilic granules in proximal epithelium, additional paraffin sections were immunohistochemically stained with rabbit polyclonal anti-human lysosomal-associated membrane protein 1 (LAMP1) antibody (ab24170, 1: 1,500 dilution, Abcam®, Tokyo, Japan), followed by rabbit Envision/HRP (DAKO Japan Co., Ltd., Tokyo, Japan). In addition, immunohistochemistry for the kidney was conducted in frozen and paraffin section using mouse monoclonal NAGL anti-dog antibody (ABS 047-17, BIOPORTO® Diagnostics) and mouse monoclonal anti-rat NAGL antibody (ABS 039-08 BIOPORTO® Diagnostics), followed by mouse Envision/HRP (DAKO Japan Co., Ltd.).

RESULTS

Clinical observation, urinalysis and blood chemistry in dogs receiving gentamicin

No changes in clinical signs, body weight or food consumption were noted in the dog given gentamicin at 25 mg/kg. In the dog given gentamicin at 50 mg/kg, loose stool was observed on Days 3, 6 and 10 and vomitus of food particles was seen on Days 8 to 10. Vomitus of whit-

ish froth and decreased activity were additionally noted on Day 10 (just before the necropsy). Food consumption decreased by 21% and 100% on Days 9 and 10, respectively but no changes in body weight of less than 10% in comparison to the pre-dosing value were observed. In urinalysis, PRO increased more than 5-fold in comparison to pre-dosing value on Days 5 (46-fold) and 10 (40-fold) and NGAL was elevated on Day 10 (141-fold) in the dog given gentamicin at 25 mg/kg. However the pre-dosing value of PRO (9.93 mg/g·CRE) was very low in comparison to the pre-dosing value of the other dog (1013.0 mg/g·CRE), and the increases in PRO were not time-dependent. In the dog given gentamicin at 50 mg/kg, fresh urine could not be collected on Day 10. NGAL increased more than 5-fold in comparison to pre-dosing value on Days 3 (32-fold) and 5 (> 225-fold), and IP was elevated on Day 5 (6-fold). ALP transiently increased on Day 3 (7-fold) (Fig. 1, Table 1). In blood chemistry, no increases more than 2-fold in comparison to pre-dosing value were noted in the dog given gentamicin at 25 mg/kg. In the dog given gentamicin at 50 mg/kg, notable changes were not seen in blood chemistry on Days 3 to 5 and serum UN and CRE increased 12.5- and 13.1-fold on day 10, respectively (Fig. 1, Table 2).

Pathological examinations in dogs receiving gentamicin

No macroscopic abnormalities were observed in any organs in the dog given gentamicin at 25 mg/kg. Discoloration and enlargement in the kidneys, edema and atrophy in the adipose tissue around the kidneys, dark red foci in the stomach and dark red coloration in subcutaneous tissue (injection site) were observed in the dog given gentamicin at 50 mg/kg. In microscopy, mild deposition of eosinophilic granules in the proximal epithelium was observed in the dog given gentamicin at 25 mg/kg. Marked swelling and necrosis in proximal epithelium and hyaline cast with moderate deposition of eosinophilic granules in proximal epithelium, and slight regeneration in proximal and distal epithelia were noted in the dog given gentamicin at 50 mg/kg (Fig. 2, Table 3). In the anti-LAMP1 immunohistochemistry, numerous granules that stained positively for LAMP1 was noted in proximal epithelium in both dogs (Fig. 3). In anti-NGAL immunohistochemistry, positive signals or suitable staining of proximal and distal epithelium without non-specific reaction could not be obtained (data not shown).

Urinalysis in naive dogs

Wider variations were seen in PRO in the fresh urine (2 hr) from males and ALP in pooled urine (24 hr) from

Fig. 1. Fold changes of blood chemistry and urinalysis parameters in comparison to pre-treatment values in dogs given gentamicin at 25 or 50 mg/kg. (A) Fold changes in the dog given gentamicin at 25 mg/kg. (B) Fold changes in the dog given gentamicin at 50 mg/kg. Solid lines: Serum CRE and UN, Dashed lines: Urinary ALP, LDH, GGT, PRO and NGAL.

