Netrin-1 and kidney injury. II. Netrin-1 is an early biomarker of acute kidney injury

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Reeves WB, Kwon O, Ramesh G. Netrin-1 and kidney injury. II. Netrin-1 is an early biomarker of acute kidney injury. Am J Physiol Renal Physiol 294: F731–F738, 2008. First published January 30, 2008; doi:10.1152/ajprenal.00507.2007.—Acute kidney injury is an important complication in hospitalized patients often diagnosed late and associated with high mortality and morbidity. Although biomarkers for nephrotoxicity are available, they often lack sensitivity and specificity for detecting tubular injury. Netrin-1 is a laminin-like molecule highly expressed in many organs including kidney. To determine the value of netrin-1 as a biomarker of renal injury, we analyzed its urinary excretion following ischemia-reperfusion-, cisplatin-, folic acid-, and endotoxin-induced renal injury in mice. Urinary netrin-1 levels increased markedly within 3 h of ischemia-reperfusion (40 ± 14-fold, P < 0.01 vs. baseline), reached a peak level at 6 h, and decreased thereafter, returning to near baseline by 72 h. Serum creatinine significantly increased only after 24 h of reperfusion. Similarly, in cisplatin-, folic acid-, and lipopolysaccharide-treated mice, urine netrin-1 excretion increased as early as 1 h and reached a peak level at 6 h after injection. However, serum creatinine was raised significantly after 6, 24, and 72 h after folic acid, lipopolysaccharide, and cisplatin administration, respectively. NGAL excretion in folic acid- and lipopolysaccharide-treated mice urinary samples could only be detected by 24 h after drug administration. Furthermore, urinary netrin-1 excretion increased dramatically in 13 acute renal failure patients, whereas none was detected in 6 healthy volunteer urine samples. Immunohistochemical localization showed that netrin-1 is highly expressed in tubular epithelial cells in transplanted human kidney. We conclude that urinary netrin-1 is a promising early biomarker of renal injury.

The purpose of the present study was to determine whether changes in the urinary excretion of netrin-1 can be used as an early biomarker of AKI. The netrins were discovered about a decade ago as neuronal guidance cues (20). Netrins are laminin-like molecules with a distinctive domain organization belonging to laminin-related family of axon-guidance molecules (1). Recent studies indicate various other roles of netrins beyond axonal guidance including development of mammary gland, lung, pancreas, and blood vessels; inhibition of leukocyte migration during sepsis; mitogenesis and chemoattraction of endothelial cells (9, 23). Although the kidney has one of the highest levels of netrin expression (9), the role of netrins in kidney injury is unknown. In the companion paper (22a), we showed that netrin-1 is highly induced in tubular epithelial cells as early as 3 h after ischemia-reperfusion and reaches a peak level at 24 h. To determine whether tubular induction is accompanied by an increase in urinary excretion, we measured netrin-1 levels in urine by Western blot analysis. We examined several different models of AKI to determine the general utility of urinary netrin-1 in detecting a broad range of kidney injury. Here, we report that netrin-1 appears in urine as early as 1 h after different renal insults. In addition, analysis of urine samples from humans with acute renal failure also showed increased netrin-1 levels in all forms of AKI.

MATERIALS AND METHODS

Urine Collection From Animals

Renal ischemia-reperfusion. C57BL/6J mice (8–9 wk of age, Jackson Laboratory) were anesthetized with pentobarbital sodium (50 mg/kg body wt ip) and were placed on a heating pad to maintain temperature at 37°C. Both renal pedicles were identified through dorsal incisions and clamped for 26 min. Reperfusion was confirmed visually upon release of the clamps. As a control, sham-operated animals were subjected to the same surgical procedure except the renal pedicles were not clamped. Surgical wounds were closed and mice were given 1 ml of warm saline (ip) and kept in a warm incubator until they regained consciousness. Urine was collected by bladder massage and stored at −80°C until the assays were done.

Cisplatin administration. Experiments were performed using 10- to 12-wk-old male C57BL/6 mice weighing ∼25–30 g. Cisplatin (MP biomedical, LLC) was dissolved in saline at a concentration of 1 mg/ml. Mice were given a single intraperitoneal injection of cisplatin (20 mg/kg body wt). Urine was collected before injection and 1, 3, 6, 24, 48, and 72 h after cisplatin administration.

Lipopolysaccharide administration. A group of mice (n = 6–8) received intraperitoneal injections of 5 mg/kg lipopolysaccharide (LPS; from Escherichia coli 0111:B4, Sigma). Urine was collected before and 1, 3, 6, 24, 48, and 72 after LPS administration.

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**Folic acid administration.** Folic acid was dissolved in sterile 0.3 mM NaHCO₃ buffer at a concentration of 10 mg/ml. Mice were given a single intraperitoneal injection of folic acid (250 mg/kg body wt). Urine was collected at various times after folic acid administration.

