Exosomal Fetuin-A identified by proteomics: A novel urinary biomarker for detecting acute kidney injury

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Urinary exosomes containing apical membrane and intracellular fluid are normally secreted into the urine from all nephron segments, and may carry protein markers of renal dysfunction and structural injury. We aimed to discover biomarkers in urinary exosomes to detect acute kidney injury (AKI), which has a high mortality and morbidity. Animals were injected with cisplatin. Urinary exosomes were isolated by differential centrifugation. Protein changes were evaluated by two-dimensional difference in gel electrophoresis and changed proteins were identified by mass spectrometry. The identified candidate biomarkers were validated by Western blotting in individual urine samples from rats subjected to cisplatin injection; bilateral ischemia and reperfusion (I/R); volume depletion; and intensive care unit (ICU) patients with and without AKI. We identified 18 proteins that were increased and nine proteins that were decreased 8 h after cisplatin injection. Most of the candidates could not be validated by Western blotting. However, exosomal Fetuin-A increased 52.5-fold at day 2 (1 day before serum creatinine increase and tubule damage) and remained elevated 51.5-fold at day 5 (peak renal injury) after cisplatin injection. By immunoelectron microscopy and elution studies, Fetuin-A was located inside urinary exosomes. Urinary Fetuin-A was increased 31.6-fold in the early phase (2–8 h) of I/R, but not in prerenal azotemia. Urinary exosomal Fetuin-A also increased in three ICU patients with AKI compared to the patients without AKI. We conclude that (1) proteomic analysis of urinary exosomes can provide biomarker candidates for the diagnosis of AKI and (2) urinary Fetuin-A might be a predictive biomarker of structural renal injury.

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Acute kidney injury (AKI) still has a high morbidity and mortality rate of 25–70%.[1] Although numerous agents are effective in animals, the translation of an effective drug therapy for AKI from laboratory bench to beside has been difficult.[1–4] Novel biomarkers for early detection and accurate diagnosis of AKI will likely help speed the translation of new treatments for AKI. To achieve this goal, the development of novel non-invasive biomarkers to predict and evaluate severity of AKI is essential.[4,5]

Serum creatinine (SCr) has poor sensitivity and specificity to detect or grade the severity of AKI: patients are not in steady state, hence SCr lags behind both renal injury and renal recovery.[3] Urine is an ideal non-invasive source of biomarkers to diagnose and classify kidney diseases. However, conventional urine markers (casts, fractional excretion of sodium) are nonspecific and insensitive. Recently, we successfully isolated exosomes from human urine by differential centrifugation and demonstrated the presence of several disease-related proteins.[6,7] Exosomes containing vesicular membranes and intracellular fluid are normally secreted into the urine from all nephron segments, and contain proteins that may be altered in abundance or physical properties in association with various renal diseases. Urinary exosomes represent a new source for the discovery of non-invasive urinary biomarkers that can overcome much of the interference from the most abundant urinary proteins (albumin, globulin, and Tamm–Horsfall protein, etc).[8]

Recently, several novel urinary biomarkers have been discovered using genomic methods as potentially useful for prediction and diagnosis of AKI. Kidney injury molecule-1[7,9,10] and neutrophil gelatinase-associated lipocalin (NGAL)[11–14] have been demonstrated as early predictors for diagnosis or outcome in AKI patients and animal models. Interleukin-18[15,16] sodium/hydrogen exchanger isoform 3[17] and cystatin C[18] have also been verified to serve as biomarkers in small numbers of patients with AKI. Cysteine rich protein 61,[19] dendrimer-enhanced magnetic resonance imaging,[20] malondialdehyde,[21] keratinocyte-derived chemokine,[22] and meprin-1-alpha[23] have been reported to early detect renal injury in AKI animal models. Because these

biomarkers have only been validated for animals with AKI or a small number of patients, their translation to clinical application still needs further development and each biomarker currently has limitations.[5] Additional new urinary biomarkers may be required to improve early detection and new drug development in AKI.

The purpose of the present study was to discover new biomarker candidates for AKI. For this purpose, we selected a *cis*-diammineplatinum (II) dichloride (cisplatin)-induced AKI rat model, which is non-oliguric despite nephrotoxic tubular injury, for discovery of biomarkers in urinary exosomes. After the identification by mass spectrometry, we validated the potential candidates using Western blotting. We selected Fetuin-A, which is synthesized in liver and secreted into blood, to examine its temporal excretion in the urinary exosomal fraction in different types of AKI animal models to verify whether it can predict the occurrence of structural renal injury. We also measured urinary exosomal Fetuin-A in a small number of intensive care unit (ICU) patients with and without AKI for a preliminary assessment of the potential of this biomarker.

RESULTS
Induction of AKI
Cisplatin injection (6 mg/kg, intravenously) induced a significant increase in SCr and renal morphological damage including extensive t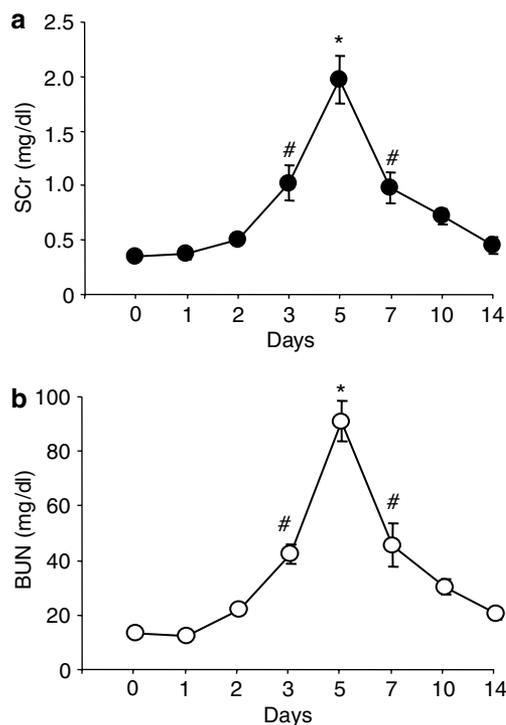ubular damage and intra-tubular cast formation in the outer stripe of the outer medulla (OSOM). SCr and blood urea nitrogen increased significantly by day 3, reached a peak at day 5, and then gradually returned to the basal value by day 14 (Figure 1a and b). Tubule morphology was normal on day 2, the brush border began to detach at day 3, tubular damage was most severe at day 5, and then gradually recovered thereafter (Figure 2a–e). However, renal function and histology was still normal at 2 days after cisplatin injection.

Bilateral ischemia for 37 min and reperfusion (I/R) increased SCr (from 0.47 ± 0.04 to 1.0 ± 0.42 mg/dl at 8 h, 1.57 ± 0.22 mg/dl at 24 h after I/R) and caused tubular damage in the OSOM in kidney detectable at 8 and 24 h (Figure 2f and g).

In contrast, a prerenal azotemia model generated by furosemide and low-sodium diet caused volume depletion (VD), weight loss ($9.69 \pm 0.99\%$), and moderately increased SCr (0.63 ± 0.04 vs 0.45 ± 0.04 mg/dl before treatment). No renal histology changes were detected (not shown).

