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RESEARCH ARTICLE

Comparison of the course of biomarker changes and kidney injury in a rat model of drug-induced acute kidney injury

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Abstract

Objective: To aid in evaluating the performance of biomarkers, we measured kidney injury biomarkers in rat models of drug-induced acute kidney injury.

Methods and results: Rats were treated with site-specific nephrotoxins, puromycin, gentamicin, cisplatin, or 2-bromoethylamine. Fifteen biomarkers (β-2-microglobulin, calbindin, clusterin, cystatin-C, KIM-1, GST-α, GST-μ, NGAL, osteopontin, EGF, TIMP-1, VEGF, albumin, RPA-1, and urinary total protein) were examined in comparison with BUN, serum creatinine, and NAG. Some biomarkers, which were different depending in each nephrotoxin, showed ability to detect the prodromal stage of drug-induced kidney injury. Characteristic changing patterns of biomarkers were also found depending on the specific lesion site in the kidney.

Conclusion: These data suggested that establishment of a suitable biomarker panel would facilitate detection of site-specific kidney injury with high sensitivity.

Keywords: renal disease, renal toxicity, cisplatin, puromycin, gentamicin, 2-bromoethylamine

Introduction

Although the kidney is known to be a primary target of drug-induced toxicity, detection of trauma before an injury becomes too severe to treat is difficult. Blood urea nitrogen (BUN) and serum creatinine (sCrea) have long been used mainly to monitor kidney damage clinically and preclinically. However, this method's lack of sensitivity is a major drawback, failing to reveal kidney damage until 70%-80% of the renal epithelial mass has been lost (Sieber et al. 2009). Because of these limitations, novel biomarkers have been long awaited which are more sensitive and reliable.

The Critical Path Institute's Predictive Safety Testing Consortium (PSTC) recently proposed the following seven biomarkers associated with nephrotoxicity: β-2-microglobulin (B2M), clusterin, cystatin-C (CysC), kidney injury molecule-1 (KIM-1, also known as T-cell immunoglobulin and mucin-containing molecule-1 [TIM-1]), albumin, urinary total protein (uTP), and trefoil factor 3 (TFF3). These biomarkers are reported to either or both have higher performance than traditional biomarkers or be able to detect site-specific injury in the kidney and were approved by the Food and Drug Administration (FDA) and European Medicines Agency (EMA) in 2008 and by the Pharmaceuticals and Medical Devices Agency (PMDA) in 2010 for detection of acute kidney injury (AKI; Dieterle et al. 2010b; Hewitt et al. 2009). Additionally, the International Life Sciences Institute Health and Environmental Sciences Institute (ILSI-HESI) also reported extensively on four urinary kidney injury biomarkers: glutathione S-transferase α (GST-α), glutathione S-transferase μ (GST-μ), renal papillary antigen-1 (RPA-1), and clusterin. Of these markers, RPA-1 and clusterin were subsequently approved by the EMA in 2010 (EMA, 2010).

Thus far, these kidney injury biomarkers have only been used in the limited context of nonclinical drug

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development, given their usefulness in detecting drug-induced AKI in rats. However, researchers expect that these markers will come to enjoy more widespread use in the near future, influencing traditional assessment not only in the preclinical stage but even in bedside diagnosis (Dieterle et al. 2010b). In addition to the above-mentioned approved biomarkers, a large number of biomarkers for monitoring kidney toxicity have been reported, allowing for a wide selection of potential biomarkers to employ in diagnoses (Aulitzky et al. 1992; Bonventre et al. 2010; Hildebrand et al. 1999; Hoffmann et al. 2010; Kishore et al. 1983; Rached et al. 2008; Sieber et al. 2009; Sistare et al. 2010; Vaidya et al. 2006; Zhou et al. 2006).

Here, we describe characteristics of a number of biomarkers to facilitate selection of suitable markers to monitor kidney damage based on the injury site in the kidney, thereby improving evaluation efforts. We used rats models of kidney injury induced by puromycin aminonucleoside (PAN), gentamicin sulfate salt (GEN), cisplatin (CSP), or 2-bromoethylamine hydrobromide (BEA), which all induce different toxicities based on the kidney lesion site. PAN damages the glomerulus (Kramer et al. 2004) and GEN and CSP the proximal tubule, but at differing lesion sites (GEN, proximal convoluted tubules [S1 and S2 segments; Ali 2003; Houghton et al. 1976; CSP, straight tubules; S3 segment; Pabla & Dong 2008; Yao et al. 2007]). BEA is used in a well-established experimental model of renal papillary necrosis (Cuttino et al. 1981; Khan et al. 1998).

We evaluated B2M, calbindin, clusterin, CysC, KIM-1 (TIM-1), GST-α, GST-μ (also known as glutathione S-transferase Yb1; GSTYb1), neutrophil gelatinase-associated lipocalin (NGAL, also known as lipocalin-2), osteopontin, epidermal growth factor (EGF), tissue inhibitor of metalloproteinase-1 (TIMP-1), vascular endothelial growth factor (VEGF), albumin, RPA-1, and uTP in total and compared effectiveness of these biomarkers to BUN, sCrea, and N-acetyl-β-D-glucosamidase (NAG) with regard to determining the prodromal stage of drug-induced kidney injury and characteristic changing patterns of the biomarkers depending on the kidney lesion site. We also evaluated the measurement values of two different devices to assess the correlation between devices.

Methods

Reagents

All chemicals and reagents were purchased from commercial suppliers. Cisplatin, gentamicin sulfate, puromycin aminonucleoside, and 2-bromoethylamine hydrobromide were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Animal studies

Male Sprague-Dawley rats (8-9 weeks of age at commencement of treatment) were purchased from Charles River Laboratories (Shiga, Japan). Rats were administered each nephrotoxin dissolved in physiological saline. For the PAN-induced model, rats were given a single intraperitoneal injection of PAN (0, 5, 15, or 50 mg/kg; 5 animals per dosing group) and sacrificed 1, 3, 7, or 10 days after administration, respectively. For the GEN-induced model, rats were given daily subcutaneous injection of GEN (0, 10, 30, or 100 mg/kg for 7 days; 5 animals per dosing group) and sacrificed on days 1, 3, or 7 after start of administration. Seven-day recovery groups were also set after 7 days' administration. For the CSP-induced model, rats were given a single intraperitoneal injection of CSP (0, 1, 3, or 6 mg/kg; 5 animals per dosing group) and sacrificed 1, 3, or 7 days after administration. For the BEA-induced model, rats were given a single intraperitoneal injection of BEA (0, 3, 10, 30, or 100 mg/kg, 5 animals per dosing group) and sacrificed 1 or 3 days after administration.

Urine samples were collected on ice under fasted condition from 24 h before the necropsy. Blood samples were collected from the abdominal aorta at necropsy under isoflurane (PAN, GEN, and CSP studies) or ether (BEA study) anesthesia. All animal experimental procedures were approved by the Committee for Animal Experiments of Astellas Pharma Inc.