**Renal function.** Renal function was assessed by measurements of blood urea nitrogen (BUN; VITROS DT60II Chemistry slides, Ortho-clinical Diagnostics) and serum creatinine (cat. no. DZ072B, Diazyme Labs).

**Collection of Human Urine**

Human subjects were recruited when a nephrology consult was requested for rising serum creatinine and/or decreased urine output. AKI was defined by a sudden rise of serum creatinine concentration to more than 2 mg/dl in patients with normal baseline renal function or a sudden rise of serum creatinine concentration by 50% or more in patients with previous mild-to-moderate chronic kidney disease (CKD; serum creatinine <3 mg/dl). Urine samples were collected on the day of enrollment.

Control samples were collected from six healthy volunteers who have normal renal function. The collection of urine samples was approved by the Institutional Review Board of the Penn State College of Medicine. Protocol number 2007-003, entitled “Netrin-1 in ischemic reperfusion injury of kidney,” was approved by the IACUC on April 6, 2007.

**Analysis of netrin-1 and NGAL by Western blot analysis.** A volume of urine containing 4 µg of creatinine for each mouse sample and 10 µg creatinine for each human sample was subjected to Western blot analysis of netrin-1. Four micrograms of creatinine equivalent urine sample were loaded onto 4–12% polyacrylamide gels, separated, and then transferred onto a PVDF membrane. The membrane was probed with rabbit anti-mouse netrin-1 antibody (Calbiochem, cat. no. PC344). Proteins were detected using enhanced chemiluminescence detection reagents (Amersham Pharmacia Biotechnology). To determine the NGAL level in mouse urine, the same blot was stripped and reprobed with a rabbit anti-mouse NGAL antibody (Santa Cruz Biotechnology). For detection of human netrin-1, blots were probed with a mouse monoclonal anti-netrin-1 antibody (Novus Biologicals, cat. no. NB600-1344). Some samples were loaded with less than 10 µg equivalent creatinine due to dilute urine and the limited well volume of the gel. However, all signals were normalized after densitometry scanning of the blots.

**Immunohistochemistry**

Immunohistochemical localization of netrin-1 was performed as described before (9) with modification. Briefly, kidney was fixed in paraformaldehyde/lysine/periodate (PLP) fixative for human kidney tissue or in 4% paraformaldehyde for mouse tissue overnight. The fixed tissues were transferred to 30% sucrose, placed in a cryomold, and frozen. Six-micrometer-thick sections were placed on glass slides. Sections were washed with PBS, permeabilized with 0.2% Triton X-100 in PBS, and then washed again and blocked with 10% goat serum containing 1% BSA. The sections were incubated with the primary chicken anti-netrin-1 polyclonal antibody (Neuromics, cat. no. CH23002) at a 1:100 dilution for 18 h. Primary antibodies were detected using a secondary antibody conjugated with Cy5 (Abcam). Slides were mounted in aqueous mounting medium (Santa Cruz Biotechnology) and viewed using Leica confocal microscope.

**Statistical Methods**

All assays were performed in duplicate. The data are reported as means ± SE. Statistical significance was assessed by an unpaired,

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**Fig. 1.** Immunohistochemical localization of netrin-1 in kidney after ischemia-reperfusion (IR) and sham operation in C57BL/6J mice. Paraformaldehyde-fixed frozen tissue sections were stained with chicken anti-netrin-1 antibody (red stain) as described in MATERIALS AND METHODS. Slides were mounted with aqueous mounting medium containing DAPI (blue stain). A: no staining was seen in primary antibody negative control. B: in sham-operated kidney, staining was mostly seen in interstitium with little tubular staining. C and D: intense tubular staining was seen at 24 h after IR. D: higher magnification.
two-tailed Student’s t-test for single comparison or ANOVA for multiple comparisons.

RESULTS

Expression and Excretion of Netrin-1 After Ischemia-Reperfusion Injury of Kidney

The findings reported in the companion paper (22a) and Fig. 1 of this report demonstrated the marked upregulation of netrin-1 expression in renal epithelial cells after ischemia-reperfusion injury. We, therefore, sought to determine whether ischemia-reperfusion resulted in the appearance of netrin-1 in the urine. As shown in Fig. 2, netrin-1 excretion in the urine gradually increased to reach a peak level by 6 h [47-fold over baseline (0 h)] and then gradually decreased but was still detectable even 72 h after reperfusion (Fig. 2). In contrast, the serum creatinine levels were significantly elevated only after 6 h and peaked at a later time, 24 h after reperfusion (Fig. 2).