Figure 1 | SCr and blood urea nitrogen in cisplatin-induced AKI in rats (*n* = 56). (**a**) SCr and (**b**) blood urea nitrogen increased significantly on day 3, peaked at day 5, and returned to baseline at day 14 after cisplatin injection. Data are means ± s.e.m. *$P < 0.01$, #$P < 0.05$ vs day 0.
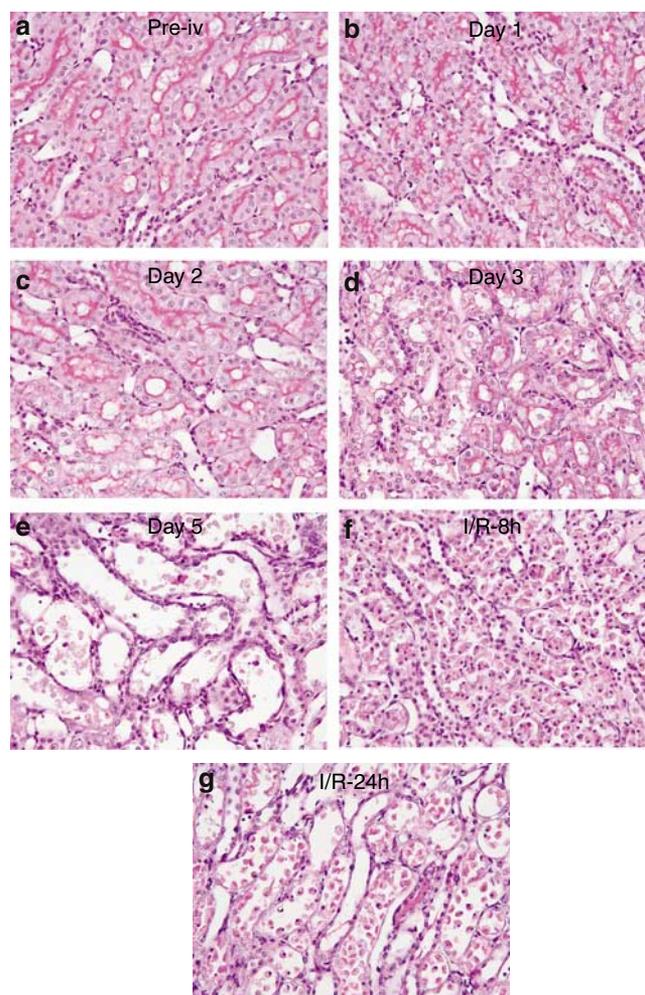

Figure 2 | Histology of the kidney sections stained with periodic acid-Schiff reagent in the OSOM after renal injury. Temporal morphologic change of OSOM (**a**) before, and at (**b**) day 1, (**c**) day 2, (**d**) day 3, and (**e**) day 5 after cisplatin injection. Kidney (**f**) 8 h and (**g**) 24 h reperfusion after 37 min of bilateral ischemia. Original magnification × 400.
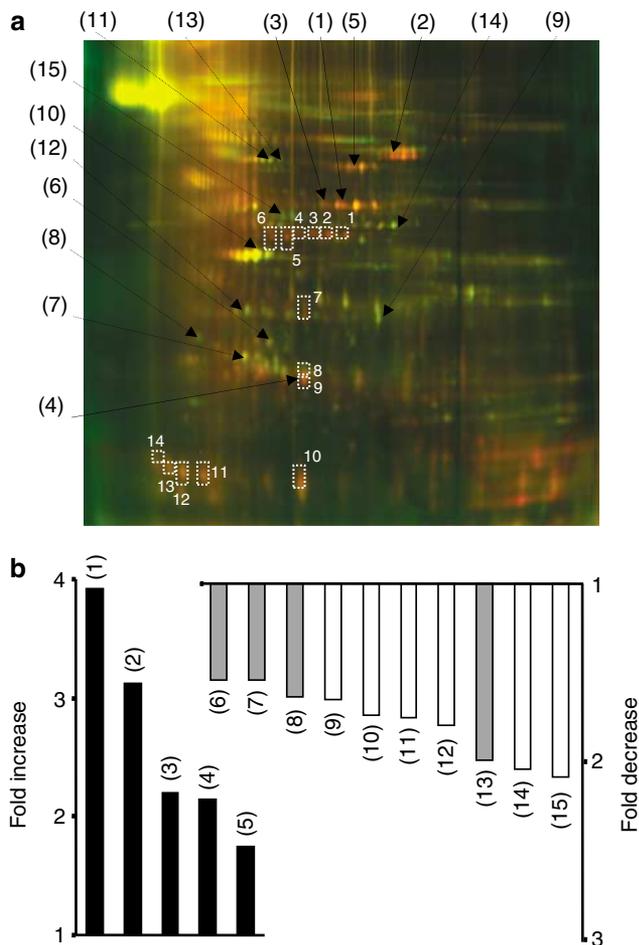
Figure 3 | 2D-DIGE and the fold change of urinary exosome-associated proteins of rats before and after cisplatin injection.
Urinary exosome-associated proteins were isolated from pooled 8 h urine samples from 12 rats before and after cisplatin injection. (**a**) 2D-DIGE shows increase (red), decrease (green), or no change (yellow) in abundance of exosome-associated proteins after cisplatin injection. Black numbers were spots identified by MALDI-TOF-TOF, white numbers were spots identified by LC-MS/MS. (**b**) Fold change of urinary exosome-associated proteins that were increased or decreased after cisplatin. The gray bars are membrane-associated proteins. (**a**) Numbers (1–15) match the black numbers in 2D-DIGE.

Identification and validation of urinary exosomes-associated proteins

Cy dye-labeled urinary exosome-associated proteins were separated by two-dimensional difference in gel electrophoresis (2D-DIGE) (Figure 3a). The DeCyder (Biological Variation Analysis) software recognized 1769 spots; 19.4% increased by at least 1.5-fold and 13.3% decreased by at least 1.5-fold after cisplatin injection. Only 74 spots had (1) a student's *t*-test *P*-value of ≤0.05, (2) a ±1.5-fold change after cisplatin injection, and (3) matched to the preparative gel (SYPRO ruby-stained gel). These 74 spots were picked from the SYPRO ruby-stained gel, and their peptide digests were analyzed by full-scan mass spectrometry (MS) followed by MS/MS using the matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF)/TOF for protein identification. Fifteen proteins were identified by MALDI-

TOF/TOF (black numbers, Figure 3a and upper portion of Table 1). Five proteins increased and 10 proteins decreased after cisplatin injection (Figure 3b). Four of the identified proteins were membrane-associated proteins (gray bar in Figure 3b). Because many of the upregulated (red) spots were not identified, we re-picked 14 red spots from the SYPRO ruby gel and the gel plugs were manually digested and peptides ziptipped for liquid chromatography-MS (LC-MS)/MS analysis. Thirteen additional proteins were identified (white numbers, Figure 3a and lower portion of Table 1). By checking the tandem MS spectra of the corresponding fragment ions from these peptides, we found that Fetuin-A had high cross-correlation score ($X_{corr} = 4$) and an excellent MS spectra feature (Figure 4).