Table 1. Urinalysis in the dogs given gentamicin at 25 or 50 mg/kg

Dose (mg/kg)	Day	-6	3	5	10
25	Urinary volume (ml/2 hr)	8	8	16	5
	CRE (mg/dl)	100.70	166.00	121.80	210.40
	GGT (U/g-CRE)	44.69	39.76	58.29	45.63
	NAG (U/g-CRE)	5.26	5.42	17.16	13.78
	ALP (U/g-CRE)	94.34	33.73	150.25	86.50
	LDH (U/g-CRE)	119.17	30.72	163.38	108.37
	PRO (mg/g-CRE)	9.93	4.82	459.77	337.45
	GLU (mg/g-CRE)	59.58	48.19	49.26	52.28
	IP (mg/g-CRE)	540.81	1002.41	718.39	1543.73
	Na (mEq/g-CRE)	45.68	34.34	29.56	54.66
	K (mEq/g-CRE)	41.41	46.33	24.96	72.43
	NGAL (µg/g-CRE)	2.14	1.58	3.38	302.41
50	Urinary volume (ml/2 hr)	22	18	16	0
	CRE (mg/dl)	99.70	143.70	118.50	ND
	GGT (U/g-CRE)	77.23	82.81	124.05	ND
	NAG (U/g-CRE)	10.03	21.22	38.82	ND
	ALP (U/g-CRE)	158.48	1050.10	174.68	ND
	LDH (U/g-CRE)	48.14	41.06	151.90	ND
	PRO (mg/g-CRE)	1013.04	1148.23	204.22	ND
	GLU (mg/g-CRE)	40.12	41.75	59.07	ND
	IP (mg/g-CRE)	189.77	796.80	1091.98	ND
	Na (mEq/g-CRE)	198.60	110.65	59.92	ND
	K (mEq/g-CRE)	96.89	95.89	98.48	ND
	NGAL (µg/g-CRE)	3.04	89.95	>675.11*	ND

ND: Not determined due to lack of urination. The data in bold indicate the values that changed more than 5-fold in comparison to pre-dosing. *: The result from the sample (with 2,000-fold dilution) exceeded the maximum detection limit (400 pg/ml).

Urinary NGAL in acute kidney injury in dogs given gentamicin

Table 2. Blood chemistry in the dogs given gentamicin at 25 or 50 mg/kg

Dose (mg/kg)	Day	-5	1	3	5	10
25	UN (mg/dl)	9.6	8.9	11.9	9.8	13.0
	CRE (mg/dl)	0.64	0.61	0.71	0.67	0.74
50	UN (mg/dl)	10.4	10.4	13.2	13.7	130.2
	CRE (mg/dl)	0.78	0.74	0.91	1.06	10.20

The data in bold indicate the values that changed more than 2-folds in comparison to pre-dosing.

Fig. 2. Light microscopy in the dogs given gentamicin at 25 or 50 mg/kg. (A) Kidney in the dog given gentamicin at 25 mg/kg. Mild deposition of eosinophilic granules in proximal epithelium (arrows). (B) Kidney in the dog given gentamicin at 50 mg/kg. Moderate to marked swelling and necrosis in proximal epithelium and hyaline casts are mainly identified at this magnification.

Fig. 3. Anti-LAMP1 immunohistochemistry in the dog given gentamicin at 25 mg/kg. Granular positive reaction is noted in proximal epithelium.

females in comparison with the values of the other sex or the other collection time. However no clear differences in the values of GGT, LDH, GLU, IP, Na, K or NGAL were noted between males or females, or between fresh and pooled urine. The values of NGAL in males ranged from 0.95 to 5.43 $\mu\text{g/g} \cdot \text{CRE}$ in fresh urine and 1.12 to 5.41 $\mu\text{g/g} \cdot \text{CRE}$ in pooled urine, respectively. For females, the values of NGAL ranged from 0.12 to 1.76 $\mu\text{g/g} \cdot \text{CRE}$ in fresh urine and 0.19 to 2.31 $\mu\text{g/g} \cdot \text{CRE}$ in pooled urine, respectively. These results indicated that apparent changes in the NGAL values were not noted between fresh and pooled urine (Table 4).

