Decreased abundance of urinary exosomal aquaporin-1 in renal ischemia-reperfusion injury

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Departments of 1Veterinary Pharmacology and 4Veterinary Pathology, Faculty of Agriculture, and 3Division of Molecular and Cellular Biology, Department of Anatomy, Faculty of Medicine, University of Miyazaki, Miyazaki; and Departments of 2Internal Medicine, 5Surgery, and 6Urology, Miyazaki Prefectural Miyazaki Hospital, Miyazaki, Japan

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Sonoda H, Yokota-Ikeda N, Oshikawa S, Kanno Y, Yoshinaga K, Uchida K, Ueda Y, Kimiya K, Uezono S, Ueda A, Ito K, Ikeda M. Decreased abundance of urinary exosomal aquaporin-1 in renal ischemia-reperfusion injury. Am J Physiol Renal Physiol 297: F1006–F1016, 2009. First published July 29, 2009; doi:10.1152/ajprenal.00200.2009.—Urinary exosomes, secreted into urine from renal epithelial cells, are known to contain many types of renal functional membrane proteins. Here, we studied whether renal ischemia-reperfusion (I/R) affects urinary exosomal aquaporin-1 (AQP1) excretion in rats subjected to renal I/R and patients who underwent renal transplantation. Immunoblotting studies demonstrated reduction of the urinary exosomal AQP1 level even at 6 h after renal I/R, and the level continued to be low over 96 h after I/R. Renal AQP1 mRNA and protein analyses revealed that the decreased excretion of urinary exosomal AQP1 is associated with renal AQP1 protein retention in the early phase and with a decreased expression level of renal AQP1 in the later phase of renal I/R injury. Decreased abundance of urinary exosomal AQP1 in a recipient patient was also observed at 48 h after renal allograft transplantation. No significant decrease in urinary exosomal AQP1 was observed in rats treated with puromycin, a model of nephropathy, or in patients with proteinuria. Our studies suggest that the renal AQP1 expression level is possibly controlled by its urinary exosomal excretion and indicate that urinary exosomal AQP1 is a novel urinary biomarker for renal I/R injury.

EXOSOMES ARE FORMED IN LATE endocytic compartments, known as multivesicular bodies and secreted from various types of cells such as antigen-presenting cells, dendritic cells, and renal epithelial cells (13, 18, 21). Although many features of exosomes remain unknown, growing evidence suggests that they play an important role in immunoregulation and tumorigenesis. Urinary exosomes are released into urine by fusion of the outer membrane of multivesicular bodies with the apical plasma membrane of renal epithelial cells (13, 18, 21). Using proteomic analysis, Pisitkun and colleagues (8, 19) have examined urinary exosomes from healthy humans and unambiguously identified 1,132 proteins in them. Urinary exosomes include membrane proteins and cytosolic proteins, which have characteristics of all renal tubule epithelial cells, podocytes, and transitional epithelia from the urinary collecting system. Importantly, at least 34 of the 1,132 proteins found in urinary exosomes have been implicated in various kidney diseases such as autosomal dominant polycystic kidney disease type 1 (polycystin-1), autosomal dominant and recessive nephrogenic diabetes (aquaporin-2), antenatal Bartter syndrome type 1 (sodium-potassium-chloride cotransporter-2), and Gitelman’s syndrome (thiazide-sensitive Na-Cl cotransporter). These findings suggest that examination of urinary exosomes could lead to the discovery of new noninvasive site-specific biomarkers for kidney disease.

Acute kidney injury (AKI) is a significant and devastating medical problem worldwide. Mortality and morbidity related to AKI remain high despite significant improvements in supportive care (15, 24). A major reason for this is the lack of an early diagnostic marker for AKI, which means that therapy is often unacceptably delayed (4, 25).

Aquaporin-1 (AQP1), a water channel protein, is abundantly expressed in renal epithelial cells of the proximal tubules and the descending thin limb. A series of experiments by Nielsen and colleagues (7, 14) has revealed that renal ischemia-reperfusion (I/R), an important cause of AKI, decreases renal AQP1 expression. Furthermore, AQP1 has been shown to be secreted into urine as an exosomal protein (22). However, it is still unclear whether renal I/R affects urinary exosomal AQP1 excretion.

