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Brief communication (Original)

Urinary exosomes from a mouse model of chronic tubulointerstitial kidney disease induced by chronic renal ischemia–reperfusion injury and nephrectomy

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Background: Biomarkers for the early diagnosis of chronic kidney disease (CKD) will lead to its better management. Rodent models will be helpful for biomarker discovery. Urinary exosomes, membrane bound vesicles, may contain interesting biomarkers.

Objective: To produce a mouse model of CKD with sufficient urine volume for exosome studies.

Methods: We characterized a mouse models of CKD induced by 55 min ischemia–reperfusion injury and contralateral nephrectomy at 1 wk after the chronic ischemia–reperfusion injury (Chr IR) in 7 mice and other 7 sham-operated control mice. We collected 24 h urine for urine exosome extraction.

Results: Blood urea nitrogen, serum creatinine, and proteinuria increased as early as 2 wk, and reciprocally lower hematocrit. The renal injury, proteinuria, and anemia persisted 12 wk after nephrectomy as consistent with uremic symptoms. The mouse model of CKD showed renal histopathology of interstitial fibrosis with mild glomerular injury, and failure to grow. At 12 wk, the volume of urine collected in 24 h was sufficient for exosome extraction. Urinary exosomes were demonstrated by western blots showing tumor susceptibility gene 101 protein.

Conclusions: Mice with renal injury induced by Chr IR showed renal injury and proteinuria as early as 2 wk with uremic symptoms and tubulointerstitial fibrosis. Most importantly, the 24 h volume of urine at 12 wk was sufficient for exosome extraction.

Keywords: Chronic ischemic reperfusion injury, chronic kidney disease, mouse model, urinary exosome

Chronic kidney disease (CKD) is an important global health care problem [1, 2]. The dialysis delivery systems for end stage kidney disease are a substantial economic burden, especially in developing countries. CKD has various etiologies, but is manifested histologically as similarly to renal fibrosis, an accumulation of excess extracellular matrix in several parts of the kidney. Renal fibrosis progresses and leads to a deterioration of renal function. Tubulointerstitial (TI) fibrosis, also referred to as interstitial fibrosis with tubular atrophy (IFTA), is an important prognostic factor in several types of CKD and is associated with a decrease in kidney function [3]. Despite the common prevalence of renal TI fibrosis in CKD regardless of etiology, known biomarkers of TI fibrosis are limited. The current well-known biomarker of CKD, albuminuria, is more associated with glomerulosclerosis than TI fibrosis. The exosome, a 35–40 nm plasma membrane enriched vesicle secreted by various cell types, is a source of interesting biomarkers [4]. The urinary exosome markers for early TI fibrosis should be beneficial as sensitive biomarkers to improve CKD prevention programs.

For biomarker discovery, animal models of disease are important. A common rodent model of CKD is 5/6 nephrectomy in rats with ligation of the anterior

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division of the renal artery, and the natural history of this model is well- established [5]. However, the rat model induced by 5/6 nephrectomy has a closer resemblance to focal segmental glomerulosclerosis, may not a good representation of TI fibrosis. Because of the greater availability of genetic manipulation in mice compared with rats, a mouse model of TI fibrosis will be useful. Unfortunately, the availability of rodent models of TI fibrosis described in the literature is very limited. There is a rat model of TI fibrosis induced by ischemia–reperfusion injury model described in the literature [6], but mouse models of TI are more scarce [7, 8]. Interestingly, Basile et al. [6] found that there is a diuresis episode after IR injury in rats. We hypothesized that ischemia–reperfusion injury in mice might also produce a diuresis episode in which urine volume might be sufficient for urinary exosome extraction. We tested our hypothesis by inducing chronic ischemia–reperfusion injury with nephrectomy (Chr IR) in CD-1 mice, a strain more susceptible to fibrosis than others [9], and extracted exosomes from 24 h collections of urine.