DISCUSSION

We investigated utilization of urinary NGAL as a sensitive biomarker of AKI using the NGAL ELISA Kit for dogs produced by BIOPORTO® Diagnostics. Our data

demonstrate that the value of NGAL was stable in naive beagle dogs regardless of pooling time. Moreover NGAL was considered to be a more sensitive biomarker of gentamicin nephrotoxicity than routine clinical chemistry parameters in blood and urine of beagle dogs.

The dose levels and treatment duration selected to induce nephrotoxicity using gentamicin were 25 mg/kg (low dose) and 50 mg/kg (high dose) for 9 days. In the low dose treatment, no abnormalities that would indicate an effect on renal function or urinary parameters were noted in clinical signs, body weight or food consumption. No clear increases in serum UN or CRE, or urinary parameters except for NGAL were observed although non-time-dependent increases in PRO were observed on Days 5 and 10. NGAL increased 141-fold in comparison with the predose value and mild deposition of eosinophilic granules in proximal epithelium was observed in the dog on Day 10 but no other lesions were noted. The eosinophilic granules in proximal epithelium were identified as lysosomes by anti-LAMP1 immunohistochem-

Table 3. Macroscopic and microscopic findings in the kidneys in the dogs given gentamicin at 25 or 50 mg/kg

Macroscopic and microscopic findings	Dose (mg/kg)	
	25	50
Macroscopic findings		
Discoloration	-	P
Enlargement	-	P
Microscopic findings		
Deposition of eosinophilic granule, proximal epithelium	+	++
Swelling, proximal epithelium	-	+++
Necrosis, proximal epithelium	-	+++
Regeneration, proximal epithelium	-	+
Regeneration, distal epithelium	-	+
Hyaline cast	-	+++

- : Within normal limits, P: Present, +: Mild, ++: Moderate, +++: Marked

Table 4. Urinalysis in naive dogs

Collection Time	Parameters	Range (Median)	
		Males (n = 6)	Females (n = 6)
2 hr	Urinary volume (ml/2 hr)	1 - 128 (50)	9 - 180 (31)
	CRE (mg/dl)	185.60 - 382.00 (230.40)	100.99 - 484.40 (265.60)
	GGT (U/g·CRE)	17.54 - 130.81 (25.84)	14.63 - 50.83 (22.37)
	ALP (U/g·CRE)	4.27 - 285.31 (75.14)	5.23 - 45.12 (10.82)
	NAG (U/g·CRE)	5.34 - 24.39 (11.60)	1.21 - 5.67 (2.49)
	LDH (U/g·CRE)	9.51 - 52.93 (22.55)	9.97 - 27.44 (17.55)
	PRO (mg/g·CRE)	8.16 - 1059.76 (190.57)	0.00 - 72.25 (33.89)
	GLU (mg/g·CRE)	36.70 - 65.20 (47.74)	37.16 - 72.15 (46.3)
	IP (mg/g·CRE)	438.84 - 1652.53 (1286.86)	773.16 - 2497.67 (1324.88)
	Na (mEq/g·CRE)	4.26 - 56.42 (25.97)	3.07 - 80.14 (41.67)
	K (mEq/g·CRE)	58.72 - 121.58 (97.40)	16.12 - 148.34 (69.67)
	NGAL (µg/g·CRE)	0.95 - 5.43 (3.53)	0.12 - 1.76 (0.63)
15 hr	Urinary volume (ml/15 hr)	44 - 118 (56)	17 - 79 (52)
	CRE (mg/dl)	156.00 - 270.60 (215.80)	104.50 - 399.00 (216.00)
	GGT (U/g·CRE)	13.05 - 118.98 (23.05)	17.04 - 58.16 (29.91)
	ALP (U/g·CRE)	17.39 - 351.48 (45.90)	7.55 - 1580.73 (21.08)
	NAG (U/g·CRE)	0.93 - 25.34 (8.54)	0.78 - 43.92 (2.00)
	LDH (U/g·CRE)	9.72 - 50.00 (15.52)	10.19 - 73.78 (15.18)
	PRO (mg/g·CRE)	13.82 - 289.07 (143.00)	0.00 - 460.07 (39.75)
	GLU (mg/g·CRE)	45.25 - 82.82 (57.96)	17.36 - 105.88 (62.50)
	IP (mg/g·CRE)	346.64 - 1505.97 (813.62)	67.70 - 3214.80 (773.27)
	Na (mEq/g·CRE)	4.30 - 138.86 (38.11)	5.55 - 177.99 (52.03)
	K (mEq/g·CRE)	93.17 - 157.51 (123.16)	21.13 - 245.17 (105.91)
	NGAL (µg/g·CRE)	1.12 - 5.41 (3.49)	0.19 - 2.31 (0.65)