Clinical chemistry and multiples assay

Four parameters (BUN, sCrea, uTP, NAG) were measured using a HITACHI 7170S (Ibaraki, Japan). Eight biomarkers (albumin, NGAL, osteopontin,KIM-1, GST-α, GST-μ, RPA-1, clusterin) were measured using Meso Scale Discovery MULTI-SPOT® Assay System (MSD). Rat-specific 96-multispot plate kits (Kidney Injury Panel 1 [rat] Assay Kit and Argutus AKI Test® [rat] Assay Kit) and 96-small spot plate kit (Rat Clusterin Assay Kit) were used according to the manufacturer's instructions. Electrochemiluminescence was detected by SECTOR® Imager 6000 (MSD; Gaithersburg, MD, USA). Additionally, frozen urine samples were sent on dry ice to Rules based medicine (Austin, TX, USA), and 12 biomarkers (B2M, calbindin, clusterin, CysC, EGF, GST-α, GST-μ, KIM-1, NGAL, osteopontin, TIMP-1, VEGF) were quantified using Rat Kidney MAP™ v1.0 multiplex immunoassay service (MAPs). The technology employed by RBM is based on the Luminex xMAP™ platform, which uses microsphere-based multiplex immunoassays in a capture-sandwich format.

Amounts of urinary biomarkers were calculated as one-day urinary excretion (normalized by urine volume). Concentration values were used only for correlation study between devices.

Histopathology

At necropsy, both kidneys (except in the BEA study, which used right kidney only) were removed and fixed in 10% phosphate buffered formalin and subsequently embedded in paraffin blocks, sectioned, stained with hematoxylin and eosin or periodic acid-Schiff, and

examined using light microscopy. Histopathology grading was established as none, minimal, mild, moderate, and severe.

Statistical analyses and data investigation

Data are presented as individual animals or mean ± SD (5 animals per group). Statistical analysis was performed by ANOVA and Dunnett's test using Graph Pad Prism 5 software package (GraphPad Software Inc., La Jolla, CA, USA). Values significantly different from control are indicated as $*p<0.05$, $**p<0.01$. Whether a measurement value was "increased" or "decreased" was determined using the individual measurement-value and control-value range for all studies in this article, as a reference, in addition to the mean value and statistical result. Data correlation for values from different devices was evaluated based on R^2 and p values.

Results

Correlation study between MSD and MAPs

Correlation between six biomarker levels measured using MAPs and MSD was evaluated by calculating the R^2 and p values (Table 1). All biomarkers except GST-μ (GSTYb1) showed good correlation between values measured by MAPs and those by MSD (Figures 1A-C), although difference were noted in absolute measurement values. In addition, the values for GST-μ seemed more consistent with the dosing and histopathological changes than GSTYb1, and GSTYb1 showed several outlier values. Given these findings, we opted to mainly show MAPs data in this article, and all data are described fully in supplementary tables.

PAN study

Albumin levels were increased from 3 days after administration at 50 mg/kg (Figure 2A), and TIMP-1 levels were increased from 7 days after administration at 50 mg/kg (Figure 2B). No meaningful changes in any other biomarkers were noted throughout the observation period (Supplementary Table 1).

With regard to histopathological observations, eosinophilic droplets were noted in the glomerular epithelium 7 days following single administration of 50 mg/kg PAN (Figure 3A and Table 2). However, this finding appeared to be resolved, with reduced incidence, 10 days after administration.

GEN study

B2M levels were increased from days 1 to 7 at 10 mg/kg or more (Figure 4K). CysC, GST-μ, and NGAL levels were also increased during this period at 100 mg/kg, but respective rates of increase on day 1 were lower than that for B2M (Figures 4C, G, and 4H). Clusterin, RPA-1, and calbindin levels were increased from day 3 at 100 mg/kg, and rate of increase rose until 7 days after cessation of administration (Figures 4E, J, and 4L). In contrast, while albumin and GST-α levels were also increased from day 3, peak values for these proteins were noted on day 7 at 100 mg/kg (Figures 4A and F). TIMP-1 and osteopontin showed the same trend of increasing levels as seen with albumin and GST-α (Figures 4B and I). KIM-1 levels were increased drastically on day 7 at 100 mg/kg and 7 days after cessation of administration at 30 and 100 mg/kg (Figure 4D). Regarding traditional biomarkers, NAG levels were found to be slightly increased on day 7 at 100 mg/kg, and a similar trend was noted for BUN 7 days after cessation of administration (Figures 4M and N). No meaningful changes in any other biomarkers were noted throughout the observation period (Supplementary Table 2).

With regard to histopathological observations, hyaline droplets were noted in proximal tubules on day 3 at 30 and 100 mg/kg (Figure 3B and Table 3), a finding which was aggravated on day 7. In addition, degeneration and necrosis of proximal and basophilic tubules and mononuclear cell infiltration were noted in the cortex and outer medulla on day 7 at 100 mg/kg (Figure 3C). Basophilic tubules, changes believed indicative of recovery, were also observed. Dilatation of distal tubules was noted in the cortex 7 days after cessation of administration at 100 mg/kg (Figure 3D).

Table 1. Correlation study between MAPs and MSD.

Biomarker	Device	Sample size	Measurement ranges	R^2	p value
KIM-1	MAPs	270	0-64 ng/mL	0.6893	**
TIM-1	MSD	270	0.1-126.2 ng/mL		
Clusterin	MAPs	270	0-30.8 ng/mL	0.5512	**
Clusterin	MSD	270	0-142.3 ng/mL		
Osteopontin	MAPs	270	0-31.8 ng/mL	0.4475	**
Osteopontin	MSD	270	0-29.4 ng/mL		
GST-α	MAPs	270	0589.0 ng/mL	0.4187	**
GST-α	MSD	270	8.1-7778.5 ng/mL		
NGAL	MAPs	270	13.4-1370.0 ng/mL	0.3925	**
Lipocalin-2	MSD	270	0-1650.2 ng/mL		
GST-μ	MAPs	270	0-66.4 ng/mL	0.0015	N.S.
GSTYb1	MSD	270	2.52–4820.2 ng/mL		

N.D. was treated as "0", N.S.: not significant, $**p<0.01$.