We used Western blot analysis to validate these identified proteins by MALDI-TOF-TOF or LC-MS/MS in urinary exosomes from individual rats 8 h before and after cisplatin injection. Of the nine commercially available antibodies (see Materials and Methods), only the Fetuin-A (increased) and Annexin V (decreased) results were consistent with the 2D-DIGE analysis after cisplatin injection. The other proteins either could not be detected by the available antibodies, or changed in the opposite direction than that predicted by 2D-DIGE (data not shown). We selected Fetuin-A as a candidate biomarker for further.

Temporal urinary excretion of exosomal Fetuin-A in different types of AKI

We selected Fetuin-A to investigate its temporal excretion normalized to urine flow rate (per hour) in several renal injury models based on our observation that it increased during the early phase of cisplatin-induced AKI. Urinary Fetuin-A increased 2.75-fold at 24 h after cisplatin injection, peaked 52.5-fold at day 2 after cisplatin injection, and remained elevated (51.5-fold) until day 5 compared to normal urine samples (Figure 5a). We found that Fetuin-A was detected 2 days before the occurrence of SCr increase and tubular damage (Figures 1 and 2). We verified that the upper two of these multiple bands were Fetuin-A when compared to bovine Fetuin-A as a positive control and pre-adsorbing antibody with Fetuin-A agarose as a negative control (Supplementary Figure 8a). Urinary exosomal Fetuin-A also increased significantly 2–8 h after 15, 30, and 40 min of I/R, then remained at an elevated level in the 8–24 h collection. And also, the abundance of urinary Fetuin-A was correlated positively to the level of SCr at 24 h after I/R (Figure 5b). In addition, a 31.6-fold of average increment was observed in urinary Fetuin-A 2–8 h after 37 min I/R (Supplementary Figure 8b). We also examined urinary Fetuin-A in prerenal azotemia rats and normal rats as controls. Fetuin-A was not detected in 1 h urine collection in both groups of rats (Figure 5c). After increasing protein loading and increasing the exposure time, Fetuin-A could be detected, but was not different between normal and prerenal azotemic rats ($P = 0.7$) (Supplementary Figure 8c). To compare the expression of urinary Fetuin-A in the different models of

Table 1 | Proteins identified by MALDI-TOF-TOF and LC-MS/MS

Spot	Protein identified by MALDI-TOF-TOF	MW	pI	
1	Salivary and hepatic alpha-amylase (α-amylase)	58	6.5	
2	β-Glucuronidase precursor	75	6.3	
3	Glucan glucanohydrolase	58	7.2	
4	Serum amyloid P-component precursor (SAP)	26	5.5	
5	Serum albumin precursor	71	6.1	
6	Annexin IV	36	5.3	
7	Chloride intracellular channel protein 1(NCC27)	27	5.1	
8	Annexin V (Anchorin CII)	36	4.9	
9	Malate dehydrogenase, cytoplasmic (MDH)	36	6.2	
10	Actin, cytoplasmic 1 (β-actin)	42	5.3	
11	Heat-shock cognate 71 kDa protein (HSP70)	71	5.4	
12	Regucalcin (Senescence marker protein 30)	34	5.3	
13	V-ATPase 69 kDa subunit 1	69	5.6	
14	Enolase 1	47	6.2	
15	(GSH-S) synthetase	53	5.5	
New hits	**Protein identified by LC-MS/MS**	**MW**	**pI**	**Spots in 2-D map**
1	Keratin, type II cytoskeletal 8	54	5.8	
	Type II cytoskeletal 1b	57	5.5	
	Type I cytoskeletal 21	49	5.3	Identified in spots 1–14
2	ANXA2_RAT Annexin A2 (Annexin II) (Lipocortin II)	38	5.7	Spots 2, 3, 5
3	EF1A2_RAT Elongation factor 1-alpha 2 (EF-1-alpha-2)	50	9.1	Spot 1
4	KPYM_RAT Pyruvate kinase, isozymes M1/M2	57	6.6	Spot 3, 5
5	RAT Histone H2B,	14	10.3	Spot 2
	Histone H4	11	11.4	Spot 8, 11
6	MYH9_RAT Myosin heavy chain, nonmuscle type A	23	5.5	Spot 2
7	NHERF_RAT Ezrin-radixin-moesin binding phosphoprotein 50	39	5.7	Spot 3
8	MUP_RAT Major urinary protein precursor (MUP)	21	5.8	Spot 10
9	PCLO_RAT Piccolo protein (Multidomain presynaptic cytoma)	55	6.1	Spot 10
10	**FETUA_RAT Alpha-2-HS-glycoprotein precursor (Fetuin-A)**	37	6.1	Spot 12, 13
11	GFAP_RAT Glial fibrillaryacidic protein, astrocyte	50	5.4	Spot 12
12	GSH0_RAT Glutamate – cysteine ligase regulatory subunit	31	5.4	Spot 8
13	Serum amyloid P-component precursor (SAP)	2.6	5.5	Spot 7, 8, 9

GSH, glutathione; LC-MS, liquid chromatography-mass spectrometry; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MW, molecular weight; pI, isoelectric point.

AKI, we selected one rat from each of the three AKI models (pre-, day 0, 1, and 2 after cisplatin injection; pre- and 2–8 h after I/R; pre- and 24–30 h after VD) and loaded 1 h collections of urinary exosomal proteins on a single gel to reduce the bias created from separate gels. Urinary Fetuin-A was only detected in the early phase of cisplatin- or I/R-induced AKI but not in VD animal. The trend of Fetuin-A is the same as the separate gels (Figure 5c).

Distribution of Fetuin-A in urine
Fetuin-A is synthesized in liver and secreted in blood. We hypothesized that blood Fetuin-A might be filtered at the glomerulus, escape tubular reabsorption, and hence appear in a soluble non-exosomal fraction. Alternatively, Fetuin-A could be excreted in detached epithelial cells and appear in the cast fraction. We checked these urinary compartments using urine samples from day 2 after cisplatin injection, which had high levels of exosomal Fetuin-A. First, we isolated proteins from the cast fraction ($17\,000 \times g$ pellet) and exosome fraction ($200\,000 \times g$ pellet) by differential centrifugation; proteins from whole urine, $17\,000 \times g$ supernatant, and $200\,000 \times g$ supernatant (exosome-free fraction) were precipitated. By Western blot analysis, most of urinary

Fetuin-A appeared in the urinary exosome fraction; only a small amount of Fetuin-A was detected in urinary casts (Figure 6a). Then, we compared the effect of washing the $200\,000 \times g$ pellets with isolation solution to remove free, soluble Fetuin-A that could possibly adhere to the outer surface of vesicles. No difference of urinary exosomal Fetuin-A was found between $200\,000 \times g$ pellets with and without washing (Figure 6b). Finally, immunoelectron microscopy (IEM) was used to determine whether urinary Fetuin-A is located inside of urine vesicles. Fetuin-A was found inside of urine vesicles (Figure 6c). In contrast, no positive stained particles were detected inside or outside of urinary vesicles in negative control samples (data not shown). Taken together, these studies demonstrated that Fetuin-A was mostly excreted in the exosome fraction of urine.