istry. A central aspect of gentamicin nephrotoxicity is its effects on renal tubules, which may range from a mere loss of the brush border in epithelial cells to overt tubular necrosis. Tubular cytotoxicity is the consequence of many interconnected actions, triggered by drug accumulation in epithelial tubular cells. Gentamicin then enters and accumulates in the endosomal compartment, the Golgi apparatus and endoplasmic reticulum (ER), causes ER stress, and unleashes the unfolded protein response. In the lysosomes, gentamicin produces membrane destabilization and lysosomal aggregation. Therefore presence of the eosinophilic granules in proximal epithelium observed in the low dose treatment was considered the initial change of gentamicin-induced nephrotoxicity without apparent elevation of serum UN and CRE. AKI could be caused by decrease of renal or intra-renal perfusion, obstructive or toxic renal tubular disorders, tubular-interstitial inflammation and edema, or decrease of glomerular filtration capacity. To describe AKI precisely and sensitively, a multidimensional classification system, which describes the severity of AKI in humans, has been developed (Adiyanti and Loho, 2012). The most frequently used reference system is RIFLE (Risk, Injury, Failure, Loss, End-stage Kidney Disease). RIFLE describes 3 severity stages of acute renal failure (Risk, Injury and Failure) and 2 output variables (Loss and End Stage Kidney Disease). The unique characteristics of the RIFLE classification include the presence of 3 variables to describe the severity of renal dysfunction based on serum CRE level, GFR changes or its duration, and the severity of reduced urine production. The “risk” category is defined as the condition when the serum CRE level increases two fold or when there is a decrease of urine output (< 0.5 ml/kg/hr for at least 12 hr). Although definitive criteria of AKI have not been established in experimental animals, the changes in the low dose treatment in our study were considered to indicate “risk” stage of AKI and urinary NGAL apparently increased. In contrast in the high dose treatment, UN and CRE increased approximately 13-fold, moderate to marked swelling and necrosis in proximal epithelium and hyaline casts in the renal tubules were observed, and slight regeneration in proximal and distal epithelium were noted on Day 10. In addition, urination did not occur in 2 hr, and vomitus, absence of food intake, and decreased activity were seen on Day 10 just before the necropsy. The nephrotoxicity of the high dose group was toxicologically and clinically severe on Day 10. In this dog, urinary NGAL increased 32-fold and > 225 -fold on Days 3 and 5, respectively. Taking these findings together, urinary NGAL was considered to be a sensitive and predictive biomarker of gentamicin-induced nephrotoxic-

ity in dogs.