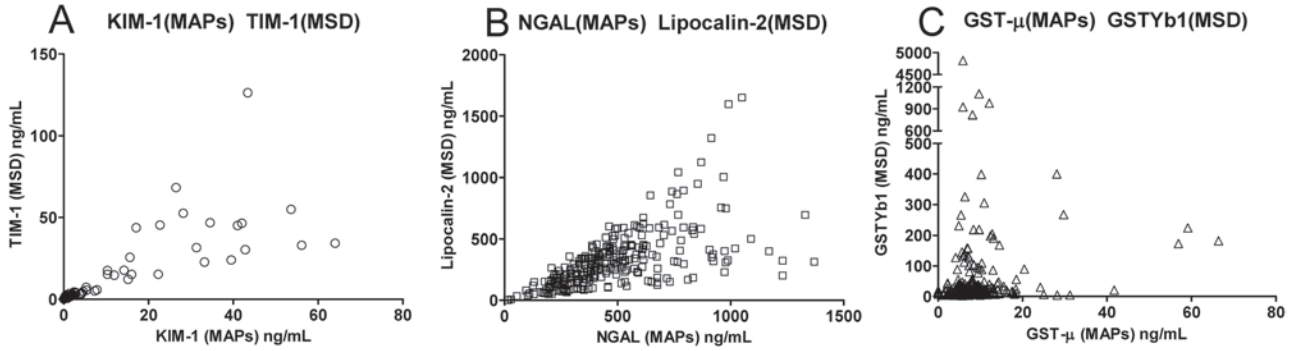

Figure 1. Correlation of the values measured by MAPs and MSD. KIM-1 and TIM-1 (A), NGAL and Lipocalin-2 (B), GST-μ and GSTYb1 (C).

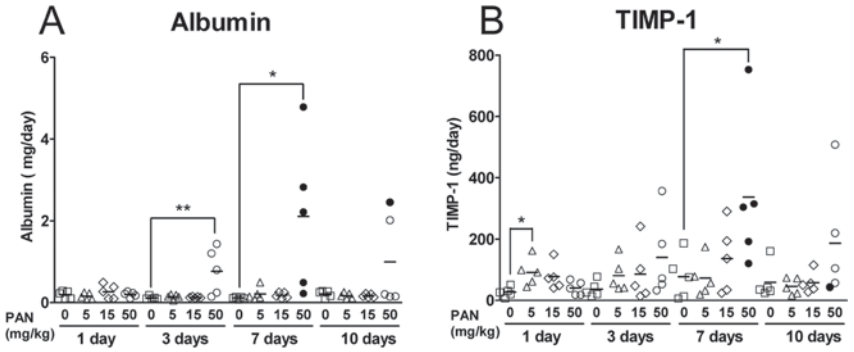

Figure 2. Excretion of albumin (A) and TIMP-1 (B) in urine of rats treated with PAN. Data are presented as values for individual animals (symbols) and means (bar; $n = 5$). Closed and open symbols indicate animals with and without histopathological findings, respectively.

CSP study

Increased levels were observed for CysC (from 1 to 7 days after administration, 6 mg/kg; Figure 5C), GST-μ (from 1 to 3 days after administration, 6 mg/kg; Figure 5G), and albumin, KIM-1, clusterin, and GST-α (from 3 to 7 days after administration, 6 mg/kg; Figures 5A, D, E, and 5F, respectively). Although the increase in albumin levels was much higher 3 days after than 7 days after administration, increases in KIM-1, clusterin, and GST-α levels were almost the same at these two points. TIMP-1, NGAL, osteopontin, RPA-1, and B2M increased at 6 mg/kg only 7 days after administration (Figures 5B, H, I, J, and 5K). In contrast to the above findings, EGF levels were decreased 1 day after at 3 mg/kg and from 1 to 7 days after administration at 6 mg/kg (Figure 5L). Regarding traditional biomarkers, levels of sCrea and BUN increased from 3 to 7 days after administration at 6 mg/kg (Figures 5M and N). No meaningful changes in any other biomarkers were noted throughout the observation period (Supplementary Table 3).

Histopathologically, only chromatin condensation with no degenerative changes was noted 1 day after 6 mg/kg CSP administration (Figure 3E and Table 4). Degeneration, necrosis, and basophilic tubules of proximal tubules were found in the cortex and outer stripe of the outer medulla from 3 days after administration at 3 mg/kg or more (Figure 3F). In addition, hyaline casts were found in tubules in the inner stripe of the outer medulla 7 days after administration at 3 mg/kg or more. Mononuclear cell infiltration was also found in the cortex and the outer medulla at 3 mg/kg or more, and dilatation of distal tubules was also observed in the cortex at 6 mg/kg.

BEA study

Albumin, TIMP-1, KIM-1, clusterin, and uTP levels were increased from 1 to 3 days after administration at 30 mg/kg or more (Figures 6A-B, 6D-E, and 6L). Further, the rates of level increase for albumin, uTP, and TIMP1 were all higher 1 day after than 3 days after administration, whereas rates for KIM-1 and clusterin were found to be increased 3 days after administration. Osteopontin levels were also increased 1 day after administration at 30 mg/kg or more, but values for all rats in the 30 mg/kg group returned to the control level 3 days after administration (Figure 6I). GST-μ and NGAL levels were increased from 1 to 3 days after at 100 mg/kg and 3 days after administration at 30 mg/kg (Figures 6G-H). CysC levels were also increased 1 day after but had returned to within the control range by 3 days after administration at 100 mg/kg (Figure 6C). EGF levels were decreased from 1 to 3 days after administration