Materials and methods

Mice, mice models, and sample collection

Animal care followed the U.S. National Institutes of Health and local Thai criteria for the use and treatment of laboratory animals. We used 6-week-old CD-1 mice purchased from the National Laboratory Animal Center, Nakhon Pathom, Thailand. The animal protocols used were approved by the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. We divided 14 mice into equal-sized sham and Chr IR surgery groups. The baseline blood chemistry tests were conducted 2 weeks before nephrectomy (–2 wk) using blood samples obtained from a tail vein. One week later a Chr IR injury was made as previously described [10], with slight modifications as follows. In brief, the surgery was conducted in 2 stages. In the first stage at 1 week after baseline blood collection (–1 wk), an abdominal incision was made under isoflurane anesthesia and the left renal artery was clamped. The abdominal wall was closed layer-by-layer with nylon suture thread (4-0). The mice were kept in a housing cage for 50 min. Subsequently, the abdominal wall was opened, and the vascular clamp removed (the total time of ischemia was 55 min). Again the abdominal wall was closed layer-by-layer with suture thread (4-0). After 1 week (0 wk), a right nephrectomy was performed under isoflurane anesthesia via an abdominal incision. Blood was collected from tail veins at 2 wk and 6 wk after nephrectomy. A 24 h urine sample was collected using a mouse metabolic cage (Hatteras Instruments, Cary, NC, USA) and preserved a protease inhibitor, then stored at –80°C until use. The urine collection and body weight measurements were done 1 day before blood collection. All mice were humanely killed at 12 wk after the nephrectomy by cardiac puncture under isoflurane anesthesia and the remaining kidney excised and stored in formalin for the renal histopathology. In sham-surgery control mice, the renal arteries were simply identified and the abdominal wall was closed layer-by-layer.

Examination of blood chemistry, proteinuria, and histology

Blood urea nitrogen (BUN) and serum creatinine (Scr) were measured using colorimetric assays (QuantiChrom kits DIUR-500 for urea assay and DICT-500 for creatinine assay; BioAssay Systems, Haywood, CA, USA), 24 h urine protein levels were measured using a Bradford assay (Bio-Rad, Hercules, CA, USA). The 24 h urine protein levels (24 h U protein) were calculated using the following equation, 24 h U protein = urinary protein × 24 h urine volume. Hematocrit (Hct) was measured using a microhematocrit method with a Hitachi 917 automated biochemistry analyzer (Roche Diagnostics, Indianapolis, IN, USA). The single kidneys removed at 12 wk were immediately fixed in 10% neutral buffered formalin solution for paraffin embedding, and then 4 μm thick sections deparaffinized, rehydrated, and stained using a Masson trichrome method to determine the fibrosis. Tubulointerstitial fibrosis was estimated at 200× magnification using the following semiquantitative criteria: 0, area of damage <5%; 1, areas of damage 5%–10%; 2, damage involving 10%–25%; 3, damage involving 25%–50%; 4, >50% of the area being affected [11-15]. The severity of the glomerular injury was determined by the percentage of the injured glomeruli [11-15].

Exosome extraction

Urine collected for 24 h at 12 wk after nephrectomy or sham surgery was centrifuged at 1000 ×*g* for 10 min to remove debris, and stored at –80°C until isolation of urinary exosomes. Urinary exosomes were isolated by differential centrifugation (17,000 ×*g* for 15 min then 200,000 ×*g* for 1 h) as described previously [16, 17]. In short, the exosome-associated

proteins isolated from urine samples were suspended using an isolation solution (10 mM triethanolamine, 250 mM sucrose, pH 7.6). Tamm–Horsfall protein was depleted by incubation with dithiothreitol (200 mg/mL) at 95°C for 2 min then samples stored at –80°C for western blot analysis. Of note, the urine of mice in the sham-surgery control group was pooled because of the inadequate urine volume for the exosome extraction for western blot analysis. By contrast, urine volumes from individual Chr IR-injured mice were sufficient for exosome extraction.