Urinary Netrin-1 is Detected Early in Cisplatin-Induced AKI

In the cisplatin model, the serum creatinine concentration began to increase between 24 and 48 h after cisplatin injection. Severe renal dysfunction was present after 72 h (Fig. 3). In contrast, urinary netrin-1 was increased 10-fold by 3 h and 30-fold by 6 h after cisplatin injection (Fig. 3). This is the first report that any molecule appears in urine at such an early time point after cisplatin administration.

Netrin-1 Appears in Urine Early After Folic Acid Administration

To test whether netrin-1 excretion in urine is detected in other forms of renal injury, we evaluated the high-dose folic acid nephrotoxicity model (Fig. 4). As noted in a previous study (2), high-dose folic acid administration (250 mg/kg body wt) resulted in severe renal failure. Netrin-1 protein excretion in the urine was detected as early as 3 h after injection, before...
the serum creatinine was significantly changed. Elevated urine netrin-1 levels persisted 48 h after folic acid administration (Fig. 4). This is the earliest reported urinary biomarker of folic acid nephrotoxicity.

**LPS Induces Massive Increase in Netrin-1 Excretion in Urine**

Administration of 5 mg/kg body wt LPS induced severe renal dysfunction by 24–48 h after injection (Fig. 5). By 72 h, most animals had recovered and serum creatinine returned to baseline. In contrast to serum creatinine, netrin-1 levels in urine were increased as early as 1 h after injection and reached a peak level (60-fold over baseline) at 6 h before gradually decreasing to baseline by 72 h (Fig. 5).

**NGAL Excretion Increased in Urine After Folic Acid and LPS Administration**

NGAL was shown to be an early marker of AKI (13, 14, 18). However, NGAL excretion in urine after folic acid and endotoxin treatment has not been reported. Unlike in the cisplatin and ischemia-reperfusion injury models, where NGAL could be detected in the urine as early as 3 h, in response to folic acid (Fig. 6A) and LPS (Fig. 6B), NGAL excretion significantly increased at 24 h. By 72 h after LPS administration, the levels were reduced significantly. In contrast, serum creatinine raised significantly by 6 h after folic acid and 24 h after LPS administration. Compared with NGAL, netrin-1 appeared in the urine earlier, suggesting that netrin-1 might be a better marker for folic acid- and endotoxin-mediated injury.

**Netrin-1 is Highly Excreted in Urine of Patients with Acute Renal Failure**

Given the complexity of the pathophysiology of AKI, diagnosing kidney dysfunction early is very challenging. The most commonly used marker, serum creatinine, often detects renal injury late, perhaps after a therapeutic window has been missed. Therefore, a sensitive early marker, which is increased...
early enough to allow intervention and may also be used to monitor recovery, would be of great value. Since netrin-1 showed these two characteristics in the animal model, we examined whether netrin-1 may be a useful biomarker for the diagnosis of renal injury in humans. Urine was collected from 16 individuals with established acute renal failure of various etiologies (Table 1) and 6 healthy volunteers. As shown in Fig. 6, netrin-1 was undetectable in the urine of normal volunteers. However, netrin-1 excretion increased over 1,000-fold in 7 of the AKI patients and over 50-fold in another 3 patients. One patient showed a 20-fold increase. Four patients showed lower, but still detectable, levels compared with healthy controls (Fig. 7, A and C). Only one renal failure patient had undetectable urinary netrin-1.

Netrin-1 is Highly Expressed in Tubular Epithelial Cells of Transplanted Human Kidney

Immunohistochemical localization of netrin-1 in human kidney sections showed that normal kidney expressed little or no netrin-1 in tubular epithelial cells but that netrin-1 was present in the peritubular matrix. However, in the posttransplant kidney collected 30 min after reperfusion, netrin-1 was highly expressed in tubular epithelial cells (Fig. 8).

DISCUSSION

The present study describes for the first time urinary netrin-1 levels in a collection of mouse models of AKI and a group of patients with acute renal failure. We found that netrin-1 is highly induced in renal tubules and associated with an increase in urinary netrin-1 levels. Netrin-1 is normally present in peritubular capillaries (22a). However, netrin-1 was highly induced in postschismic kidney tubular epithelial cells. To explore the generality of this induction, we investigated netrin-1 protein excretion in three distinct models of nephrotoxicant-induced renal injury: cisplatin, folic acid, and LPS. Despite the difference in mechanism, timing, and progression of tubular injury among these models, netrin-1 excretion was increased in all four models and preceded, in some cases by over 24 h, any increases in either BUN or creatinine.