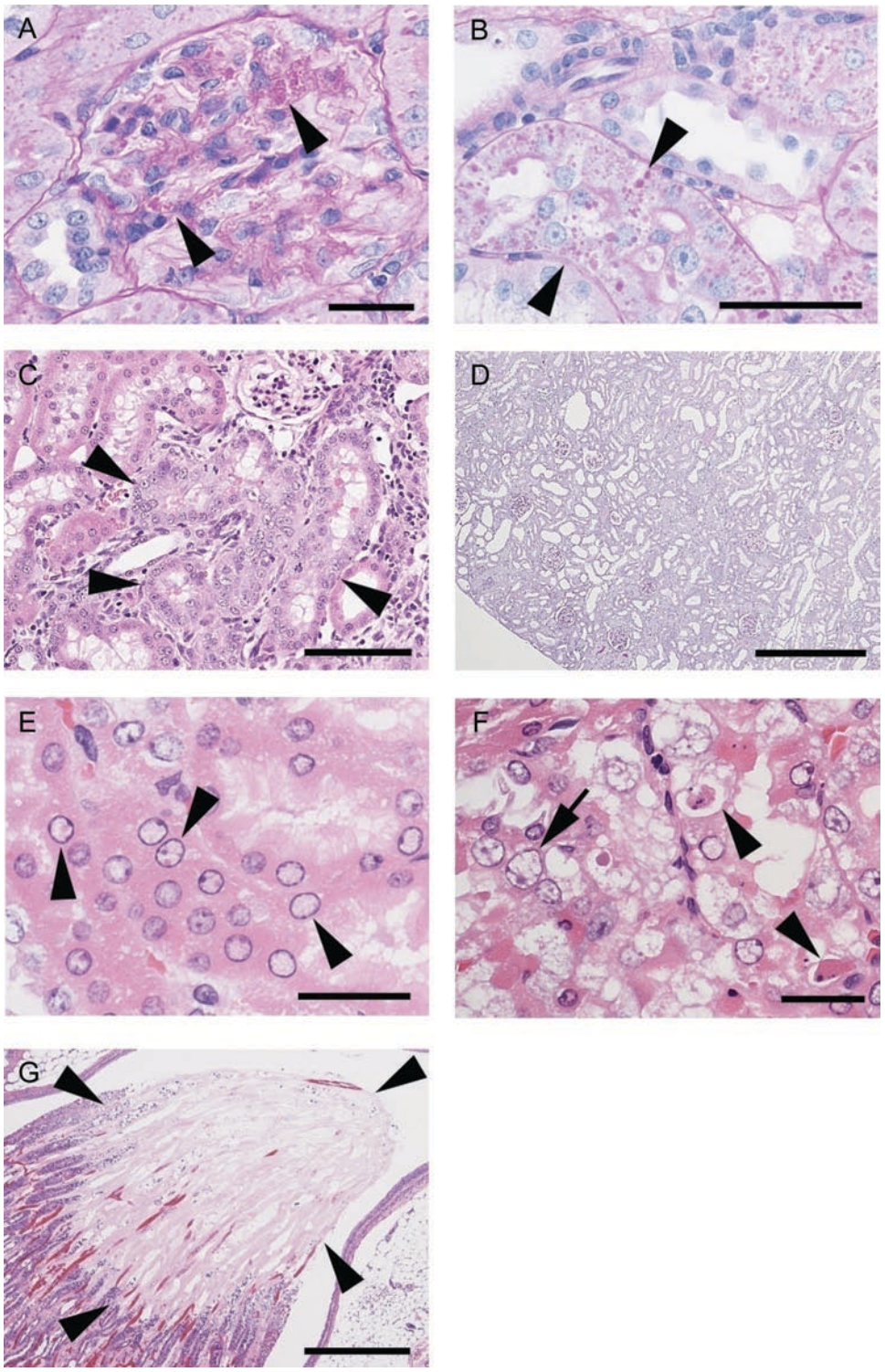

Figure 3. (A) Eosinophilic droplets in the glomerulus in rats treated with PAN (arrowhead, 50 mg/kg, bar = 25 μm, PAS). (B) Hyaline droplets in proximal tubules in rats treated with GEN (arrowhead, 100 mg/kg, bar = 50 μm, PAS). (C) Basophilic tubules in the outer medulla in rats treated with GEN (arrowhead, 100 mg/kg, bar = 100 μm, HE). (D) Dilatation of tubules in rats treated with GEN (100 mg/kg, bar = 500 μm, PAS). (E) Chromatin condensation in proximal tubules in rats treated with CSP (arrowhead, 6 mg/kg, bar = 25 μm, HE). (F) Degeneration (arrow) and necrosis (arrowhead) in proximal tubules in rats treated with CSP (6 mg/kg, bar = 25 μm, HE). (G) Papillary necrosis in rats treated with BEA (arrowhead, 100 mg/kg, bar = 500 μm, HE).

at 100 mg/kg (Figure 6K), whereas RPA-1 levels were increased 1 day after administration at 30 mg/kg and 3 days after administration at 30 mg/kg or more (Figure 6J). GST-α levels were decreased almost completely 3 days after administration at 100 mg/kg (Figure 6F). Regarding traditional biomarkers, BUN levels were increased from 1 day at 30 mg/kg or more, but all values in the 30 mg/kg group except for one rat had returned

Table 2. Histopathological changes in rat kidney after single administration of puromycin aminonucleoside.

Days after dosing (day)	1				3				7				10			
Dose (mg/kg)	0	5	15	50	0	5	15	50	0	5	15	50	0	5	15	50
Eosinophilic droplets in the glomerular epithelium	–	–	–	–	–	–	–	–	–	–	–	+ to ++ (5/5)	–	–	–	+ (1/5)

Histopathological grade: –, lesion not observed; +, minimal severity; ++, mild severity; +++, moderate severity of lesion.
Numbers in parentheses describe "number of animals with findings"/"number of animals examined."

nearly to the control level by 3 days after administration (Figure 6N). A clear increase in sCrea levels was noted only in the 100 mg/kg group 3 days after administration (Figure 6M). No meaningful changes in any other biomarkers were noted throughout the observation period (Supplementary Table 4).

Histopathologically, papillary necrosis, infiltration of inflammatory cells, and eosinophilic droplets in collecting ducts were found in the papilla 1 day after administration at 30 mg/kg or more (Figure 3G and Table 5). Dilatation of proximal tubules was also observed at 30 mg/kg or more. Papillary necrosis and infiltration of inflammatory cells were still observed in the papilla 3 days after administration. Basophilic tubules in the papilla and cortex, hyperplasia of surface epithelium in the papilla, and proximal tubule dilatation in the cortex were all observed at 30 mg/kg or more. In addition, hyaline casts were found only at 100 mg/kg in the papilla.

Discussion

To evaluate biomarker usefulness, we assessed the accuracy of the relationship between biomarker changes and histopathological findings, comparing findings between 15 selected biomarkers and several traditional biomarkers, using four rat models of kidney injury induced by lesion site-specific nephrotoxins.

In the PAN study, increased levels of albumin can be used to detect prodromal or earlier stages of glomerular injury, a result partially consistent with findings by Thielemans et al. (1994). In their report, albumin levels were found to be increased earlier than noticeable changes in B2M and CysC in rats treated with PAN. The low degree of toxicity in our PAN study compared with this previous study might have been due to our lack of observed increases in B2M and CysC levels, which are correlated with relatively severe glomerular damage (Dieterle et al. 2010a). Our findings here suggest that albumin may exhibit sensitivity superior to other biomarkers in detecting glomerular injury caused by PAN.

In the GEN study, B2M, CysC, GST-μ, and NGAL levels were increased prior to proximal tubular injury by GEN. GEN inhibits proximal tubular epithelial cell lysosomal function due to the GEN accumulation, subsequently produces tubular degeneration (Ali 2003). These biomarkers may detect the phase of lysosomal accumulation of GEN. Among these biomarkers, B2M showed excellent prodromal detection potential. Reabsorption deficiency due to proximal tubular injury is known to result in increases in these urinary levels of low molecular proteins, such as B2M and CysC (Dieterle et al. 2010a). Their values may therefore be excellent indicators of function loss in early phases of kidney injury prior to observed histopathological changes. In addition, the extra-high sensitivity of B2M was considered not only due to reduced function of proximal tubules but also to the characteristic profile of GEN. Polycationic drugs such as GEN are known to dose dependently inhibit B2M reabsorption, even at the clinically relevant dose (Bernard et al. 1986; Dieterle et al. 2010a).

A mechanism identical to that of B2M and CysC was considered to occur with mid-sized molecular weight proteins. Levels of albumin, one such mid-sized molecular protein, increased from the same time point as proximal tubular injury induction by GEN. Albumin is filtrated partially by glomeruli and reabsorbed by epithelial cells lining the proximal tubules through receptor-mediated endocytosis and degraded (Clavant et al. 2003; Iglesias et al. 2001). It is also known that albumin is cut into low molecular weight fragments by proteases in the urine (Kania et al. 2010). The lower sensitivity of albumin than B2M, CysC in GEN study might be due to the difference rate of leakage from the glomeruli and albumin degradation in the urine.