Western blot analysis

Exosome-associated proteins, isolated from individual mouse urine samples, were separated by 1-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes. Then the membranes were probed overnight at 4°C with rabbit polyclonal antibody to tumor susceptibility gene 101 (TSG101) (Abcam, Cambridge, MA, USA). Peroxidase conjugated, affinity-purified donkey anti-rabbit immunoglobulin G (Jackson Immuno Research Laboratories, West Grove, PA, USA) was used as the secondary antibody. The antigen–antibody reactions were visualized using enhanced chemiluminescence (Amersham Biosciences, Little Chalfont, Bucks, UK) and light-sensitive film (BioMax XAR, Kodak, Rochester, NY, USA). The density of western blotting results was determined by ImageJ, version 1.36b (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

All data are expressed as mean ± SD. Differences between groups were analyzed using a *t* test or ANOVA. $P < 0.05$ was accepted as significant.

Results

Uremia and renal fibrosis in the Chr IR-induced mouse model of CKD

We evaluated renal function of the Chr IR-induced model of renal injury using the time course of BUN and Scr (**Figure 1A** and **B**). At baseline, 1 week before IR injury of the left kidney, BUN and Scr were 13 ± 6 and 0.2 ± 0.1 mg/dL, respectively. Then renal function was rapid deteriorated at 2 wk consistent with the renal mass reduction from the two-stage Chr IR surgery. BUN and Scr at 2 wk were 58 ± 22 and 0.6 ± 0.2 mg/dL, respectively. Subsequently, elevated levels of both BUN and Scr persisted, BUN and Scr at 12 wk were 59 ± 14 and 0.6 ± 02 mg/dL, respectively. TI fibrosis in renal histopathology, as evidence of renal injury, was seen as areas of blue connective tissue after Masson trichrome staining (**Figure 2**). Of note, there was a mild TI injury score without different glomerular injury compared with mice in the sham surgery group (**Figure 2**) demonstrating that the Chr IR-induced model of CKD was predominantly a model of TI fibrosis. In addition, we found that the Chr IR-induced model of CKD showed anemia as early as 2 wk with approximately 50% reduction from baseline, Hct reduced from 50 ± 7% to 26 ± 5% (**Figure 1D**). Subsequently, Hct improved, but failed to return to baseline levels; Hct at 12 wk was 38 ± 7%.

Failure to thrive and diuresis in the Chr IR-induced mouse model of CKD

To determine whether there was another presentation of uremia, we evaluated the time course of mouse body weight (**Figure 3A**). Despite ischemia–reperfusion-injury of the left kidney with right nephrectomy, there was no weight reduction in the mice. However, body weight of mice affected by Chr IR did not increase within 4 weeks of the perioperative period from –2 wk to 2 wk; 33 ± 4 g to 33 ± 2 g, respectively. By contrast, the body weight of sham-operated mice increased significantly from 32 ± 2 g to 37 ± 3 g at –2 wk and 2 wk, respectively. After 2 wk, there was weight gain by both Chr IR-injured and sham-operated mice, but the gain differed. Weight gain from 2 wk to 12 wk in mice with Chr IR-injury was approximately 7.8 g (from 33 ± 2 g to 41 ± 5 g; 23% increase) and was significantly less than the gain in the mice in the sham-surgery group, which was approximately 10 g (from 37 ± 3 g to 47 ± 2 g; 27% increase). To explore whether there was sufficient urine volume for exosome extraction using the double centrifugation method, we measured the time course of 24 h urine volume (**Figure 3B**).

Exosome extraction from 24 h urine of mice with Chr IR-injury

At 2 wk and 12 wk after surgery, 24 h urine volume was greater than at baseline and sufficient for exosome extraction. We selected urine volume at 12 wk to extract urine exosomes. Of note, the urine volume at 2 wk postoperatively was also sufficient for exosome extraction, but no fibrosis was seen in renal histopathology at 2 wk (data not shown). We found TSG101, a structural molecule of exosomes, in urine from mice with Chr IR-injury at 12 wk (**Figure 4**).