We therefore examined urinary exosomal AQP1, renal AQP1 mRNA, and renal AQP1 protein expression in rats that had been subjected to renal I/R. Furthermore, the amount of exosomal AQP1 in urine from an allograft transplant patient was studied. The urinary exosomal AQP1 level was also examined in rats treated with puromycin, a model of nephropathy, and in patients with proteinuria.

MATERIALS AND METHODS

Chemicals and antibodies. Imidazole was from MP Biomedicals (Solon, OH), DTT and platinum Taq DNA polymerase High Fidelity were from Invitrogen Japan (Tokyo, Japan). 3’, 3’-Diaminobenzidine tetrahydrochloride was from Sigma (Tokyo, Japan), and complete protease inhibitor cocktail tablets were from Roche Diagnostics (Tokyo, Japan). Anti-AQP1 or β-actin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). HRP-conjugated anti-rabbit IgG was from Cell Signaling Technology. Other chemicals were from Bio-Rad Laboratories (Hercules, CA) or Wako Pure Chemical Industries (Osaka, Japan).

Animal models. All animal studies were in accordance with the Guide for the Care and Use of Laboratory Animals in the University of Miyazaki and were conducted in compliance with Law Concerning the Protection and Control of Animals (Japanese Law No. 105, October 1, revised on June 22, 2005), Standards Regarding to the Care and Management of Laboratory Animals and Relief of Pain (Notification No. 88 of the Ministry of the Environment, Japan, April 28, 2005).
Male Sprague-Dawley (SD) rats were purchased from Kyudo (Saga, Japan) and beige rats and their genetic control rats, Dark-Agouti rats, were purchased from Japan SLC (Shizuoka, Japan). All animals were kept in metabolic cages and given free access to water during the study period.

In most parts of this study, we employed unilateral renal I/R, because we would avoid the effect of change in urinary volume excretion on the systemic circulation. SD rats (347–504 g) were subjected to unilateral renal I/R or sham operation as described previously (12). For unilateral I/R, the right renal pedicle was clamped using two microvascular clamps (Roboz, Gaithersburg, MD) for 1 h. Control (sham operation) rats were subjected to identical treatment without clamping of the renal pedicles. When the effect of bilateral renal I/R on urinary exosomal AQP1 excretion was examined (see Fig. 2), the bilateral renal pedicles were clamped for 35 min. Blood and kidney samples were collected under ether anesthesia. Plasma creatinine and urea nitrogen concentrations were measured using an autoanalyzer (FUJI DRICHEM 3500i, Fuji Film Medical, Tokyo, Japan).

For creation of a nephrotic syndrome rat model, SD rats (260–275 g) were injected intraperitoneally with puromycin aminonucleoside (PAN; 150 mg/kg) (6). Control rats were injected with saline. At 6 days after injection, blood and urine samples were collected. Total urinary protein concentration was measured using a quick start Bradford protein assay kit (Bio-Rad Laboratories).

Urinary creatinine concentration, mg/ml Sham 0.99

Spot urine samples were collected from a donor patient at 1 day before and at 2 and 6 days after renal living-donor transplantation and also from the recipient patient at 2 days before and 2 and 6 days after. Also, spot urine samples were obtained from patients with proteinuria. The patients were diagnosed as having membranous nephropathy (2.5 g/day for daily urinary protein excretion, 0.6 mg/dl for serum creatinine concentration, 78.2 ml/min for creatinine clearance); minimal change disease (4.2 g/day for daily urinary protein excretion, 1.3 mg/dl for serum creatinine concentration, 42.8 ml/min for creatinine clearance); antineutrophilic cytoplasmic antibody (ANCA)-associated and rapidly progressive glomerulonephritis (1.2 g/day for daily urinary protein excretion, 4.9 mg/dl for serum creatinine concentration, 1.5 ml/min for creatinine clearance); Henoch-Schönlein purpura (4.7 g/day for daily urinary protein excretion, 1.3 mg/dl for serum creatinine concentration, 73.9 ml/min for creatinine clearance); or IgA nephritis (1.0 g/day for daily urinary protein excretion, 0.5 mg/dl for serum creatinine concentration, 108.8 ml/min for creatinine clearance) on a kidney biopsy. We collected all urine samples in collection tubes containing a protease inhibitor mixture (1 mM EDTA, 1 mM p-amidinophenyl methanesulfonyl fluoride hydrochloride, and 10 µg/ml leupeptin).