In the CSP study, it was noteworthy that CysC and GST-μ levels increased and EGF decreased before the degenerative changes were observed (1 day after administration). These three biomarkers might be prognostic biomarkers of proximal tubular injury. One day after administration, only chromatin condensation was found, which was observed as an initial degeneration change induced by CSP (Falkenberg et al. 1996). Degenerative changes were found from 3 days after administration even though the severity and incidence were lesser than previous report (Gautier et al. 2010).

Increasing levels of CysC were also observed in the GEN study, the mechanism for which was considered the same as described above (GEN study). In contrast, B2M levels increased only 7 days after administration; this difference in B2M sensitivity to proximal tubular injury induced by CSP and GEN may be due to the reason described above (GEN study) or due to differing lesion sites.

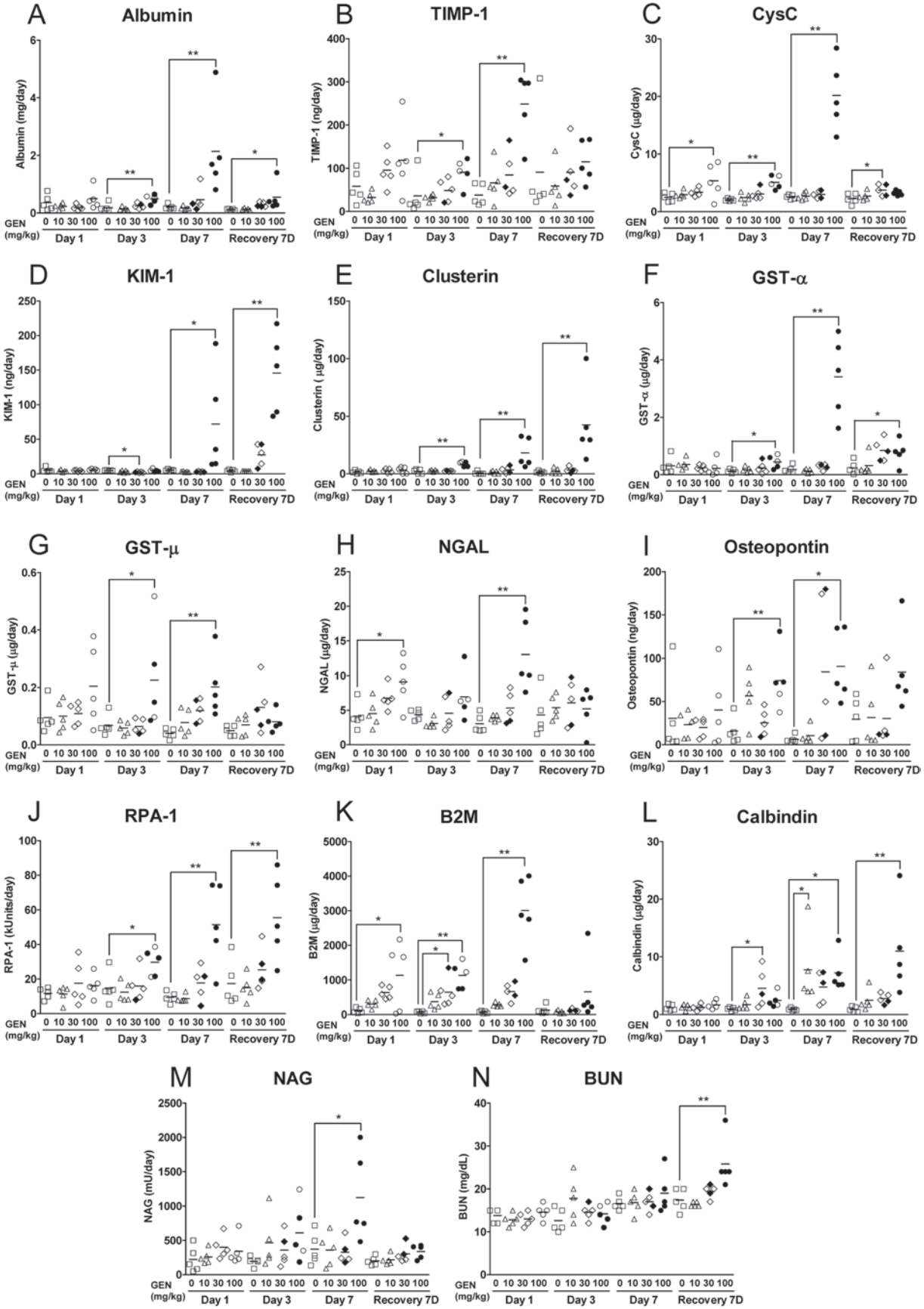

Figure 4. Excretion of albumin (A), TIMP-1 (B), CysC (C), KIM-1 (D), clusterin (E), GST-α (F), GST-μ (G), NGAL (H), osteopontin (I), RPA-1 (J), B2M (K), calbindin (L), and NAG (M) into urine and concentration of BUN (N) in serum of rats treated with GEN. Data are presented as values for individual animals (symbols) and means (bar; $n = 5$). Closed and open symbols indicate animals with and without histopathological findings, respectively.

Table 3. Histopathological changes in rat kidney after repeated administration of gentamicin sulfate salt.

Period (day)	1				3				7				7 + recovery 7			
Dose (mg/kg)	0	10	30	100	0	10	30	100	0	10	30	100	0	10	30	100
Hyaline droplets in proximal tubules in the cortex and the outer medulla	–	–	–	–	–	–	+ (1/5)	+ (3/5)	–	–	+ (2/5)	++ to +++ (5/5)	–	–	–	+ (1/5)
Dilatation of distal tubules in the cortex	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+ to +++ (4/5)
Degeneration and necrosis of proximal tubules in the cortex and the outer medulla	–	–	–	–	–	–	–	–	–	–	–	+ to ++ (5/5)	–	–	–	+ (3/5)
Basophilic tubules in the cortex and the outer medulla	–	–	–	–	–	–	–	–	–	–	–	+ to ++ (4/5)	–	–	+ (2/5)	++ to +++ (5/5)
Mononuclear cell infiltration in the cortex and the outer medulla	–	–	–	–	–	–	–	–	–	–	–	+ to ++ (2/5)	–	–	–	+ to ++ (4/5)

Histopathological grade: –, lesion not observed; +, minimal severity; ++, mild severity; +++, moderate severity of lesion.
Numbers in parentheses describe "number of animals with findings"/"number of animals examined."

Observation of decreased levels of EGF after CSP administration was consistent with findings of Safirstein et al. (1989) EGF inhibits growth of several types of cells (Barnes 1982; Safirstein et al. 1989), and levels may be reduced in preparation for repairing proximal tubular injury, although the timing of the decrease in the present study may have been too sudden to consider this possibility.