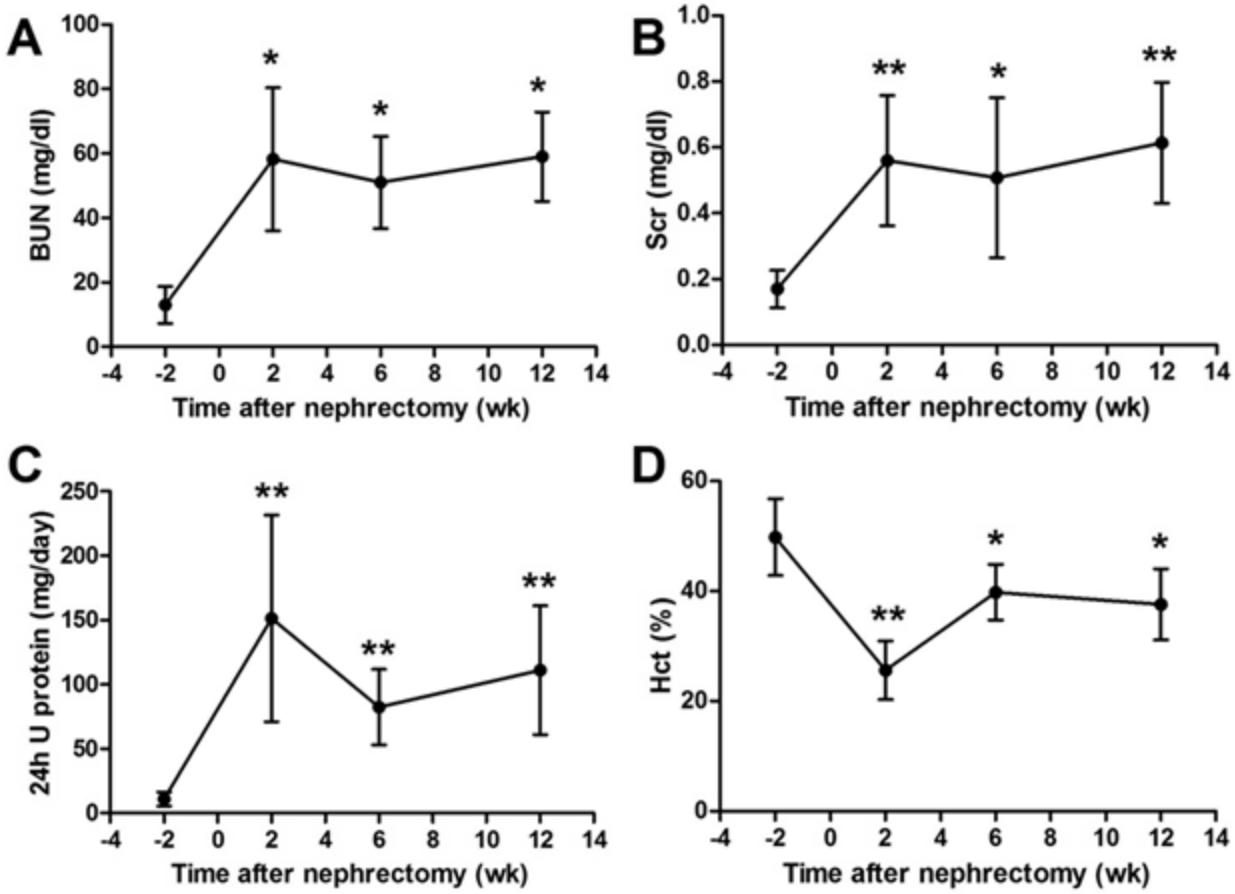

Figure 1. Characteristics of the renal injury caused by the chronic ischemia–reperfusion injury with nephrectomy in the mouse model. Renal injury and anemia as demonstrated by (**A**) blood urea nitrogen (BUN), (**B**) serum creatinine (Scr), (**C**) 24 h urinary protein levels, and (**D**) hematocrit (Hct). (n = 5/time point) **P* < 0.05, ***P* < 0.01 vs. baseline