Table 1. Changes in urine volume, urinary creatinine and electrolyte excretion, urinary osmolality, plasma creatinine, and urea nitrogen concentration after renal ischemia-reperfusion

<table>
<thead>
<tr>
<th>Group</th>
<th>6 h</th>
<th>30 h</th>
<th>96 h</th>
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<tbody>
<tr>
<td>Urine volume, ml/6 h</td>
<td>Sham</td>
<td>6.90±1.16 (n = 28)</td>
<td>5.89±0.71 (n = 18)</td>
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<tr>
<td></td>
<td>I/R</td>
<td>7.29±0.62 (n = 31)</td>
<td>5.62±0.67 (n = 19)</td>
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<td>Urinary creatinine concentration, mg/ml</td>
<td>Sham</td>
<td>0.99±0.10 (n = 28)</td>
<td>0.93±0.19 (n = 18)</td>
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<td></td>
<td>I/R</td>
<td>0.75±0.06 (n = 31)</td>
<td>0.73±0.08 (n = 19)</td>
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<tr>
<td>Urinary creatinine excretion, mg·6 h⁻¹·kg⁻¹</td>
<td>Sham</td>
<td>13.20±1.16 (n = 28)</td>
<td>13.52±3.06 (n = 18)</td>
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<tr>
<td></td>
<td>I/R</td>
<td>12.22±0.84 (n = 31)</td>
<td>11.16±2.50 (n = 19)</td>
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<td>Urinary electrolyte excretion</td>
<td>Sodium, meq/6 h</td>
<td>Sham</td>
<td>0.21±0.06 (n = 8)</td>
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<td></td>
<td>I/R</td>
<td>0.16±0.01 (n = 7)</td>
<td>0.65±0.08 (n = 7)</td>
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<td>Potassium, meq/6 h</td>
<td>Sham</td>
<td>0.82±0.13 (n = 8)</td>
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<td>I/R</td>
<td>0.83±0.05 (n = 7)</td>
<td>0.60±0.10 (n = 6)</td>
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<tr>
<td></td>
<td>Chloride, meq/6 h</td>
<td>Sham</td>
<td>0.41±0.06 (n = 7)</td>
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<td>I/R</td>
<td>0.72±0.03 (n = 7)</td>
<td>0.65±0.03 (n = 7)</td>
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<tr>
<td>Urinary osmolality, mosmol/kgH₂O</td>
<td>Sham</td>
<td>1,287.13±197.1 (n = 8)</td>
<td>881.5±133.31 (n = 6)</td>
</tr>
<tr>
<td></td>
<td>I/R</td>
<td>876.78±116.64 (n = 9)</td>
<td>819.29±109.62 (n = 7)</td>
</tr>
<tr>
<td>Plasma creatinine concentration, mg/dl</td>
<td>Sham</td>
<td>0.35±0.02 (n = 16)</td>
<td>0.31±0.01 (n = 14)</td>
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<tr>
<td></td>
<td>I/R</td>
<td>0.49±0.01* (n = 18)</td>
<td>0.43±0.01* (n = 15)</td>
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<tr>
<td>Plasma urea nitrogen concentration, mg/dL</td>
<td>Sham</td>
<td>14.43±0.99 (n = 16)</td>
<td>14.17±0.73 (n = 14)</td>
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<tr>
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<td>I/R</td>
<td>21.53±1.07* (n = 16)</td>
<td>18.95±0.81* (n = 15)</td>
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</table>

Values are means ± SE. I/R, ischemia-reperfusion; sham, sham operation. *P < 0.01 vs. sham rats.
at 200,000 g for 1 h. The pellet obtained from ultracentrifugation was suspended in an isolation solution, and total protein concentration was determined using a Pierce BCA Protein Assay reagent Kit (Pierce, Rockford, IL) before addition of 4× sample buffer. The mixture of protein sample with 4× sample buffer was incubated at 37°C for 30 min.

