Urinary exosomal transcription factors, a new class of biomarkers for renal disease

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Urinary exosomes are excreted from all nephron segments and constitute a rich source of intracellular kidney injury biomarkers. To study whether they contain transcription factors, we collected urine from two acute kidney injury models (cisplatin or ischemia-reperfusion), two podocyte injury models (puromycin-treated rats or podocin-Vpr transgenic mice) and from patients with focal segmental glomerulosclerosis, acute kidney injury and matched controls. Exosomes were isolated by differential centrifugation and found to contain activating transcription factor 3 (ATF3) and Wilms Tumor 1 (WT-1) proteins detected by Western blot. These factors were found in the concentrated exosomal fraction, but not in whole urine. ATF3 was continuously present in urine exosomes of the rat models following acute injury at times earlier than the increase in serum creatinine. ATF3 was found in exosomes isolated from patients with acute kidney injury but not from patients with chronic kidney disease or controls. Urinary WT-1 was present in animal models before significant glomerular sclerosis and in 9/10 patients with focal segmental glomerulosclerosis but not in 8 controls. Our findings suggest that transcription factor ATF3 may provide a novel renal tubular cell biomarker for acute kidney injury while WT-1 may detect early podocyte injury. Measurement of urinary exosomal transcription factors may offer insight into cellular regulatory pathways.

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Much effort is being expended in discovering noninvasive biomarkers for acute and chronic kidney injury.[1,2] Several promising biomarker candidates are being evaluated for their effectiveness for early detection, disease classification, predicting severity/outcome, and/or predicting response to treatment.[2–22] Urinary exosomes are a rich source of biomarkers because they are released from every segment of the nephron, including podocytes.[23] Exosomes are right-side-out vesicles that originate from endocytic vesicles that fuse with multivesicular bodies (MVB). Invagination of the MVB membrane can form internal vesicles within the MVB that have a right-side-out orientation. When an MVB fuses with the plasma membrane, the internal vesicles enter the extracellular space as exosomes.[24] Membrane proteins such as transporters and ion channels are expected to be highly enriched in exosomes;[23,25] however, exosomes can also contain cytosolic proteins.[23,26] Transcription factors (TF) are found in the cytosol as well as the nucleus and should theoretically be present in exosomes. However, TFs have not been detected in exosomes using proteomics discovery techniques, perhaps because TFs are usually expressed at such low levels inside of cells.

Transcription factors can be activated by developmental, physiological, pathological, and therapeutic stimuli. Because they orchestrate the mobilization of several genes, TFs often play central roles in the initiation and development of many kidney diseases. Therefore, measuring TFs might be more informative than measuring downstream proteins. In microarray experiments, mRNA levels of some TFs were markedly induced during the early phase of acute kidney injury (AKI)[27–32] TFs also play important roles in the development of glomerulosclerosis and tubulointerstitial fibrosis.[33,34] Changes in mRNA levels do not always correspond to changes in protein levels for a given gene;[29,35] therefore, if candidate genes are identified by microarray experiments, the genes are best evaluated at the protein level before entering a biomarker development pipeline.[1]

Transcription factors are expressed at low levels inside of cells and are difficult to detect outside of cells; indeed,

transcription factor proteins have not been detected in urine. The purpose of this study was to determine whether TFs can be detected in urine by examining the exosomal fraction and whether TFs can serve as novel urinary biomarkers for acute and chronic renal injury. We chose two transcription factors previously identified by microarray studies: activating transcription factor 3 (ATF3) for AKI[29,31,36] and Wilms' Tumor 1 (WT-1) for focal segmental glomerulosclerosis (FSGS).[37] We examined temporal expression of ATF3 in ischemia/reperfusion (I/R) and cisplatin animal models of AKI and a small number of human samples. We evaluated the temporal expression of WT-1, which is often used as a molecular marker for podocytes,[34,38] in urinary exosomes from puromycin-induced FSGS in rats and doxycycline-induced collapsing glomerulopathy (CG) in podocin/Vpr transgenic mice, and FSGS patients. This novel discovery strategy of using the unique properties of urinary exosomes to sample the intracellular and/or exported compartment may allow new insights to be gained about cellular regulatory pathways without an invasive renal biopsy.