Location of Fetuin-A in rat kidney during development of AKI
Fetuin-A was not detected by immunohistochemical examination in normal kidney on day 1 after cisplatin injection and was barely detectable in the cytoplasm of tubule epithelial cells in the OSOM staining on day 2 after cisplatin injection (Figure 7a and b). The staining of Fetuin-A increased significantly in proximal tubular cells with normal
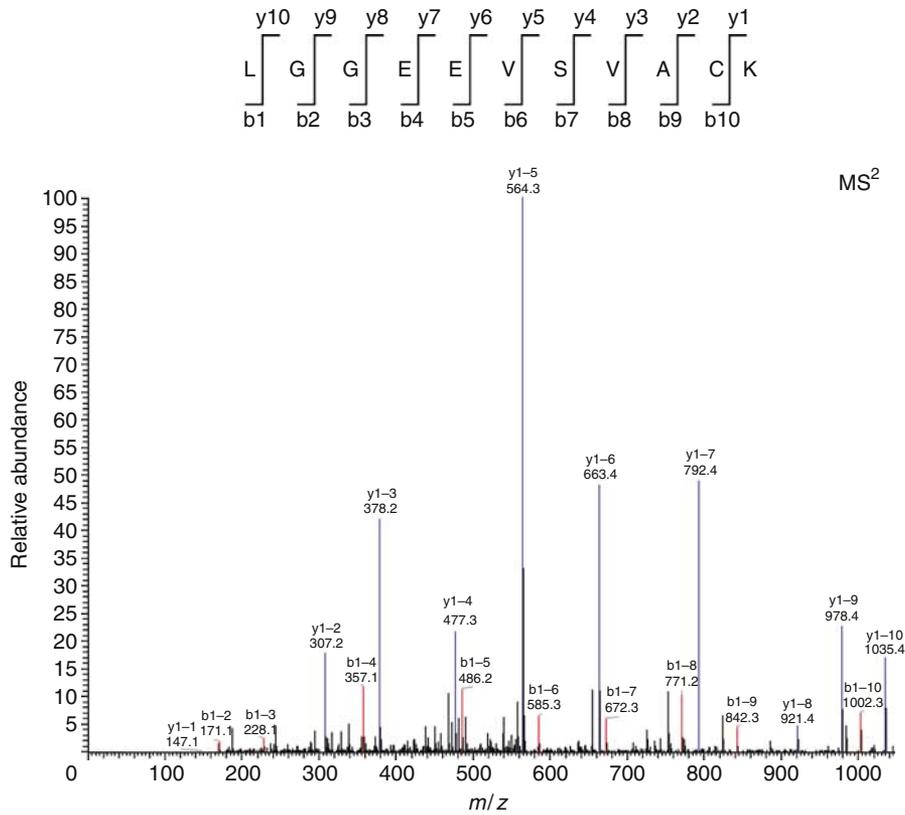
Figure 4 | Identification of Fetuin-A protein by LC-MS/MS. MS^2 spectra of a Fetuin-A peptide (sequence: LGGEEVSVACK). The red peaks indicate matched b-ion series, the blue peaks indicate matched y-ion series, and the black peaks indicated unmatched ions. The majority of peaks are matched in agreement with the high cross-correlation score ($X_{corr} = 4$).
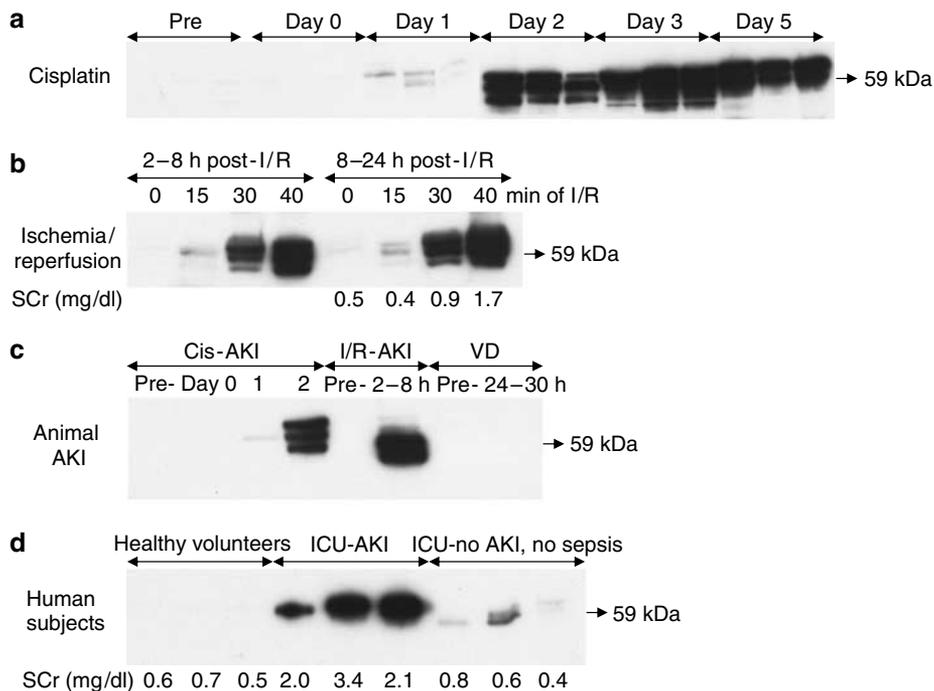