**Immunoblot analysis and silver-staining.** Immunoblot analysis was performed as previously reported (11). Antibody-associated protein on the membrane was detected by a Super Signal chemiluminescence detection system (Pierce).

To evaluate total protein level of urinary exosomes, after separation by SDS-PAGE, the gel was stained with a Silver Stain Plus Kit (Bio-Rad Laboratories).

**Immunohistochemistry.** The paraffin-embedded kidney blocks were cut at 2-μm thickness, deparaffinized, and rehydrated. Antibodies were retrieved by incubating the specimen in distilled water at 121°C for 5 min, and the endogenous peroxidase was consumed using a 3% H2O2 solution. After washing, the specimen was incubated with anti-AQP1 antibody diluted to 1:100 for 1 h at 37°C. To visualize the protein-antibody complex, the specimen was reacted with Envision System Labelled Polymer reagent (Dako Japan, Tokyo, Japan) for 1 h at 37°C, followed by treatment with 3, 3'-diaminobenzidine tetrahydrochloride. The specimen was counterstained with hematoxylin.

**RT-PCR analysis.** A total RNA was isolated from each region of kidney using an RNaseasy Protect Minikit (Qiagen) with DNase digestion. Total RNA was transcribed to cDNA by using an iScript cDNA Synthesis kit (Bio-Rad Laboratories), according to the respective manufacturer’s instructions. Rat GAPDH and AQP1 were amplified with the following primers: forward (5'-actcccatcctccaccttt-3') and reverse (5'-taacctttgaggacat-gt-3') for GAPDH and forward (5'-gctcaatctacgga-3') and reverse (5'-ttgtatccacggctgta-3') for AQP1.

**Densitometric analysis and statistical analysis.** The resultant bands of RT-PCR or immunoblotting analysis were scanned and quantified by WinRoof software V5.7 (Mitani, Tokyo, Japan). Quantitative data are represented as means ± SE. Statistical comparisons were accomplished by t-test or Pearson analysis. P values <0.05 were considered statistically significant.

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**Fig. 1.** Urinary exosomal aquaporin-1 (AQP1) abundance after renal ischemia-reperfusion (I/R). **A:** typical immunoblots with anti-AQP1 antibody of urinary exosomal samples obtained at 0–6 h (6 h), 24–30 h (30 h), 90–96 h (96 h), and 474–480 h (480 h) after renal I/R. Two bands can be seen, the top being glycosylated (35–50 kDa) and the bottom nonglycosylated (<30 kDa) AQP1. Each lane was loaded with 100 μg of creatinine. **B** and **C,** summarized quantitative data obtained by immunoblot analyses of urinary exosomal AQP1 at 0–6 (n = 9), 24–30 (n = 7), 90–96 (n = 8), and 474–480 h (n = 4) after renal I/R. Each value after normalization to the urinary creatinine content (B) or time of collection (C) is expressed as % of the mean value of the urinary AQP1 from sham rats at the corresponding time points. Values are means ± SE. *P < 0.05, **P < 0.01 vs. sham rats.

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RESULTS

Changes in urine and plasma data after unilateral renal I/R. Unilateral renal I/R (I/R group) or a sham operation (sham group) was performed in rats, and urine samples were collected at 0–6, 24–30, 90–96, and 474–480 h after the operation. Plasma samples were also collected at 6, 30, and 96 h after renal I/R. As shown in Table 1, plasma creatinine and urea nitrogen concentrations in the I/R group were slightly but significantly increased compared with the sham group. These mild increases in plasma creatinine and urea nitrogen in a unilateral renal I/R model are in good agreement with previous observations (2, 23). In contrast, urinary volume, creatinine, osmolality, and electrolyte excretion in the I/R group did not differ from those in the sham group. These data show that unilateral renal I/R in the present study caused AKI without change in urinary volume and electrolyte excretion, indicating that this model minimally affects systemic circulation.