Discussion

We produced a Chr IR-injury in mice as previously described with a few modifications in the time of ischemia and method of anesthesia [10]. We describe the characteristics of the model of CKD in terms of renal injury with serum markers, urine protein, and renal histology. We determined whether the 24 h urine production at 12 wk after nephrectomy, when renal interstitial fibrosis could be seen in histopathology, was sufficient for urinary exosome extraction using a centrifugation method. We found that Chr IR-injury resulted in persistent renal injury (as measured by BUN, Scr, and anemia), tubular proteinuria, failure to thrive, and renal interstitial fibrosis resembling that in patients with early TI fibrosis injury. Additionally, 24 h urine from mice with Chr IR injury at 12 wk was sufficient to extract exosomes as demonstrated by detection of TSG101 using western blotting.

Uremic symptoms in the Chr IR-induced mouse model of chronic tubulointerstitial fibrosis

The Chr IR-induced model of CKD followed the strategy of the remnant kidney model by reducing kidney function to <50% that of normal function [5]. Among various rodent models of CKD, the remnant kidney models are the most favorable. Other models of CKD, such as podocyte injury models, are generally used for genetic manipulation to examine glomerulopathy [18-20]. Mouse models of TI CKD are limited. We recently developed a Ch IR-induced model of CKD [10]. In the present study, we modified our previous protocol to use a longer time of ischemia to ensure development of renal injury. After Chr IR, mouse renal function rapidly deteriorated as early as 2 wk with high BUN and Scr compared with baseline renal function (**Figure 1A and B**). However, the