Figure 5 | Temporal excretion of urinary exosomal Fetuin-A in AKI animal models and human subjects by Western blotting analysis. (**a**) Cisplatin-induced AKI rats (typical result from three replicates), (**b**) I/R-induced AKI rats (in 15, 30, and 40 min of bilateral I/R), (**c**) one blot of above three types of AKI rats (one rat pre-, day 0, day 1, and day 2 after cisplatin injection; one rat from pre- and 8 h after I/R; one rat from pre- and 24–30 h after VD), and (**d**) ICU patients with and without AKI compared to healthy volunteers.
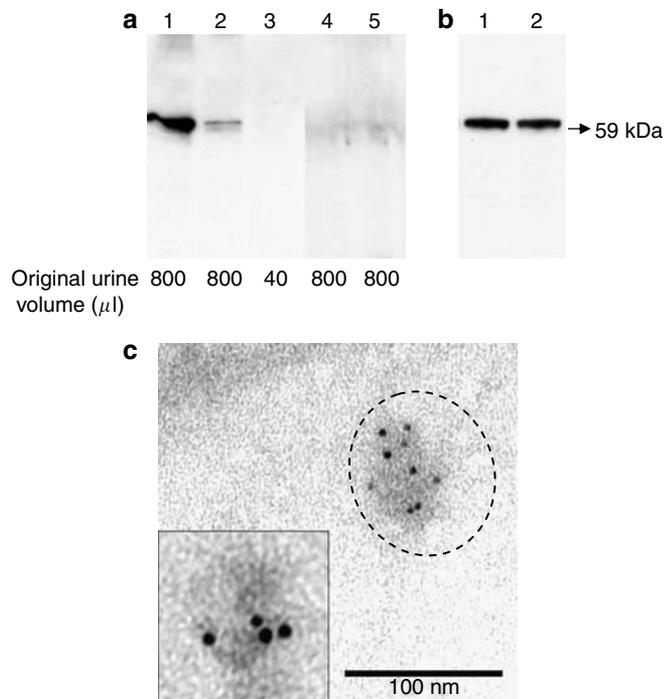
Figure 6 | **The distribution of Fetuin-A in rat urine 2 days after cisplatin injection.** (**a**) The secretion of Fetuin-A in different urinary fractions from the same urine sample (lane 1: 200 000 × *g* pellets, lane 2: 17 000 × *g* pellets, lane 3: whole urine, lane 4: 17 000 × g supernatant, and lane 5: 200 000 × g supernatant) by Western blot analysis. The lower label indicates the original volume of urine used for each lane. (**b**) Comparison of urinary exosomal Fetuin-A in the 200 000 × *g* pellets without and with washing by isolation solution (lane 1: unwashed pellet; lane 2: washed pellets). (**c**) Fetuin-A located inside of urinary vesicles in 200 000 × *g* pellets by IEM. Bar = 100 nm. Inset shows a magnified image of a urinary vesicle labeled with gold-conjugated anti-Fetuin-A.

Figure 7 | **Location of Fetuin-A in OSOM of rat kidney.** Left panels: Temporal immunohistochemistry staining for Fetuin-A in OSOM of kidneys (**a**) before and at (**b**) day 2, (**c**) day 3, and (**d**) day 5 after cisplatin injection in rats. Right panel: (**e**) Negative control (injured kidney section at day 3 after cisplatin injection), Sham surgery for (**f**) I/R, I/R rats at (**g**) 8 h, and (**h**) 24 h after I/R. Original magnification × 400.

structure and few damaged tubules on day 3 (Figure 7c); no positive staining was detected in the day 3 kidney sections with renal structural injury when primary Fetuin-A antibody was used instead of normal donkey serum (Figure 7e); and then was expressed highly in the detached tubular cells in the tubule lumen on day 5 after cisplatin injection (Figure 7d). After I/R, positive staining of Fetuin-A increased significantly in partially damaged proximal tubule cells at 8 h and was restricted to the detached cells in the lumen by 24 h after the surgery compared to the sham kidney (Figure 7f–h).

Urinary exosomal Fetuin-A in ICU patients

Spot urine samples were collected and stored at −80°C from three healthy volunteers (age 34–43 years), three ICU patients with AKI, and three ICU patients without AKI. The clinical details of the ICU patients are listed in Table 2. We isolated exosome-associated proteins from these urine samples and examined the abundance of Fetuin-A normalized by urine creatinine using Western blot analysis. Urinary exosomal Fetuin-A increased significantly in ICU patients with AKI compared to either healthy volunteers or ICU patients without AKI (Figure 5d).
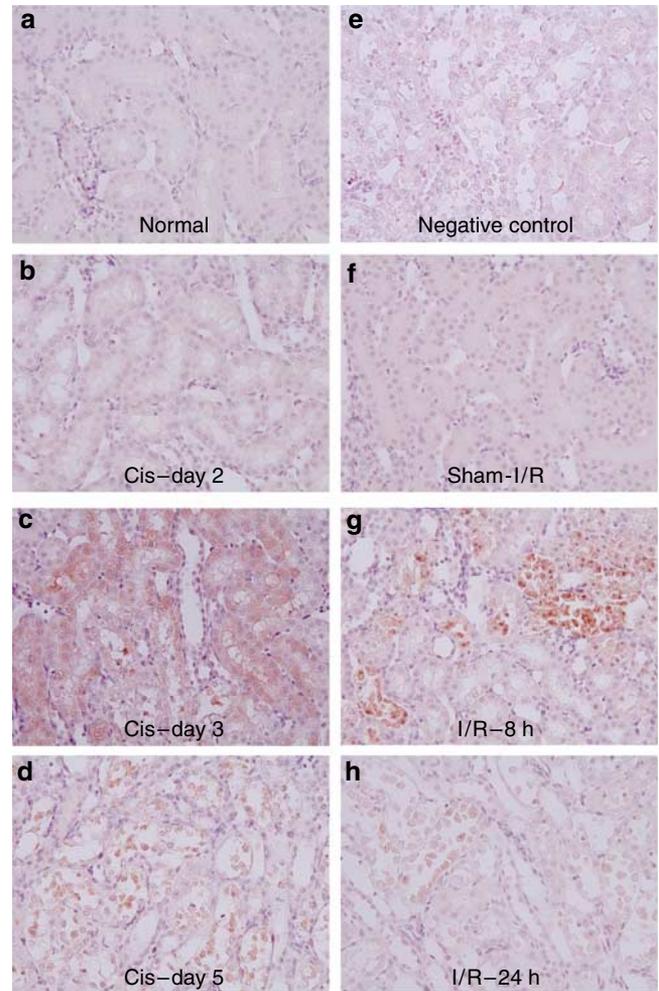
DISCUSSION

Urine is an ideal source for discovery of non-invasive biomarkers and it is likely that multivariable assessment tools that incorporate both biomarkers and clinical information can enhance the diagnostic and predictive accuracy in a complex disease such as AKI.[5] Recently, it has been suggested that proteomic techniques including high-throughput identification of proteins may be useful to discover urinary biomarkers for various kidney diseases.[23,24] These methods have thus far identified that abundant proteins or well-known proteins such as albumin, transferrin, and α-1-antitrypsin are increased in glomerular diseases;[24] and urinary β2 microglobulin, retinol-binding protein, and carbonic anhydrase are increased in acute renal allograft rejection.[25] However, these abundant proteins lack specificity for diagnosis of disease and impede the discovery of new biomarkers, especially in glomerular diseases. It has been

Table 2 | Characteristics of ICU patients in the study

Patients	Age/gender	Primary diagnosis	APACHE II score	AKI	Serum creatinine (mg/dl)	Blood urea nitrogen (mg/dl)	Urine output (ml/day)	Furosemide (mg)	Blood culture	SIRS	Sepsis	WBC	Temperature
1	86/M	MI, AKI, pneumothorax	22	Yes	2.0	36	207	100	No	No	No	11.79	37.3
2	67/M	COPD lung cancer AKI	14	Yes	3.4	89	477	80	No	No	No	17.86	37.4
3	37/M	Pneumonia, ARDS	21	Yes	2.1	54	2381	None	No	Yes	Yes	2.9	38.2
4	50/F	Meningitis	10	No	0.8	11	2468	None	Yes (+)	Yes	Yes	12.46	37.8
5	42/M	Pneumonia	9	No	0.6	11	2135	20	No	Yes	No	14.77	38.5
6	41/F	DM, necrotizing, fascitis	17	No	0.4	16	1617	None	Yes (+)	Yes	Yes	10.76	38.7

AKI, acute kidney injury; APACHE II, acute physiology and chronic health evaluation; ARDS, acute respiratory distress syndrome; COPD, chronic obstructive pulmonary disease; DM, diabetes mellitus; F, female; ICU, intensive care unit; M, male; MI, myocardial infarction; SIRS, systemic inflammatory response syndrome; WBC, white blood cell.

hypothesized that the low abundance urinary proteins might be better targets for biomarker discovery.[26] However, the removal of interfering abundant proteins might also remove less abundant proteins that bind to the abundant proteins.