Urinary exosomal AQP1 excretion after renal I/R. To determine exosomal AQP1 levels in the urine after unilateral renal I/R, exosomes were obtained by differential centrifugation and immunoblot analysis was performed (19, 27). The loading volume for immunoblotting was normalized to urinary creatinine content. As shown in Fig. 1A, immunoblots of urine exosomal samples collected at 0–6 h after sham operation showed two distinct bands migrating below 30 and 35–50 kDa. This corresponds to nonglycosylated and glycosylated AQP1, respectively (17). Exosomal glycosylated AQP1 was significantly reduced even 0–6 h urine after renal I/R operation, compared with the sham group (Fig. 1B). In urine collected at 24–30 and at 90–96 h after unilateral renal I/R, in addition to glycosylated AQP1, nonglycosylated AQP1 abundance was also decreased, and these reductions were recovered to the normal level in urine collected at 474–480 h.

It is known that urinary creatinine excretion is affected by factors such as muscle mass, gender, and age. Therefore, we also evaluated the urinary exosomal AQP1 levels after normalization to the time of collection (6 h). As shown in Fig. 1C, virtually similar results were obtained with normalization to urinary creatinine content and to time of collection.

Fig. 2. Urinary exosomal AQP1 abundance after bilateral renal ischemia-reperfusion. A: typical immunoblots with anti-AQP1 antibody of urinary exosomal samples obtained at 24–34 h (34 h) after bilateral renal I/R. Each lane was loaded with 50 μg of creatinine. B: summarized quantitative data obtained by immunoblot analysis of urinary exosomal AQP1 in A. Each value after normalization to the urinary creatinine content is expressed as % of the mean value of the urinary AQP1 from sham rats. Values are means ± SE. **P < 0.01 vs. sham rats.

Fig. 3. Silver-stained gel for urinary exosomal proteins. Typical examples of silver-staining with urinary exosomal proteins at 0–6 (A), 24–30 (B), and 90–96 h (C) after renal I/R and the corresponding immunoblots with anti-AQP1 antibody are shown. Each lane was loaded with 50 μg of creatinine. The numbers on the right indicate the molecular mass (in kDa).
Under unilateral renal I/R conditions, it is conceivable that urinary excretion is not only related to the injured kidney but also to the contralateral, intact kidney. We therefore investigated exosomal AQP1 levels in urine after bilateral renal I/R. At 34 h after bilateral renal I/R operation, plasma creatinine (6.6 ± 0.2 mg/dl for I/R, n = 3; 0.28 ± 0.03 mg/dl for sham, n = 4) and urea nitrogen (178.7 ± 6.5 mg/dl for I/R, n = 3; 14.3 ± 2.3 mg/dl for sham, n = 4) concentrations were significantly increased (P < 0.001) compared with those in sham-operated rats. All the rats subjected to bilateral renal I/R operation did not produce urine until 24 h after the insult, and only three of four rats produced urine until 34 h after the procedure. Thereafter, three rats died at 52, 54, and 90 h after the insult. The urinary volume collected during 6 h (24–30 h after the operation) was too low to allow us to obtain urinary exosomes. Therefore, we evaluated urinary exosomal AQP1 excretion in urine collected at 24–34 h after the insult. Figure 2, A and B, show representative immunoblots of urine exosomes collected at 24–34 h after sham or bilateral renal I/R operation and the summarized data, respectively. Urinary exosomal AQP1 excretion was dramatically reduced after bilateral renal I/R, and this reduction was more pronounced compared with that in urinary exosomes collected at 24–30 h after unilateral renal I/R (Fig. 1), suggesting that the decrease in urinary exosomal AQP1 after unilateral renal I/R is strongly related to the injured kidney.

Urinary exosomal proteins after unilateral renal I/R. We examined whether a decrease in urinary exosomal AQP1 after renal I/R was caused by a nonspecific reduction of total exosomal protein excretion. Silver-staining was carried out with the urinary exosome samples used in Fig. 1. As shown in Fig. 3, no decrease in total urinary exosomal protein excretion was observed. On the other hand, the corresponding immunoblots with anti-AQP1 antibody showed the significant decrease in urinary exosomal AQP1 abundance. We also measured total urinary protein excretions of 0- to 6- and 24- to 30-h samples using the Bradford method. Total urinary protein excretion of urine collected at 0–6 h in the I/R group was significantly higher than that in the sham group (I/R group; 15.3 ± 2.1 mg/6 h, sham group; 3.6 ± 0.3 mg/6 h, n = 3). In 24–30-h samples, total protein excretion did not differ between the two groups (I/R group; 2.4 ± 0.7 mg/6 h, sham group; 2.0 ± 0.2 mg/6 h, n = 3). These data indicate that the reduction of exosomal AQP1 excretion after renal I/R was not due to a nonspecific reduction of total exosomal protein excretion.