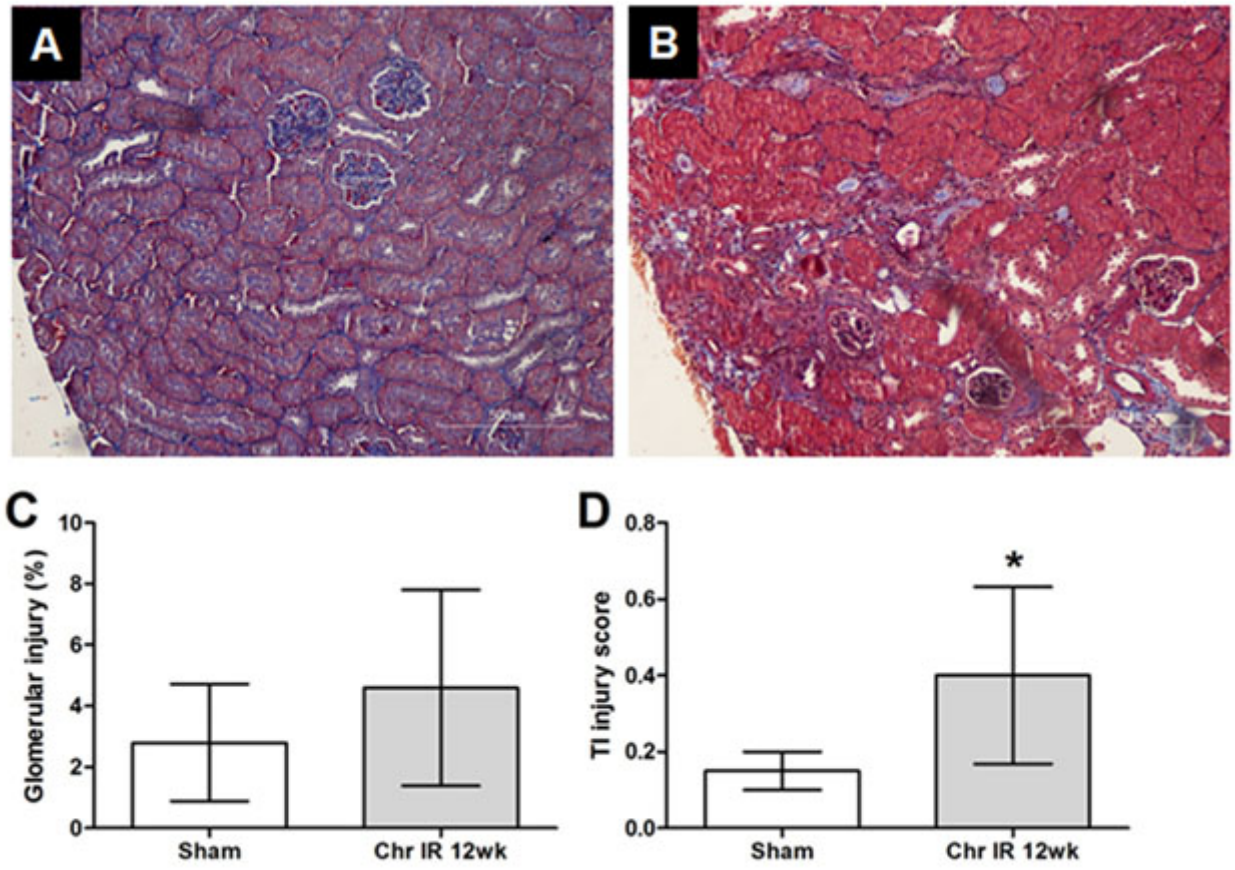

Figure 2. Characteristics of renal histopathology caused by chronic ischemia–reperfusion injury with nephrectomy (Chr IR) in the mouse model of CKD. Representative Masson trichrome staining at an original magnification of 200× of the left kidney at 12 wk in (**A**) sham and (**B**) Chr IR (scale bars 500 μm). Semiquantitative comparison of kidneys from mice in terms of (**C**) glomerular injury and (**D**) tubulointerstitial (TI) injury in mice with sham surgery (n = 3) and Chr IR injury (n = 5) is shown. **P* < 0.05 vs. sham-surgery controls.

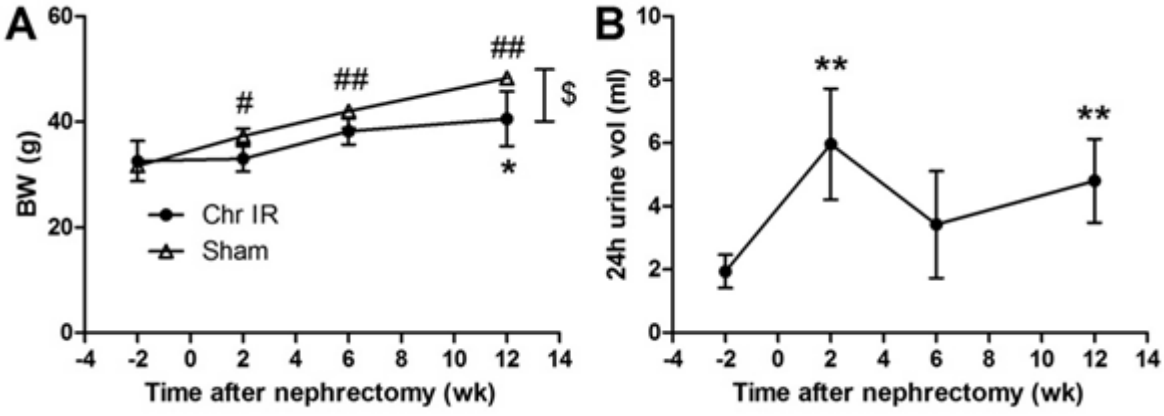

Figure 3. Body weight and 24 h urine volume of mice with chronic ischemia–reperfusion injury with nephrectomy (Chr IR). (**A**) The body weight of sham-operated mice (n = 3/time point) and Chr IR-injured mice (n = 5/time point) is shown. (**B**) The 24 h urine volume is shown (n = 5/time point). **P* < 0.05, ***P* < 0.01 vs. baseline in Chr IR-injured mice, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs baseline in sham-operated mice, $^{\$}P < 0.05$.