NGAL is a 25-kDa lipocalin that was originally described as being covalently bound to matrix metalloprotein-9 in animal neutrophils (Kjeldsen *et al.*, 2000). NGAL is synthesized in renal tubular, intestinal, hepatic, and pulmonary tissue, and the expression of NGAL is low in these tissues under physiologic conditions. Circulating NGAL is filtered by the glomerus, and reabsorbed in the proximal epithelium. It is secreted in low concentrations by the thick ascending limb of the renal tubule (Shemin and Dworkin, 2011; Kuwabara *et al.*, 2009). In proximal tubular injury, NGAL synthesis is increased and reabsorption may decrease so that urinary levels increase. In distal tubular injury, synthesis and secretion of NGAL is increased (Schmidt-Ott *et al.*, 2007; Shemin and Dworkin, 2011). In our study, marked increases NGAL was observed in urine, and necrosis and swelling of proximal epithelium and regeneration of proximal and distal epithelium were noted in the dog given the high dose of gentamicin. Although immunohistochemistry of frozen and paraffin section in the dog kidney using commercially available anti-NGAL antibodies was failed to identify NGAL expression and its distribution in this study, it was reported that NGAL mRNA was highly upregulated after kidney injury such as renal ischemia-reperfusion, and cisplatin or gentamicin nephropathy in rodents (Mishra, 2003; Rouse *et al.*, 2011). In the high dose treatment of this study, reabsorption of NGAL in the proximal epithelium was considered to be the main reason for the increase in urinary NGAL due to severe proximal tubular lesions, and elevation of NGAL synthesis and/or secretion in injured tubular epithelium may also contribute to the changes. In gentamicin-treated rats, increases in urinary NGAL and cystatin were most sensitive in comparison to KIM-1, clusterin or β_2 -microglobulin in urine (Hoffmann *et al.*, 2010). However NGAL did not always demonstrate most sensitive reactions to other drug-induced AKI models in rats (Tonomura *et al.*, 2010; Fuchs *et al.*, 2012). Cystatin C is suggested as an indicator of disturbance of glomerular filtration, reabsorption and lysosomal degradation by proximal tubule cells (Hoffmann *et al.*, 2010). A critical aspect of tubular cytotoxicity from gentamicin is its concentration in the cytosol and accumulation in lysosomes. In comparison, a small amount of gentamicin directly enters the cytosol and nucleus independently from the endocytosis mediated by the megalin/cubilin complex (Quiros *et al.*, 2011). The molecule that acts as a receptor for NGAL is the well-characterized multiprotein receptor megalin/cubulin. Megalin is expressed by proximal tubule cells in the kidney, which are known target cells of NGAL, and mice that are genetically deficient in

megalin excrete NGAL in the urine (Schmidt-Ott *et al.*, 2007). It is speculated that the sensitive elevation of urinary NGAL in the dog treated with low dose gentamicin may have been caused by accumulation of gentamicin in lysosomes of the proximal epithelium or dysfunction of the endocytosis due to membrane destabilization mediated by the megalin/cubilin complex. This hypothesis needs to be evaluated with further studies using models of the “risk” stage of AKI induced by gentamicin or other agents to disrupt megalin endocytosis.

NGAL is emerging as an excellent biomarker in the urine and plasma, for the early prediction of AKI, for monitoring clinical trials in patients, and for the prognosis of AKI in several common clinical scenarios (Devarajan, 2011; Shemin and Dworkin, 2011). The value of urinary NGAL in dogs was stable in naive beagle dogs regardless of pooling time, also indicating that NGAL was a very sensitive marker for detection of slight AKI in this dog model. Our data suggested that NGAL in the urine would be a sensitive and predictable biomarker of AKI in dogs given gentamicin as well as in rats. However the changes in urinary NGAL in comparison to conventional biomarkers in blood and urine of beagle dogs need to be fully characterized using other AKI models induced by known nephrotoxic agents. Further studies will provide sufficient information to set NGAL or other biomarkers as preclinical platform to predict nephrotoxicity.

In conclusion, repeated injections (s.c.) of gentamicin for 9 days in beagle dogs induced numerous lysosomal granules in the proximal tubule without no apparent nephrotoxicity at 25 mg/kg, and marked nephrotoxicity at 50 mg/kg. Urinary NGAL is considered to be a more sensitive AKI biomarker than the classic detection markers such as serum CRE or BUN, and conventional urinary biomarkers to predict nephrotoxicity induced by gentamicin.