Recently, exosomes containing both membrane and cytosolic proteins were isolated from normal human urine by differential centrifugation, and exosomal proteins related to kidney diseases were identified by LC-MS/MS.[7] Exosomal protein biomarkers might be clinically useful biomarkers; Du Cheyron *et al.*[17] reported that sodium/hydrogen exchanger isoform 3, a membrane protein, increased in acute tubule necrosis patients but not in prerenal azotemia or acute glomerular diseases. We thought that this urinary compartment, containing a reduced protein complexity and depleted predominant proteins, might be used for biomarker discovery, particularly for proteins that might indicate structural renal disease.

We investigated urinary exosomes from cisplatin-induced AKI rats for biomarker discovery by 2D-DIGE. We could identify only 20% of the picked spots, and only 26% of the identified proteins were membrane-associated proteins, potentially caused by insufficient solublization, limited access to trypsin sites, or low abundance of membrane proteins. We also had great difficulty validating the DIGE results, perhaps owing to the pitfalls inherent in 2D analysis, including changes in spot position owing to post-translational modification or the presence of multiple proteins per spot, or to insensitive antibodies. Further research to more effectively solubilize membrane proteins, optimize analytical protocols, and improve the detection of post-translational modifications will be required to overcome these current limitations in the proteomics field.

Nevertheless, we did find that one excreted exosomal protein, Fetuin-A (P24090), that was increased after renal injury and could be validated by Western blotting. Two red spots (spots 12 and 13) that were increased after cisplatin injection were identified as Fetuin-A by LC-MS/MS on the third survey of the gel. However, the position of these spots on the 2D gel did not match the predicted molecular weight and isoelectric point. We hypothesize that cleavage of Fetuin-A occurred before 2D electrophoresis and that intact Fetuin-A with correct molecular weight and isoelectric point was not seen because Fetuin-A is highly glycosylated. Fetuin-A, also known as alpha-2-HS-glycoprotein (NP_001613), was identified by MALDI-TOF MS in a previous proteomic analysis of human urinary proteins isolated by acetone precipitation.[27] However, Fetuin-A was not identified by MALDI-TOF MS or LC-MS/MS in normal urinary exosomal proteins isolated by ultracentrifugation from either human[7,27] or male Sprague—Dawley rats.[28]

We found that most of urinary Fetuin A was detected in the $200\,000 \times g$ pellet. Only a small amount was in the $17\,000 \times g$ pellet, and none was detected in the supernatant of $200\,000 \times g$ after AKI. Washing the $200\,000 \times g$ pellet to remove bound Fetuin-A that could adhere to the outer surface of vesicles, did not affect the abundance of urinary Fetuin-A. Moreover, IEM revealed Fetuin-A located inside of urinary vesicles; a previous study also demonstrated that Fetuin-A is located in the vesicles from culture medium of human vascular smooth muscle cells.[29]

The quantification of Fetuin-A identified by 2D DIGE was validated by the low abundance or lack of Fetuin-A in normal urine and kidney when compared to the large increase in urine and kidney in animal models of cisplatin and I/R, and in human ICU patients with AKI. Fetuin-A increased 2 days earlier than SCr in cisplatin-induced AKI and before SCr increased in I/R-induced AKI. Urinary Fetuin-A was detectable 1 day before recognition of morphological injury and remained high, whereas structural renal injury was present morphologically. Fetuin-A did not increase following VD (prerenal azotemia), which is sometimes difficult to differentiate from structural renal injury in the clinic. Urinary Fetuin-A showed a similar spectrum compared to the other current early urinary biomarkers in AKI models, such as kidney injury molecule-1, NGAL, interleukin-18, and NAG.[5] However, Fetuin-A has two interesting characteristics: (1) it has a very large dynamic range, similar to NGAL, but much larger than kidney injury molecule-1, and (2) the modestly delayed onset in cisplatin, compared to the immediate increase of other markers such as NGAL, suggests that Fetuin-A might be useful to detect structural injury, not early renal response to injury. Taken together, the pre-clinical animal data suggest that urinary exosomal Fetuin-A might serve as an additional urinary biomarker to predict structural renal injury.

We also found that urinary exosomal Fetuin-A was much higher in ICU patients with AKI compared to faint

expression of Fetuin-A in ICU patients without AKI, and was not detected in the urine exosome fraction of healthy volunteers, consistent with the previous study.[7] Mortality and morbidity of sepsis-induced AKI in ICU remains high. A simple urinary biomarker has limitations in a complex population such as ICU patients with AKI. Sepsis-induced AKI is often difficult to diagnose because patients are volume expanded, which depresses SCr, and the fractional excretion of sodium may be low despite irreversible renal injury.[30] Recent studies have found that urinary NGAL increased 2 h after cardiac surgery and can accurately predict AKI in children; however, it does not perform as well in adult ICU patients with multifactorial AKI.[12] Urinary interleukin-18 increased at 4–6 h after cardiac surgery[31] and also increased 24 h before SCr in ICU patients with AKI. Both urinary NGAL and interleukin-18 could also partially predict the outcome of AKI.[16] Du Cheyron et al.[17] reported that urinary sodium/hydrogen exchanger isoform 3 isolated by ultracentrifugation could distinguish acute tubule necrosis from other forms of AKI. It is likely that a panel of biomarkers, along with associated clinical information, will be required for early and accurate diagnosis of ICU patients with AKI. Our current studies suggest that urinary exosomal Fetuin-A might be useful in human AKI; however, additional studies will be needed to determine whether urinary Fetuin-A can predict, diagnose, track treatment effects, evaluate severity or prognosis in a large number of ICU patients and patients with typical acute tubule necrosis.

Possible mechanism of why urinary exosomal Fetuin-A is increased in AKI

Fetuin-A is synthesized in liver and secreted into the blood stream, where it is a negative acute-phase response protein and plays its anti-inflammatory role via suppressing release of tumor necrosis factor-alpha induced by lipopolysaccharide *in vitro* and *vivo*.[32] Fetuin-A is also a systemically acting inhibitor of ectopic calcification,[33] and decreased serum Fetuin-A concentration is associated with a higher mortality rate in dialysis patients and can predict mortality of chronic kidney diseases.[34,35] High plasma Fetuin-A levels are correlated with insulin resistance and fatty liver in human[36] and are also independently associated with metabolic syndrome in non-diabetic outpatients with coronary artery disease.[37] However, the function of Fetuin-A in AKI is unknown.