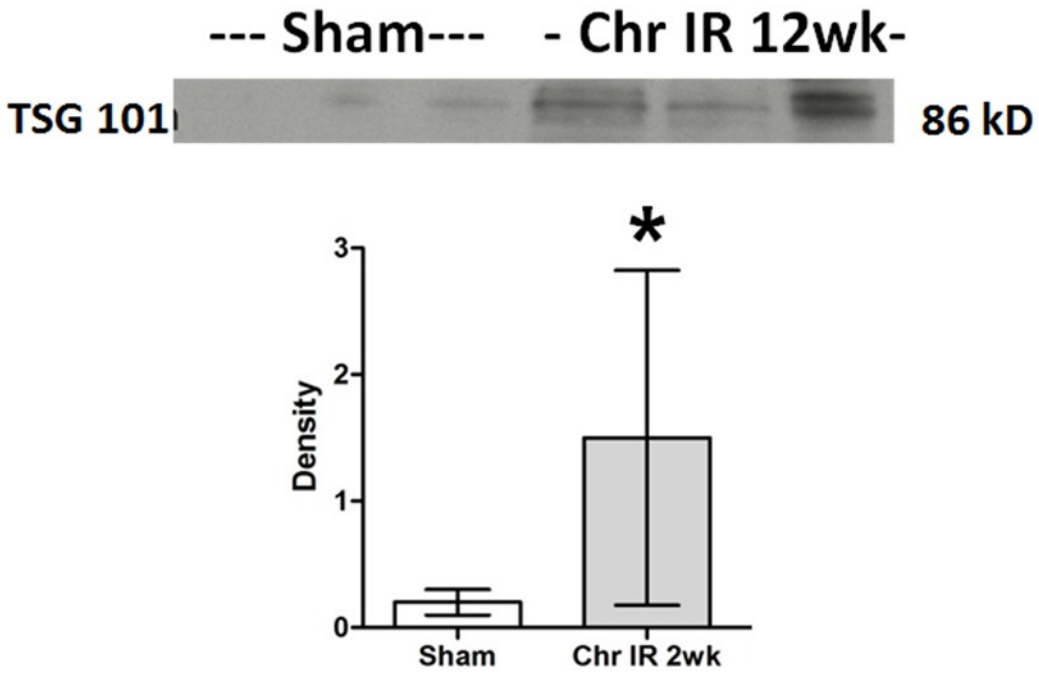

Figure 4. Western blot detection of urinary exosome tumor susceptibility gene 101 (TSG101) in 24 h urine collection from mice with chronic ischemia–reperfusion injury with nephrectomy (Chr IR). Urinary exosome TSG101 from individual mice was analyzed by western blotting (rabbit polyclonal primary antibody; Abcam, Cambridge, MA, USA) with enhanced chemiluminescence detection (Amersham Biosciences) of peroxidase-conjugated secondary antibody using light-sensitive film (Kodak BioMax XAR) followed by densitometry (ImageJ, version 1.36b; National Institutes of Health, Bethesda, MD, USA). *P < 0.001 vs sham-operated controls at 2 wk.