Levels of AQP1 protein expression in rat kidneys after unilateral I/R. Next, we examined AQP1 protein expression levels in the cortex, outer medulla, and inner medulla of the kidney by immunoblotting. As shown Fig. 4, compared with that seen in the sham group, levels of glycosylated AQP1 protein expression in all regions at 6 h after renal I/R tended to be increased and the differences did not achieve significance.

At 30 h after renal I/R, glycosylated and nonglycosylated AQP1 in the cortex and nonglycosylated AQP1 abundance in the outer medulla were significantly increased. In contrast, glycosylated AQP1 protein expression was decreased in the contralateral kidney.
inner medulla at 30 h after the insult. At 96 h after renal I/R, AQP1 protein expression levels in the cortex and the outer medulla were lower than those in the sham group. In the contralateral kidney (intact kidney), AQP1 protein expression levels at 30 h after the operation were not significantly altered in any kidney regions except for glycosylated AQP1 in the inner medulla compared with the sham group (Fig. 3).

Next, we evaluated the relationship between urinary exosomal AQP1 and renal AQP1 abundance in each region. As shown in Fig. 5, there was a significant negative correlation between urinary exosomal AQP1 excretion (24- to 30-h urine) and renal AQP1 abundance (30 h after renal I/R) in the cortex and outer medulla. On the other hand, at 96 h after renal I/R a significant positive correlation between urinary exosomal AQP1 excretion (90- to 96-h urine) and renal AQP1 abundance (96 h after renal I/R) in the cortex was obtained, and the correlations in the outer and inner medulla tended to be positive. Similar correlations were observed when we analyzed separately glycosylated and nonglycosylated AQP1.

Kidney immunohistochemistry. Figures 6 and 7 show the results of immunohistochemistry for AQP1 in kidneys with and without unilateral I/R. In the kidney of the sham group at 6 h after the operation, AQP1 was abundantly expressed in the brush border and weakly expressed in the basolateral membrane of the proximal tubular cells. The apical membrane of descending thin limb cells was also stained with anti-AQP1 antibody. Similar staining patterns were observed in kidneys at 30 and 96 h after the operation in the sham group (data not shown). In kidneys at 6 and 30 h after I/R, AQP1 expression was increased in the proximal tubules and descending thin limb cells. AQP1 was abundant in the brush border and the cytoplasm of these epithelial cells. In contrast, at 96 h after I/R, AQP1 expression was markedly decreased in these segments and all three regions showed an increase in the number of cells, and regeneration characterized by large nuclei. In the inner medulla, AQP1 expression level was somewhat increased at 6 h after reperfusion, but were decreased at 30 and 96 h after I/R compared with the sham group. These immunohistochemistry data supported the above-mentioned immunoblotting data.

Renal AQP1 mRNA expression after unilateral I/R. To determine renal AQP1 mRNA levels, semiquantitative RT-PCR was performed. Each region of kidney (cortex, outer medulla, and inner medulla) was prepared at 6, 30, and 96 h after I/R. As shown in Fig. 8, renal AQP1 mRNA expression in all regions at most time points examined was significantly reduced by renal I/R.

AQP1 levels in urine from rats with Chediack-Higashi syndrome. It is considered that exosomes are produced via a pathway of endocytosis and lysosomal trafficking (13, 18, 21). To examine whether disorder of lysosomal trafficking affects the secretion of exosomal AQP1 into urine, we studied urinary exosomal AQP1 excretion in rats with Chediack-Higashi syndrome (beige rats), which are known to have impaired lysosomal trafficking (16). Urinary exosomal AQP1 excretion in beige rats did not differ from that in their genetic control rats (data not shown), indicating that this type of disorder of lysosomal secretion was not related to the regulation of urinary exosomal AQP1 excretion.