We hypothesized that increased urinary exosomal Fetuin-A might be secreted from proximal tubule cells. A previous study reported that Fetuin-A was located in the developing kidney tubule in rat embryo by immunohistochemical staining.[38] In this study, Fetuin-A was located in the cytoplasm of damaged proximal tubules that were attached on the basement membrane and at even higher concentration in proximal tubule cells that had detached from the tubule basement membrane. Although exosomal Fetuin-A may be synthesized by the kidney, it may also appear in the urine as a result of incomplete proximal tubule processing in protein-

uria states (a form of overflow proteinuria) or released during tubular cell apoptosis. Fetuin-A was detected in apoptotic vascular smooth muscle cells,[29] and apoptotic cells have been demonstrated in tubular cells in cisplatin- or I/R-induced AKI in animals.[39–41] We did not find evidence for free Fetuin-A that was filtered at the glomerulus, at least in the settings examined.

Conclusion

Proteomic methods can be used to discover candidate urinary biomarkers for AKI, although the process in the present study is more difficult because of inefficiencies at the proteome identification and antibody recognition steps. Urinary exosomal Fetuin-A is increased in AKI, but not in prerenal azotemia. Urinary exosomal Fetuin-A is elevated in patients with AKI in ICU. Determination of the clinical value of urinary Fetuin-A requires further study in a large number of patients.

MATERIALS AND METHODS
AKI models and urine collection

Male Sprague–Dawley rats were purchased from Harlan Sprague—Dawley Inc. (Indianapolis, IN, USA). All animals had free access to water and standard food, and were treated in accordance with the National Institute of Health guidelines for use and care of research animals. All urine samples from animals were collected using protease inhibitors in metabolic cages and then stored at −80°C, until the isolation of exosome-associated proteins as described previously.[6]

Cisplatin-induced AKI (n = 151): Rats (250–280 g; 8 weeks) were given by a single intravenous injection of cisplatin (6 mg/kg body weight) (Sigma Corp, St Louis, MO, USA). Blood samples from the abdominal aorta were collected in 56 rats under isoflurane anesthesia before cisplatin (day 0) and at 1, 2, 3, 5, 7, 10, and 14 days after cisplatin injection. Urine samples were collected from 12 rats before (−24 to −16 h) and 0–8 h after cisplatin injection and pooled for biomarker discovery by proteomics analysis. We then collected urine samples from four individual cisplatin-treated rats for validation of the identified proteins. A second set of temporal urine samples were collected on 0–5 days from three additional rats for validation of Fetuin-A. Urine samples were also collected at day 2 after cisplatin injection for examining urinary IEM.

I/R-induced AKI (n = 12): Rats (180–210 g; 7 weeks) were subjected to respective 15, 30, and 40 min bilateral I/R and sham surgery (n = 4) as described previously.[19] Urine samples were collected at 2–8 and 8–24 h after I/R. Additional eight rats were subjected to 37 min I/R for temporal urine collections at −24 to −18 h before I/R and 2–8 h and 8–24 h after I/R. The rats were killed at 8 or 24 h after I/R for the collection of blood samples and kidney tissues.

VD (n = 4): Rats (380–400 g; 14 weeks) were fed a low-salt diet (0.03%) (Diet Test Inc., Philadelphia, PA, USA) 18 h before intraperitoneal injection of furosemide as described previously.[19] We collected 6 h urine samples from four rats at −24 to −18 h before and 24–30 h after VD. Rats were killed at 33 h after furosemide injection for blood collection.

Human samples: To verify the clinical potential of Fetuin-A, we also collected spot human urine samples from three healthy volunteers, three ICU patients with AKI, and three ICU patients

with and without AKI and sepsis. Samples were collected at George Washington University Intensive Care Unit under approval of the GW Institutional Review Board (Protocol 090013ER).

Examination of renal function

SCr was measured by picric acid-based colorimetric autoanalyzer (Astra 8 autoanalyzer; Beckman Instruments, Fullerton, CA, USA) and blood urea nitrogen by an autoanalyzer (Hitachi 917, Boehringer Mannheim, Indianapolis, IN, USA). The kidneys were examined for histology and immunohistochemistry. The harvested left kidney from each animal was immediately fixed in 10% neutral buffered formalin solution. The kidney tissue block was dehydrated through a graded alcohol series, embedded in paraffin, and cut into 4-μm sections and then stained with periodic acid Schiff reagent.

Isolation of urinary exosome-associated proteins and depletion of Tamm–Horsfall protein

Urine samples were extensively vortexed immediately after they thawed. Urinary exosome-associated proteins were isolated by differential centrifugation ($17\,000 \times g$ 15 min to remove urinary sediment then $200\,000 \times g$ 1 h pellet) as described previously.[6,7] The exosome-associated proteins isolated from the pooled urine samples were suspended by isolation solution ($10\,\text{mM}$ triethanolamine, $250\,\text{mM}$ sucrose (pH 7.6)). To partially deplete the abundant Tamm–Horsfall protein, the suspensions were incubated with reducing agent dithiothreitol ($200\,\text{mg/ml}$) at 95°C for 2 min, and the samples were ultracentrifuged again. This procedure was repeated a second time, allowing the denatured Tamm–Horsfall protein to be released into the supernatant.[7] The high-spin pellets were solubilized in 2D lysis buffer ($7\,\text{M}$ urea, $2\,\text{M}$ thiourea, 4% CHAPS, and $15\,\text{mM}$ Tris (pH 8.5)) for 2D proteomic analysis. The concentration of protein was measured by 2D quant protein assay (Amersham, Piscataway, NJ, USA). For Western blot analysis, exosome-associated proteins were isolated from individual rat urine samples and suspended by isolation solution. For comparison with urinary exosome fraction, acetone precipitation was used to concentrate the soluble protein in whole urine, $17\,000 \times g$ supernatant, and $200\,000 \times g$ supernatant.[6]

Proteomics methods

Two-dimensional difference in gel electrophoresis. 2D gels were run in triplicate. Four hundred picomoles of CyDye were used to label $50\,\mu$g of protein as suggested in the manufacturer's protocol: pre-cisplatin proteins were labeled with Cy 3; post-cisplatin proteins with Cy 5; a mixture of both proteins were labeled with Cy 2 as an internal standard. After quenching the reaction with $10\,\text{mM}$ of lysine, the labeled samples were mixed together and rehydration buffer was added ($7\,\text{M}$ urea, $2\,\text{M}$ thiourea, 4% CHAPS, 1% ampholytes pH 3-10NL (Amersham), $40\,\text{mM}$ dithiothreitol, and 0.0002% bromophenol blue). We also added $750\,\mu$g of unlabeled protein mixture in another gel for SYPRO ruby staining in order to obtain protein identification. We normalized the loading proteins with and without cisplatin treatment by collection time. The mixed samples were run in triplicate, loaded on an immobilized pH gradient strip ($24\,\text{cm}$; pH 3-10NL) for isoelectric focusing according to the following protocol: 30 V for 14 h (active rehydration), 500 V for 500 V h, 1000 V for 1000 V h, and 8000 V for 66 667 V h. Each strip was equilibrated for 15 min in a equilibration solution containing $50\,\text{mM}$ Tris-HCl (pH 8.8), $6\,\text{M}$ urea, 30% glycerol, 2% sodium dodecyl sulfate, 0.002% bromophenol blue with 0.5% dithiothreitol followed

by a second 15 min equilibration with iodoacetamide (4.5%). Strip were briefly rinsed in $1\times$ sodium dodecyl sulfate-polyacrylamide gel electrophoresis buffer and applied to a 12.5% polyacrylamide gel for electrophoresis at 5 W/gel for 30 min followed by 17 W/gel for 4 h, 15°C.