ACKNOWLEDGMENTS

The authors thank Dr. Mitsuya Yasuda at Medical Safety Research Laboratories, Daiichi Sankyo Co., Ltd. for his support and scientific advice throughout the animal experiments. We also appreciate Ms. Takako Kato and Ms. Yoko Suzuki at Medical Safety Research Laboratories, Daiichi Sankyo Co., Ltd. for their technical assistance of histopathological specimen preparation.

REFERENCES

- Adiyanti, S.S. and Loho, T. (2012): Acute Kidney Injury (AKI) Biomarker. *Acta. Med. Indones.*, **44**, 246-255.
- Bonventre, J.V., Vaidya, V.S., Schmolander, R., Feig, P. and Dieterle, F. (2010): Next-generation biomarkers for detecting kidney toxicity. *Nat. Biotechnol.*, **28**, 436-440.
- Devarajan, P. (2011): Biomarkers for the early detection of acute kidney injury. *Curr. Opin. Pediatr.*, **23**, 194-200.
- Dieterle, F., Sistare, F., Goodsaid, F., Papaluca, M., Ozer, J.S., Webb, C.P., Baer, W., Senagore, A., Schipper, M.J., Vonderscher, J., Sultana, S., Gerhold, D.L., Phillips, J.A., Maurer, G., Carl, K., Laurie, D., Harpur, E., Sonee, M., Ennulat, D., Holder, D., Andrews-Cleavenger, D., Gu, Y.Z., Thompson, K.L., Goering, P.L., Vidal, J.M., Abadie, E., Maciulaitis, R., Jacobson-Kram, D., Defelice, A.F., Hausner, E.A., Blank, M., Thompson, A., Harlow, P., Throckmorton, D., Xiao, S., Xu, N., Taylor, W., Vamvakas, S., Flamion, B., Lima, B.S., Kasper, P., Pasanen, M., Prasad, K., Troth, S., Bounous, D., Robinson-Gravatt, D., Betton, G., Davis, M.A., Akunda, J., McDuffie, J.E., Suter, L., Obert, L., Guffroy, M., Pinches, M., Jayadev, S., Blomme, E.A., Beushausen, S.A., Barlow, V.G., Collins, N., Waring, J., Honor, D., Snook, S., Lee, J., Rossi, P., Walker, E. and Mattes, W. (2010): Renal biomarker qualification submission: a dialog between the FDA-EMEA and Predictive Safety Testing Consortium. *Nat. Biotechnol.*, **28**, 455-462.
- Dobyan, D.C., Cronin, R.E. and Bulger, R.E. (1982): Effect of potassium depletion on tubular morphology in gentamicin-induced acute renal failure in dogs. *Lab. Invest.*, **47**, 586-594.
- Fuchs, T.C., Frick, K., Emde, B., Czasch, S., Landenberg, F. and Hewitt, P. (2012): Evaluation of novel acute urinary rat kidney toxicity biomarker for subacute toxicity studies in preclinical trials. *Toxicol. Pathol.*, **40**, 1031-1048.
- Gautier, J.C., Riefke, B., Walter, J., Kurth, P., Mylecraine, L., Guilpin, V., Barlow, N., Gury, T., Hoffman, D., Ennulat, D., Schuster, K., Harpur, E. and Pettit, S. (2010): Evaluation of novel biomarkers of nephrotoxicity in two strains of rat treated with Cisplatin. *Toxicol. Pathol.*, **38**, 943-956.
- Greaves, P. (2012): Urinary tract. In *Histopathology of Preclinical Toxicity Studies*, 4th Edition. Academic Press, London, pp.537-614.
- Hoffmann, D., Fuchs, T.C., Henzler, T., Matheis, K.A., Herget, T., Dekant, W., Hewitt, P. and Mally, A. (2010): Evaluation of a urinary kidney biomarker panel in rat models of acute and subchronic nephrotoxicity. *Toxicology*, **277**, 49-58.
- Kjeldsen, L., Cowland, J.B. and Borregaard, N. (2000): Human neutrophil gelatinase-associated lipocalin and homologous proteins in rat and mouse. *Biochim. Biophys. Acta.*, **1482**, 272-283.
- Kuwabara, T., Mori, K., Mukoyama, M., Kasahara, M., Yokoi, H., Saito, Y., Yoshioka, T., Ogawa, Y., Imamaki, H., Kusakabe, T., Ebihara, K., Omata, M., Satoh, N., Sugawara, A., Barasch, J. and Nakao, K. (2009): Urinary neutrophil gelatinase-associated lipocalin levels reflect damage to glomeruli, proximal tubules, and distal nephrons. *Kidney Int.*, **75**, 285-294.
- Mishra, J., Ma, Q., Prada, A., Mitsnefes, M., Zahedi, K., Yang, J., Barasch, J. and Devarajan, P. (2003): Identification of Neutrophil Gelatinase-Associated Lipocalin as a Novel Early Urinary Biomarker for Ischemic Renal Injury. *J. Am. Soc. Nephrol.*, **14**, 2534-2543.
- Mori, K., Lee, H.T., Rapoport, D., Drexler, I.R., Foster, K., Yang, J., Schmidt-Ott, K.M., Chen, X., Li, J.Y., Weiss, S., Mishra, J., Cheema, F.H., Markowitz, G., Suganami, T., Sawai, K., Mukoyama, M., Kunis, C., D'Agati, V., Devarajan, P. and Barasch, J. (2005): Endocytic delivery of lipocalin-siderophore-iron complex rescues the kidney from ischemia-reperfusion injury. *J. Clin. Invest.*, **115**, 610-621.