In the BEA study, renal papillary necrosis was clearly observed from one day after administration. Albumin was believed to be a comparatively useful biomarker as it exhibited the highest rate of increase and therefore was the most sensitive. Although its pattern of increase was similar to TIMP-1, osteopontin, and uTP, rates of increase in these latter biomarkers were lower than that for albumin. Histopathologically, other secondary changes, such as basophilic tubules and dilatation of proximal tubules in the cortex, were also observed. These findings were consistent with previous reports (Murray et al. 1972; Thukral et al. 2005). The levels of KIM-1, CysC, and Clusterin might be increased because of these proximal tubular injuries. Biomarkers associated with RPN were identified in this BEA study. However, histopathological changes were too severe, that we could not deny the possibility that there were some factors results from plasma and blood component entering the urine at the site of injury. Thus, further investigation including rats with early lesions of RPN will be needed to evaluate biomarkers on prodromal stage.

RPA-1 is an uncharacterized high molecular weight membrane-bound glycoprotein expressed in the collecting ducts and can be detected at high levels in rat urine following exposure to compounds that induce renal papillary necrosis (Falkenberg et al. 1996; Price et al. 2010). However, we noted a delay in increase in RPA-1 levels in our study, a finding consistent with that of Hildebrand et al. (1999). In this manner, we confirmed that RPA-1 could not function as a prognostic marker for BEA-induced renal papillary necrosis. In addition, necrotic changes are not necessary to increase RPA-1 levels: simply by inducing functional tubular changes by administering a vasopressin analog (dDAVP), RPA-1 levels increased in urine and the collecting duct with hypertrophic/hyperplastic changes (Tabata et al. 2009). Additionally, RPA-1 levels were also increased in GEN and CSP study, though the timing of the increase was at a time or later than degenerative changes. It is known that RPA-1 expressed in the epithelial cells of collecting duct in the cortex or in the medulla, when rats were treated with GEN or CSP (Zhang et al. 2008, Zhang et al. 2009). Increasing of RPA-1 levels might result from the hypertrophic changes of collecting duct.

We also noted that levels of GST-α had decreased to an undetectable level, 3 days after administration in the highest dose group. This phenomenon was also reported by ILSI-HESI, and GST-α was not approved due to this issue (Harpur et al. 2011). We were unable to determine

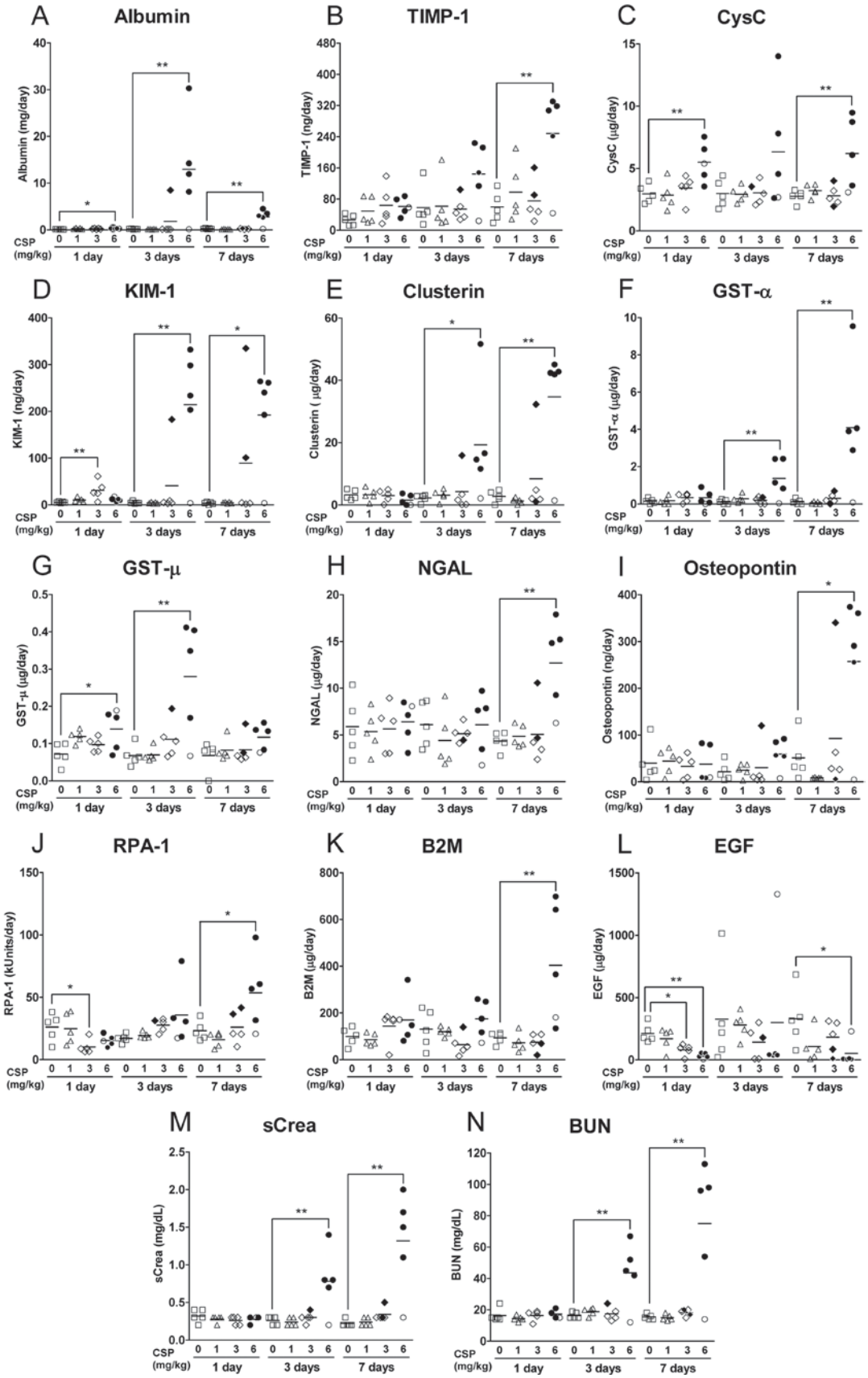

Figure 5. Excretion of albumin (A), TIMP-1 (B), CysC (C), KIM-1 (D), clusterin (E), GST-α (F), GST-μ (G), NGAL (H), osteopontin (I), RPA-1 (J), B2M (K), and EGF (L) into urine and concentration of sCrea (M), BUN (N) in serum of rats treated with CSP. Data are presented as values for individual animals (symbols) and means (bar; $n=5$). Closed and open symbols indicate animals with and without histopathological findings, respectively.

Table 4. Histopathological changes in rat kidney after single administration of cisplatin.