Image acquisition and analysis. CyDye-labeled gels were scanned on a Typhoon 9400 variable mode imager (Amersham Biosciences, Piscataway, NJ, USA) at an excitation wavelength of 520/40 (maximal/bandwidth) for Cy2- (laser-blue), 580/30 for Cy3- (laser-green), or 670/30 for Cy5- (laser-red) labeled samples. The photo multiplier tube (PMT) setting was adjusted to avoid signal saturation. The image resolution was $100\,\mu$m. Images were then processed with Decyder Biological Variation Analysis module v5.01 software (Amersham Biosciences) to match multiple 2D-DIGE gels for comparison and statistical analysis of protein abundance changes by spot fluorescence intensities. After protein spots were designated to be picked, the gel with $750\,\mu$g protein was fixed in 30% methanol and 7% acetic acid for 1 h and incubated in SYPRO ruby protein gel stain (Bio-Rad Laboratories, Hercules, CA, USA) overnight. The pick gel was then washed three times with ultrapure water (Millipore, Billerica, MA, USA) for 10 min each wash. Next, the gel was scanned at an excitation wavelength of 610/30 to visualize all proteins in the gel. CyDye- and SYPRO-derived images were matched using Biological Variation Analysis software, from which a pick list was generated.

Spot picking and protein identification. An automated Ettan Spot Handling Workstation (GE healthcare, Piscataway, NJ, USA) was used for gel-spot picking, in-gel protein digestion with trypsin, and spotting on MALDI plates, as described previously.[42] Peptides were analyzed using the Proteomics Analyzer (ABI 4700 MALDI-TOF/TOF). Full-scan MS spectra were obtained first, followed by MS/MS spectra. Protein identification was carried out using the search engine, MASCOT (Matrix Science, Boston, MA, USA). An additional 14 spots not identified by MALDI-TOF-TOF were picked with the Ettan Spot Handling Workstation then manually digested with trypsin and desalted with C18 Ziptips (Millipore, Billerica, MA, USA). The extracted peptides were analyzed using the Finnigan LTQ mass spectrometer (Thermo Electron, San Jose, CA, USA), according to the published procedure.[43]

Western blot analysis

Exosome-associated protein samples (from urine samples of the same time for each rat) were separated by 1D sodium dodecyl sulfate/polyacrylamide gel electrophoresis and then gels were transferred to polyvinylidene difluoride membranes. After blocking with 5% milk (1 h), membranes were probed overnight at 4°C with antibodies: rabbit polyclonal antibodies to Fetuin-A (1:500) (Novus Biologicals, Littleton, CO, USA), α-Amylase (1:5000) (Rockland, Gilbertsville, PA, USA), serum Amyloid P (1:1000) (Abcam Inc., Cambridge, MA, USA), Annexin V ($2\,\mu$g/ml) (Abcam), neuron specific enolase (1:1000) (Biogenesis Inc., Kingston, NH, USA); mouse monoclonal antibodies to β-Glucuronidase (1:50) (Cell Sciences, Canton, MA, USA), Regucalcin (1:500) (Cell Sciences), Annexin IV (1:1000) (BD Biosciences Pharmingen, San Diego, CA, USA); sheep polyclonal anti-MDH (1:4000) (Rockland). Peroxidase-conjugated, affinity-purified donkey anti-rabbit, anti-mouse, or anti-sheep immunoglobulin G (1:100 000) (Jackson Immuno-Reseach, West Grove, PA, USA) were used for 90 min at room temperature. The antibody–antigen reactions were visualized by using enhanced chemiluminescence plus Western blotting detection system (Amersham Biosciences) and light-sensitive film (Kodak

BioMax XAR, Rochester, NY, USA). For quantification of band intensity, National Institute of Health Image software was used. To verify the true bands of Fetuin-A, we loaded duplicate of serial dilution of bovine Fetuin-A (Sigma Aldrich, St Louis, MO, USA) as a positive control and urinary exosomal proteins from a cisplatin-treated rat at day 3 on one gel and then gel was transferred to polyvinylidene difluoride membrane. The membrane was cut into two parts with same protein samples. One was probed with Fetuin-A antibody and another was probed with pre-adsorbed Fetuin-A antibody as a negative control with Fetuin-A agarose, immobilized on cross-linked 4% beads agarose (Sigma Aldrich) and then followed by the above procedure.

IEM of urinary Fetuin-A

A $200\,000 \times g$ pellet was obtained from 8 h urine samples of rats 2 days after cisplatin injection. The suspension was mixed 1:1 with 4% paraformaldehyde and then applied to 200-mesh nickel grids. After blocking with 1% bovine serum albumin and washing, the grid was incubated with polyclonal rabbit anti-Fetuin-A (1:500) (Novus) containing 0.02% Triton X-100 (to permeabilize the vesicle membrane) and only antibody solution for 45 min at room temperature. Then, the grids were exposed to goat anti-rabbit immunoglobulin G (1:40) conjugated with 6 nm colloidal gold particles (12 nm) (1:40, Jackson ImmunoResearch) for 60 min at room temperature. After washing, membranes were negatively stained with 0.5% uranyl acetate. After drying, the grids were examined with a JEOL 1200 EX electron microscope operated at 60 kV.

Immunohistochemical examination of Fetuin-A

The paraffin-embedded kidney blocks were cut at 4 μm thickness, deparaffinized, and rehydrated. After consuming endogenous peroxidase with 3% H_2O_2, slides were pre-incubated with 10% normal donkey serum to block nonspecific reactions and then incubated with polyclonal rabbit anti-Fetuin-A (1:150) (Novus) overnight at 4°C. The slides were followed by incubation with biotin-conjugated donkey anti-rabbit immunoglobulin G (1:1000) (Jackson ImmunoReseach) for 30 min at room temperature, and then reacted with streptavidin-conjugated peroxidase (Dako Corp., Carpinteria, CA, USA) for 30 min at room temperature. The reaction products were visualized using a DAB kit (Vector Laboratories Inc., Burlingame, CA, USA).

Statistical analysis

All data are expressed as mean ± s.e.m. Differences between groups were analyzed for statistical significance by *t*-test. A *P*-value < 0.05 was accepted as statistically significant.

SUPPLEMENTARY MATERIAL
Figure S8. Blot of purified Fetuin-A and changes of Fetuin-A in group animals subjected to ischemia/reperfusion or volume depletion.

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