RESULTS

Temporal urinary excretion of exosomal ATF3 in animal models of AKI

Induction of AKI in cisplatin and I/R models was confirmed by increases in serum creatinine (SCr; Figure 1) and histological evidence of renal tubule damage (data not shown).[14]

Urinary exosomal ATF3 increased significantly as early as 0–2 h after 35 min of ischemia and reperfusion, then remained elevated in the 2–8 h and the 8–24 h collection periods (Figure 1a). Urinary ATF3 also increased 24 h after cisplatin injection, peaked at day 2 after cisplatin injection, decreased to a very low level at day 3, and reappeared at day 5 (Figure 1b). Urinary ATF3 was detected 2 days before the SCr increase and tubular damage (Figures 1b and 2). The biphasic pattern was confirmed in two independent experiments, although ATF3 was detected weakly in some animals at day 3 after cisplatin injection (data not shown). In contrast, ATF3 was not detected in urine exosomes from either normal or volume-depleted rats (Figure 1c), or puromycin-treated rats, an animal model of FSGS (data not shown).

Renal expression of ATF3 after cisplatin-induced AKI

To determine whether the changes in urinary exosomal ATF3 content reflected histological changes and/or changing content of ATF3 in kidneys, we examined renal ATF3 protein expression by immunohistochemistry. ATF3 was not detected by immunohistochemical examination in normal kidney but was increased in the nuclei of tubule epithelial cells in the outer stripe of the outer medulla from days 1–5 after cisplatin (Figure 2). ATF3 staining increased significantly in normal-appearing proximal tubular cells on day 1 (Figure 2b) and peaked on day 2 when renal function and histology remained normal (Figure 2c). On day 3, when tubular injury became evident, ATF3 was still detected in nuclei of cells attached to the tubule basement membrane, but faint staining could be detected in the cytoplasm as well. (Figure 2d). As cells detached from the basement membrane on day 5 after cisplatin, positive staining was detected in nuclei of detached cells, but there was also staining in cellular debris (Figure 2e). The primary antipeptide antibody was incubated with the immunogenic peptide, to confirm the absence of nonspecific staining (Figure 2f). Urinary ATF3 paralleled the initial rise in renal ATF3 expression, but preceded the increase in SCr and histological changes, by 2 days (Figures 1 and 2).

Urinary exosomal ATF3 in patients with AKI

Spot urine samples were collected from two healthy volunteers and four ICU patients with AKI, including urine collections at two different time points during the course of AKI in two patients (Table 1), and four patients with chronic kidney disease (Supplementary Table 1). We isolated exosomes from these urine samples and examined the abundance of ATF3 normalized by urine creatinine. Urinary

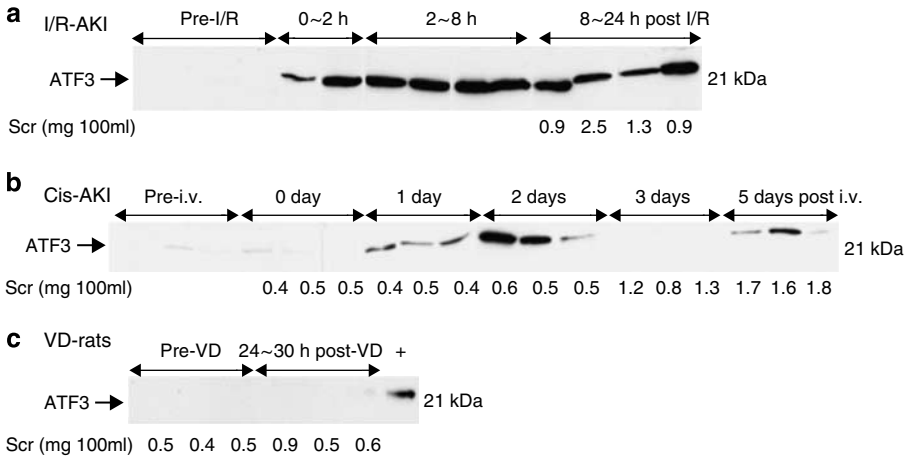

Figure 1 | Time course of excretion of urinary exosomal ATF3 in ischemic and nephrotoxic AKI rat models. Urinary exosomal ATF3 from individual rats analyzed by western blotting. (**a**) I/R-induced AKI rats after 35 min of bilateral ischemia and reperfusion, (**b**) Cisplatin-induced AKI rats, (**c**) volume depleted rats. +, ATF3 positive control.