Urinary exosomal AQP1 level in allograft transplant patients. Renal I/R injury is inevitable in renal transplantation. We had an opportunity to examine human urinary exosomal
Fig. 6. Immunohistochemistry in kidney at low magnification. Representative stainings in 3 regions of the kidney (cortex, outer medulla, and inner medulla) at 6 h after sham operation and 6, 30, and 96 h after unilateral renal I/R are shown. Brown staining indicates the presence of AQP1. No brown staining was observed if the primary antibody was omitted (control staining; data not shown). *, Glomerulus. Positive regions in the glomerulus are erythrocytes. Bars = 100 μm.
AQP1 after renal transplantation. Spot urine samples were collected from both donor and recipient patients in a case of renal transplantation (nonidentical twins, 38 yr old, ABO-compatible, cold ischemic time for 84 min in the operation). Urinary exosomes were isolated from those urine samples, and the abundance of AQP1 and fetuin-A, which had been identified as a predictive exosomal biomarker for AKI (26), was studied by immunoblot analysis with normalization by urine creatinine content. As shown in Fig. 9, urinary exosomal AQP1 markedly decreased in the recipient patient at 2 and 6 days after renal transplantation even though the plasma creatinine concentration was dramatically decreased following the operation. Interestingly, urinary exosomal AQP1 was not detected in the recipient patient at 2 days before renal transplantation, suggesting that end-stage kidneys no longer produce exosomal AQP1 in urine. Urinary exosomal fetuin-A increased in the recipient patient at 2 days after renal transplantation. The increased level of exosomal fetuin-A was also observed in the donor patient at 2 days after nephrectomy.

The urinary exosomal AQP1 in five patients with proteinuria was also examined. Compared with control samples made from three normal male volunteers (aged 21–45), a >50% decrease in urinary exosomal AQP1 was not observed in all patients (330% for a patient with membranous nephropathy, 280% for a patient with minimal change disease, 93% for a patient with ANCA-associated and rapidly progressive glomerulonephritis, 600% for a patient with Henoch-Schönlein purpura, 94% for a patient with IgA nephritis).

We also examined urinary exosomal AQP1 excretion in a rodent model of nephrotic syndrome (6). Rats were injected intraperitoneally with puromycin aminonucleoside (150 mg/kg), whereas control rats were given saline. Six days after the treatment, the total urinary protein excretion in rats injected with PAN was >10 times higher than that in control rats. Plasma creatinine in PAN-treated rats was not different from that in control rats (PAN-treated rats 0.33 ± 0.03 mg/dl; control 0.3 ± 0.0 mg/dl, n = 3). Urinary exosomal AQP1 excretion did not differ between the two groups (data not shown).

DISCUSSION

The major findings of this study were as follows. 1) Urinary exosomal AQP1 decreased at 6 h after unilateral renal I/R and remained lowered up to 96 h after the insult; this was not accompanied by a dramatic increase in plasma creatinine. 2) The reduction of urinary exosomal AQP1 was independent of the total urinary exosomal protein level. 3) The decreased exosomal AQP1 level was not associated with the expression level of renal AQP1 protein up to 30 h after renal I/R. 4) The decreased urinary exosomal AQP1 was also observed in an allograft renal transplant recipient. 5) No decrease in urinary
Exosomal AQP1 excretion was observed in a model of nephropathy and in patients with proteinuria. These findings indicate that renal I/R specifically reduces urinary exosomal AQP1 excretion, leading to a detection of I/R injury. The mechanism underlying the reduction of urinary exosomal AQP1 is not thought to be simply dependent on renal AQP1 expression level.

Our data showed that AQP1 mRNA in all regions of the kidney up to 30 h after renal I/R was reduced, suggesting that AQP1 protein synthesis was suppressed in response to renal I/R. However, immunoblotting and immunohistochemistry data showed that AQP1 protein expression in most regions of the kidney at the corresponding time points was not decreased. On the other hand, urinary exosomal AQP1 excretion was decreased and there was a significant negative correlation between urinary exosomal AQP1 excretion and renal AQP1 abundance in the cortex and outer medulla (Fig. 4A), where these regions are known to be rich in AQP1 expression (17). These observations suggest that renal AQP1 retention occurs through inhibition of urinary exosomal AQP1 excretion as an early event after renal I/R. Recently, Verkman and colleagues (9, 20) have observed that migration ability of AQP1-deficient proximal tubular cells is reduced and AQP1 null mice have more severe tubular injury after renal I/R than kidneys of wild-type mice. Based on the present study and the reported evidence, it is conceivable that AQP1 plays an important role in cell migration and renal AQP1 retention by inhibition of urinary exosomal AQP1 excretion may be an active process to heal wounds. Furthermore, exosome release by reticulocytes has been reported to clear unwanted proteins and to allow net loss of the cell surface membrane, thus contributing to red blood cell differentiation (21). Urinary exosomes excretion may play an important role in regulating renal epithelial protein content.