Days after dosing (day)	1				3				7			
Dose (mg/kg)	0	1	3	6	0	1	3	6	0	1	3	6
Degeneration and necrosis of proximal tubules in the cortex and the outer stripe of the outer medulla	–	–	–	+[a] (4/5)	–	–	++ (1/5)	++ to +++ (4/5)	–	–	+ to ++ (2/5)	+ to ++ (4/5)
Basophilic tubules of proximal tubules in the cortex and the outer stripe of the outer medulla	–	–	–	–	–	–	++ (1/5)	++ (4/5)	–	–	++ (2/5)	+++[b] (4/5)
Hyaline casts (proteinaceous) in tubules in the inner stripe of the outer medulla	–	–	–	–	–	–	–	–	–	–	+ (1/5)	++ (4/5)
Dilatation of distal tubules in the cortex	–	–	–	–	–	–	–	–	–	–	–	+ (3/5)
Mononuclear cell infiltration in the cortex and the outer medulla	–	–	–	–	–	–	–	–	–	–	+ (1/5)	++ (3/5)

[a]Only nuclear degenerative changes (chromatin condensation), [b]with mitoses.
Histopathological grade: –, lesion not observed; +, minimal severity; ++, mild severity; +++, moderate severity of lesion.
Numbers in parentheses describe "number of animals with findings"/"number of animals examined."

why this phenomenon occurred and further investigation is required.

We evaluated whether biomarker performance "increased" or "decreased," by groups. However, there are individual animal variability (for example, Figures 2A-B, 4F-H, 5C, and 5G). Individual variability is caused by the variable severity of lesions induced by each nephrotoxin. There are a lot of considerable reasons, such as variable receptivity in each animal, technical issues, environmental factors, and so on. Especially in early stage of histopathology, the variability of early lesion tends to be strongly reflected in the degrees of biomarker changes. In contrast, the individual variability of biomarker levels might become smaller in late stage of histopathology because of the higher severity of histopathology with smaller qualitative and quantitative variation in addition to the high-fold biomarker changes. Late biomarkers with larger increasing rate, such as KIM-1 and clusterin, tend to be of high specificity.

A radar graph of the four studies in this article is shown in Figure 7, indicating the characteristic changing patterns of biomarkers depending on lesion site in the kidney. For example, albumin levels were increased in the prodromal stage of PAN-induced glomerular injury and at or after GEN-, CSP-, and BEA-induced tubular injury. We therefore noted two distinct aspects of biomarker potential for albumin: albumin can not only be used to detect prodromal glomerular injury but also to detect lesion-site nonspecific injury in the kidney.

In light of its FDA/EMA/PMDA approval, albumin has recently been recognized as an important biomarker of AKI (Bolisetty & Agarwal 2011). Urinary albumin levels have been found to be increased not only due to abnormal glomerular filtration and reduced tubular reabsorption, but also due to the induction of albumin gene expression in the kidney, which is normally silent, with AKI (Ware et al. 2011). This observation suggests that albumin levels may increase in a number of kidney injury situations. Levels may therefore also be increased for nonlesion-related reasons, albumin may come to be accepted as a first-line biomarker together with BUN and sCrea in the near future. KIM-1 and clusterin, were common among the studies with tubular injury (in GEN, CSP, and BEA study), with high specificity for tubular injury but an inability to detect the prodromal stage.

Although a difference in absolute measurement values was noted in the correlation study, all correlation values except for GST-μ (GSTYb1) were acceptable. We can compare the data from different devices by recognizing differences in measurement of absolute values. GST-μ can be divided into three isozymes with homo or heterodimeric forms: Yb1Yb1, Yb1Yb2, and Yb2Yb2 (Hayes 1983). MSD adopted polyclonal antibodies against Yb1 and Yb2. In contrast, MAPs adopted a monoclonal antibody against Yb1. Additional protein recognition via the MSD antibody may have caused the noncorrelation of GST-μ observed here.

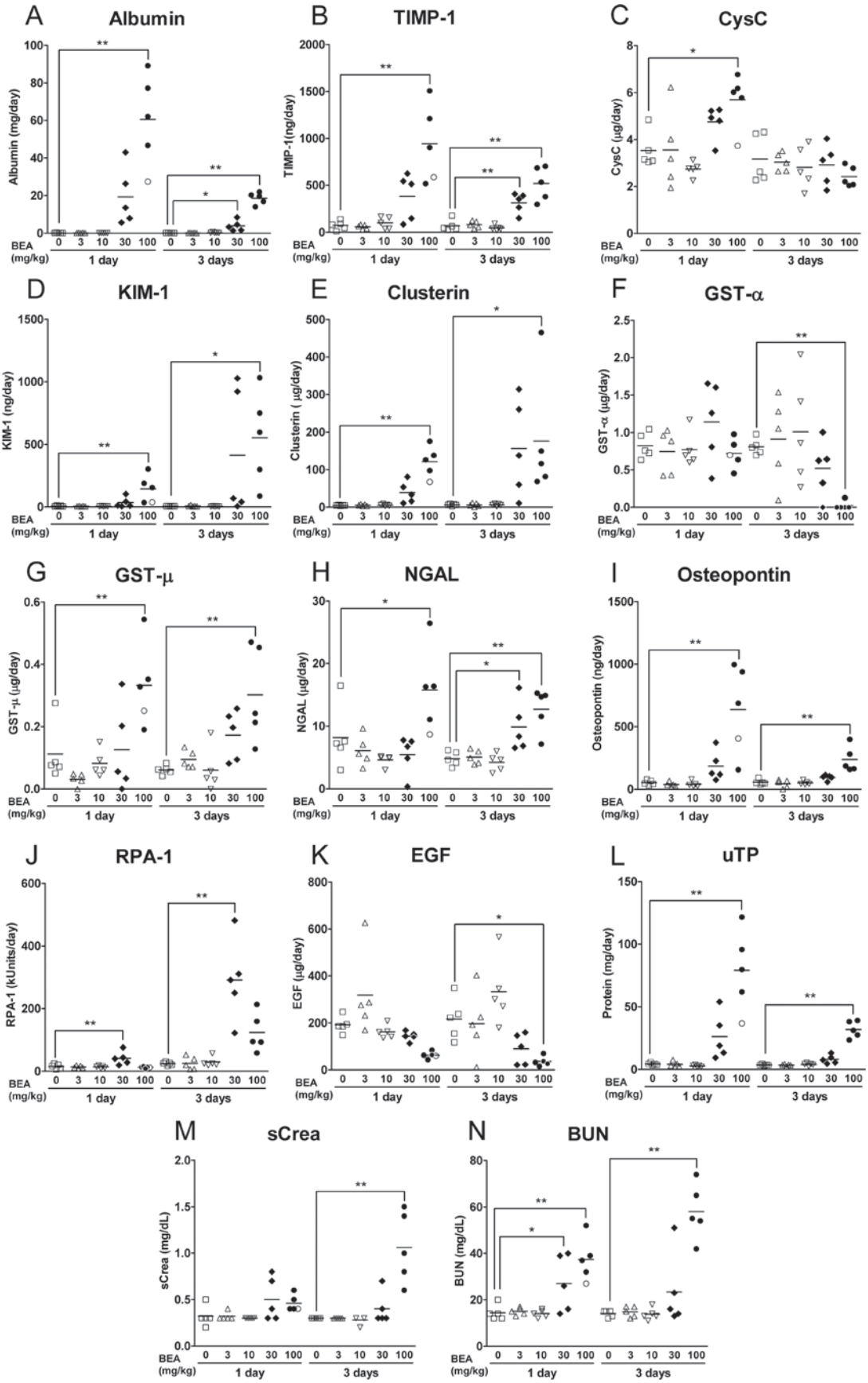

Figure 6. Excretion of albumin (A), TIMP-1 (B), CysC (C), KIM-1 (D), clusterin (E), GST-α (F), GST-µ (G), NGAL (H), osteopontin (I), RPA-1 (J), EGF (K), and uTP (L) into urine and concentration of sCrea (M), BUN (N) in serum of rats treated with BEA. Data are presented as values for individual animals (symbols) and means (bar; $n=5$). Closed and open symbols indicate animals with and without histopathological findings, respectively.