Urinary NGAL in acute kidney injury in dogs given gentamicin

- Mori, K. and Nakao, K. (2007): Neutrophil gelatinase-associated lipocalin as the real-time indicator of active kidney damage. *Kidney Int.*, **71**, 967-970.
- Quiros, Y., Vicente-Vicente, L., Morales, A.I., Lopez-Novoa, J.M. and Lopez-Hernandez, F.J. (2011): An integrative overview on the mechanisms underlying the renal tubular cytotoxicity of gentamicin. *Toxicol. Sci.*, **119**, 245-256.
- Rouse, R.L., Zhang, J., Stewart, S.R., Rosenzweig, B.A., Espandari, P. and Sadrieh, N.K. (2011): Comparative profile of commercially available urinary biomarkers in preclinical drug-induced kidney injury and recovery in rats. *Kidney Int.*, **79**, 1186-1197.
- Schmidt-Ott, K.M., Mori, K., Li, J.Y., Kalandadze, A., Cohen, D.J., Devarajan, P. and Barasch, J. (2007): Dual action of neutrophil gelatinase-associated lipocalin. *J. Am. Soc. Nephrol.*, **18**, 407-413.
- Shemin, D. and Dworkin, L.D. (2011): Neutrophil gelatinase-associated lipocalin (NGAL) as a biomarker for early acute kidney injury. *Crit. Care Clin.*, **27**, 379-389.
- Tonomura, Y., Tsuchiya, N., Torii, M. and Uehara, T. (2010): Evaluation of the usefulness of urinary biomarkers for nephrotoxicity in rats. *Toxicology*, **273**, 53-59.
- Varzi, H.N., Esmailzadeh, S., Morovvati, H., Avizeh, R., Shahriari, A. and Givi, M.E. (2007): Effect of silymarin and vitamin E on gentamicin-induced nephrotoxicity in dogs. *J. Vet. Pharmacol. Ther.*, **30**, 477-481.
- Waikar, S.S., Sabbiseti, V.S. and Bonventre, J.V. (2010): Normalization of urinary biomarkers to creatinine during changes in glomerular filtration rate. *Kidney Int.*, **78**, 486-494.