In contrast to early events, at 96 h after renal I/R expression of both AQP1 mRNA and AQP1 protein was decreased, along with a decrease in urinary exosomal AQP1 excretion. These data indicate that the decreased excretion of urinary exosomal AQP1 is associated with renal AQP1 protein expression in the later phase of renal I/R injury. At this time point, histological analyses clearly showed an increase in cell number and appearance of cells with large nuclei. These histological features correspond to regeneration in these segments. Therefore, AQP1 appears not to be required for cell proliferation and regeneration after completion of cell migration. We note that the cell proliferation rate is similar in wild-type and AQP1-deficient cells (9).

Previous studies have demonstrated the changes in excretion of urinary exosomal Na+/H+ exchanger isoform 3 (NHE3) (5) and fetuin-A (26) after renal I/R. NHE3, a sodium transporter protein in the proximal tubule, was present in urine within 24 h after renal allograft transplantation, and it returned to an undetectable level at 48 h after transplantation, indicating that it is a temporal marker of renal I/R injury. Using a proteomic analysis technique, Zhou and colleagues (26) have clarified that urinary exosomal fetuin-A is increased after renal I/R. In the present study, we observed that urinary exosomal fetuin-A level was increased in both recipient and donor patients. This indicates that urinary exosomal fetuin-A level may be changed in response to change in renal hemodynamics (e.g., due to nephrectomy). Therefore, although urinary exosomal NHE3 and fetuin-A

Fig. 8. AQP1 mRNA expression in kidney. Representative pictures of RT-PCR and quantified data for the cortex (A), outer medulla (B), and inner medulla (C) are shown. Each value is expressed as % of the mean value for sham rats. Values are means ± SE (n = 4). *P < 0.05, **P < 0.01 vs. sham rats.
are sensitive markers for detecting renal I/R injury, NHE3 may be silent in the later phase of renal I/R injury and fetuin-A may not be specific to renal I/R. In contrast, urinary exosomal AQP1 excretion decreased to a trough at 6–96 h after renal I/R in rats, showing a strong correlation with both renal AQP1 retention in the early phase and decreased renal AQP1 expression in the later phase after I/R. Furthermore, a dramatic decrease in exosomal AQP1 was observed in a recipient patient compared with those in a donor patient. These suggest that urinary exosomal AQP1 excretion might be a better indicator for early to late detection of renal cellular states after renal I/R.

Beige rats are a model for Chediak-Higashi syndrome, which is characterized by a defect in the ability of cells to secrete lysosomes in specialized cells such as cytotoxic T cells and platelets (1, 10). The protein bearing the mutation responsible is lysosomal trafficking regulator (LYST) protein (1, 10). Although the exact role of LYST protein in the biology of lysosomes is still unknown, the enlargement of lysosomes and the defect in its secretion upon stimulation in LYST- and LYST homolog-deficient cells, suggest that LYST protein is involved in determining the size of lysosomes and regulating their movement within cells (3). In the present study, we compared urinary exosomal AQP1 excretion in beige rats with that in their genetic control counterparts. Both types of rats excreted AQP1 via urinary exosomes to a similar degree. This suggests that LYST protein is not involved in the secretion of exosomal AQP1 into urine and that the importance of LYST protein for lysosomal trafficking is limited to certain cell types.

In conclusion, we have demonstrated that urinary exosomal AQP1 may allow early to late detection of renal cellular states after renal I/R, such as I/R-induced cell injury and the subsequent regeneration, and may be used to predict posttransplant AKI (delayed graft failure) caused by I/R. Additional studies will be necessary to better evaluate urinary exosomal AQP1 in a large number of patients.

REFERENCES