severity of the renal injury was just moderate compared with the 5/6 nephrectomy mouse model of CKD induced by the partial renal excision [13]. However, the renal injury was sufficiently severe to maintain renal injury (**Figure 1A and B**) and showed renal TI fibrosis at 12 wk after surgery (**Figure 2**). This model might therefore be appropriate for early TI fibrosis as seen in patients with early CKD. Moreover, we observed anemia (**Figure 1D**) and failure to thrive (**Figure 3A**) consistent with clinical presentations in patients with CKD. The body weight of mice with sham surgery increased more than it did in mice with Chr IR injury. In parallel, Hct was lowest at 2 wk after nephrectomy, which might the result of a perioperative injury affecting interstitial erythropoietin synthesis (**Figure 3B**). The Hct then improved at 6 wk, but was persistently lower than baseline at 12 wk. Therefore, the Chr IR-induced model of CKD might also be a good model of CKD induced anemia and malnutrition.

There was less glomerular injury, but more prominent TI injury in the Chr IR-induced model (**Figure 2**) and a compatible level of proteinuria. It is interesting that proteinuria was detectable as early as 2 wk after Chr IR. The proteinuria at 2 wk tended to be higher than at 12 wk after nephrectomy, which might be explained by a postischemic effect. The Bradford protein assay detects both low-molecular-weight protein and albumin. Therefore is difficult to determine whether the proteinuria is prominently of tubular or glomerular dysfunction. Further studies of tubular function in this model will be of interest.

Postischemic diuresis and urinary exosome extraction

Urinary exosomes are 35–40 nM membrane bound vesicles with unknown function, excreted by several types of cells along the urinary system [21]. Because the components of the exosome and cell membranes are the same hydrophobic molecules, urinary exosomes are a method of sampling the hydrophobic membrane molecules from hydrophilic urine [22]. Moreover, the exosome membrane protects internal cytosolic molecules. Not surprisingly, urine exosomes are a good source of mRNA, miRNA, and transcriptional factors that usually deteriorate rapidly in extracellular environments [23, 24]. Therefore, urinary exosomes are a new source of biomarkers

[25]. However, the discovery of biomarkers requires appropriate animal models that mimic specific diseases and conditions. The Chr IR-induced mouse model of CKD appears representative of early chronic TI fibrosis. We took advantage of the presentation of diuresis in the Chr IR-induced model to extract exosomes from urine. Mouse urine volume increased approximately 1.5 times from baseline at 12 wk after nephrectomy (**Figure 3B**). The standard double centrifugation method for exome extraction requires at least 4.5–5 mL urine [16, 17]. To detect urinary exosomes, we chose TSG101, a molecule found in the exosome production pathway [21]. With time normalized to 24 h, the level of TSG101 was greater at 12 wk after nephrectomy than it was at baseline (**Figure 4**). However, we could not conclude that there were more exosomes at 12 wk after nephrectomy because there might be more TSG101 in individual exosomes at 12 wk than in urinary exosomes at baseline. Further experiments to determined exosome numbers are needed. However, urinary exosome TSG101 seems to increase with the progression of CKD in the Chr IR-induced model of CKD, and TSG101 itself is a candidate biomarker. Further studies are required to determine whether TSG101 is a biomarker.

Conclusion

We demonstrated that Chr IR injury in a male CD-1 mouse induces a model of TI fibrosis because: (1) there were persistent kidney injury as shown by an increase BUN and Scr, (2) TI fibrosis is seen in renal histopathology, (3) proteinuria is consistent with that in patients with TI injury, and (4) anemia and reduced growth rate resemble uremic symptoms. We found that 24 h urine from mice with the Chr IR-induced injury was sufficient for urinary exosome extraction and contained TSG101. The Chr IR-induced mouse model of CKD is appropriate for the study of several topics such as tubulointerstitial CKD, uremic malnutrition, and CKD induced anemia.

Disclosures

The authors declare no competing interests.

Author contributions

AL, WC, SE designed the study, analyzed, and interpreted the data. AL and WC drafted the manuscript. AL, WC, and SE reviewed the data and critically revised the manuscript. All authors approved the final version of the manuscript.

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