Table 5. Histopathological changes in rat kidney after single administration of bromoethylamine hydrobromide.

Days after dosing (day)	1					3				
Dose (mg/kg)	0	3	10	30	100	0	3	10	30	100
Papillary necrosis	–	–	–	+ to ++ (5/5)	+ to ++ (4/5)	–	–	–	+ to ++ (3/5)	+ to +++ (4/5)
Infiltration of inflammatory cells in the papilla	–	–	–	+ (3/5)	+ (4/5)	–	–	–	+ to ++ (4/5)	+ to ++ (5/5)
Eosinophilic droplets in collecting ducts in the papilla	–	–	–	+ (3/5)	+ to ++ (4/5)	–	–	–	–	–
Basophilic tubules in the papilla	–	–	–	–	–	–	–	–	+ to +++ (4/5)	++ to +++ (5/5)
Hyaline casts (proteinaceous) in the papilla	–	–	–	–	–	–	–	–	–	+ (4/5)
Hyperplasia of surface epithelium in the papilla	–	–	–	–	–	–	–	–	+ (4/5)	+ to +++ (4/5)
Basophilic tubules in the cortex	–	+ (1/5)	–	–	–	–	–	–	+ (1/5)	+ (5/5)
Dilatation of proximal tubules in the cortex	–	–	–	+ to ++ (3/5)	+ (4/5)	–	–	–	++ (1/5)	++ to +++ (5/5)

Histopathological grade:– lesion not observed; +, minimal, ++ mild, +++ moderate severity of lesion.
Numbers in parentheses describe "number of animals with findings" / "number of animals examined."

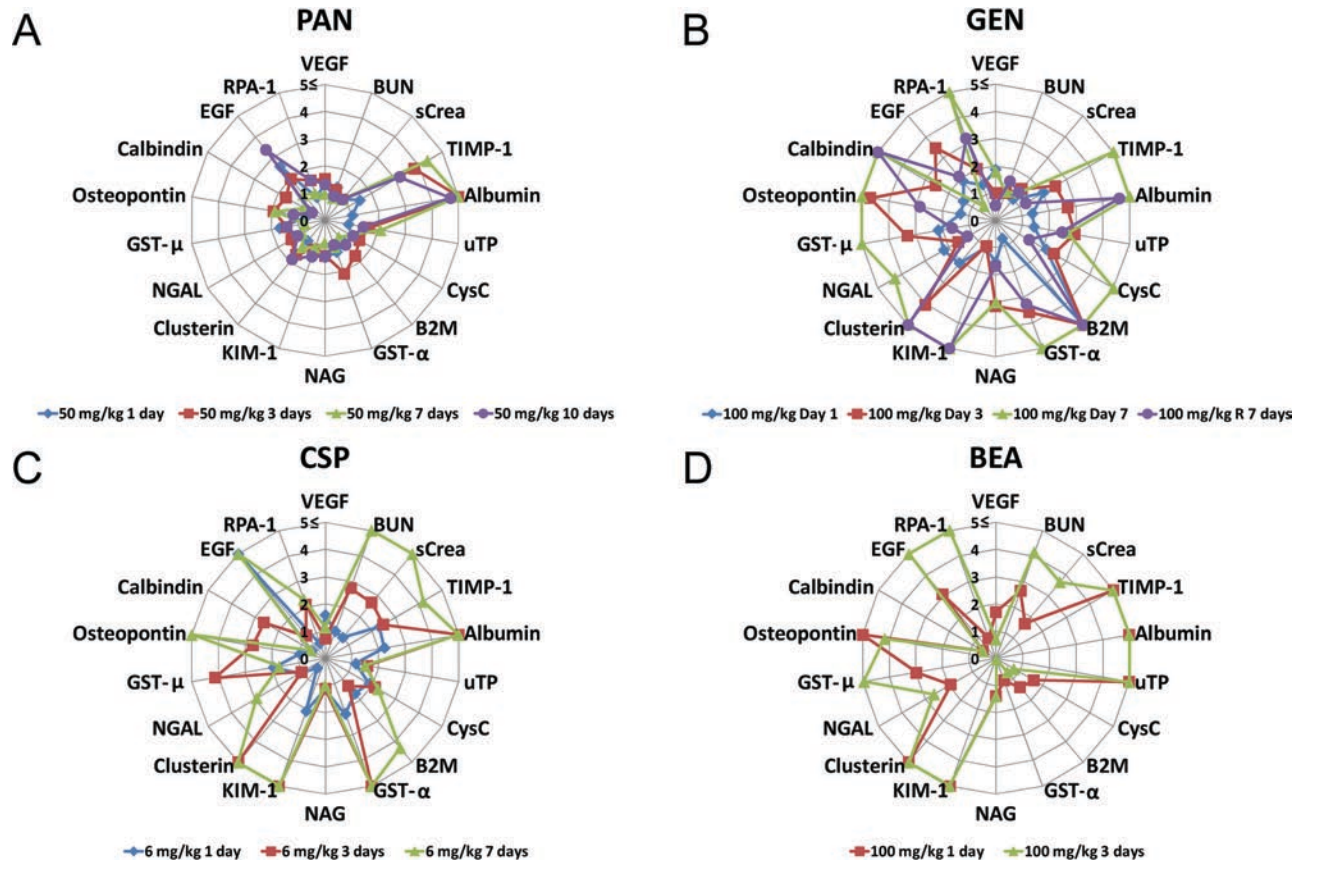

Figure 7. Fold changes of biomarkers in radar graphs. Graph values were fold changes from control mean value. Reciprocal value of fold change was plotted for EGF. (A) On 1, 3, 7, and 10 days after administration of 50 mg/kg PAN-treated rats. (B) On days 1, 3, and 7 during and 7 days after "cessation of" administration (R7 days) of 100 mg/kg GEN-treated rats. (C) On 1, 3, and 7 days after administration of 6 mg/kg CSP-treated rats. (D) On 1 and 3 days after administration of 30 mg/kg BEA-treated rats.

Conclusion

We examined 15 lesion-specific biomarkers and compared results with those for three traditional biomarkers using models of drug-induced acute renal toxicity with respect to ability to detect the prodromal stage of drug-induced kidney injury and characteristic changing patterns of biomarkers depending on the specific lesion site in the kidney. Selection of suitable markers should be based on injury site for better evaluation of kidney injury.

Declaration of interest

The authors report no declaration of interest.

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