The signal for the rapid increase in netrin-1 expression and excretion is not clear. Since netrin-1 mRNA expression was decreased rather than increased after reperfusion, netrin-1 production may be regulated at the level of translation. Urinary levels decreased at later time points except in the folic acid model where sustained levels of netrin-1 were present. The reason for this difference in the folic acid model is not clear. It is possible that it may be related to the mechanism of tubular injury as injection of high dose of folic acid causes an intense proliferative response in the absence of significant necrosis (3). Overexpression of netrin-1 in the mouse intestine has been shown to cause spontaneous formation of hyperplastic and neoplastic lesions. Moreover, in the adenomatous polyposis coli mutant background associated with adenoma formation, enforced expression of netrin-1 engenders aggressive adenocarcinomatous malignancies suggesting that netrin-1 can promote intestinal tumor development, probably by regulating cell survival (11). Also, addition of netrin-1 to mouse proximal tubular epithelial cells (TKPTS) increased the proliferation rate of these cells (unpublished data, G. Ramesh), suggesting that netrin-1 may facilitate tubular epithelial cell proliferation and regeneration in response to injury.

Several other proteins have been shown to have increased expression and increased excretion in the urine in response to tubular injury. Some of these have been validated in human samples (5, 17, 24). Urinary netrin-1 showed a similar spectrum compared with other previously reported early urinary biomarkers such as NGAL, IL-18, KIM-1, NAG, and Fetuin-A. However, unlike KIM-1 and IL-18, netrin-1 has a very large dynamic range, similar to NGAL. However, urinary netrin-1 increased at an earlier time point than NGAL in two of the murine models studied here. Compared with Fetuin-A, levels of netrin-1 increased at an earlier time in cisplatin nephrotoxicity. Urinary levels of netrin-1 drop to near normal once renal function recovers, suggesting that netrin-1 may serve as both an early injury marker and a prognostic marker of renal recovery.

We also found that the urinary level of netrin-1 was elevated in patients with AKI compared with faint or no excretion of netrin-1 in normal healthy volunteers. We saw a 50-kDa major

Table 1. ARF data

<table>
<thead>
<tr>
<th>Patients</th>
<th>Age/Gender</th>
<th>Potential Causes of ARF</th>
<th>Serum Creatinine at Time of Urine Sample Collection</th>
<th>Urine Output, ml/day</th>
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<tr>
<td>1</td>
<td>81/F</td>
<td>Postschismic ARF, CHF</td>
<td>2.1</td>
<td>380</td>
</tr>
<tr>
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<td>50/M</td>
<td>Postschismic ARF</td>
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<tr>
<td>3</td>
<td>83/F</td>
<td>Prerenal azotemia</td>
<td>4.9</td>
<td>30</td>
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<tr>
<td>4</td>
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<td>Sepsis, hyotension, prerenal</td>
<td>2.7</td>
<td>439</td>
</tr>
<tr>
<td>5</td>
<td>74/M</td>
<td>Sepsis, multiple myeloma</td>
<td>7.7</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>65/M</td>
<td>Pneumonia, CHF, hypotension</td>
<td>5.7</td>
<td>70</td>
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<tr>
<td>7</td>
<td>30/M</td>
<td>Fulminant hepatitis, polysubstance abuse</td>
<td>8.7</td>
<td>304</td>
</tr>
<tr>
<td>8</td>
<td>61/M</td>
<td>Hepatic failure, sepsis</td>
<td>4.8</td>
<td>305</td>
</tr>
<tr>
<td>9</td>
<td>58/M</td>
<td>HTN, CAD, OSA, OA</td>
<td>5.5</td>
<td>2,870</td>
</tr>
<tr>
<td>10</td>
<td>70/M</td>
<td>IV radiocontrast</td>
<td>3.3</td>
<td>6,400</td>
</tr>
<tr>
<td>11</td>
<td>40/F</td>
<td>S/p cholecystectomy</td>
<td>2.4</td>
<td>2,000</td>
</tr>
<tr>
<td>12</td>
<td>68/M</td>
<td>CAD, DM, leg cellulitis, sepsis</td>
<td>4.3</td>
<td>3,525</td>
</tr>
<tr>
<td>13</td>
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<td>Cardiogenic shock, IABP, hypotension</td>
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<td>5,765</td>
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<tr>
<td>14</td>
<td>41/M</td>
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<td>7,560</td>
</tr>
<tr>
<td>15</td>
<td>70/M</td>
<td>Hypotension, cardiac arrest</td>
<td>6.4</td>
<td>4,400</td>
</tr>
<tr>
<td>16</td>
<td>44/M</td>
<td>Aortic dissection, repair; cardiac arrest</td>
<td>6.1</td>
<td>3,695</td>
</tr>
</tbody>
</table>

CHF, congestive heart failure; CAD, coronary artery disease; DM, diabetes mellitus; IABP, intra-aortic balloon pump.
band on Western blot, as in mouse urine, and in addition, some samples showed bands at \( \sim 75 \) and \( \sim 25 \) kDa. These two bands were not detected in mouse urine samples or in most human urine samples. Based on the amino acid sequence and Western blot analysis of rat embryonic spinal cord, it was determined that the molecular weight of netrin-1 is \( \sim 75 \) kDa (10). Therefore, it is possible that the 75-kDa band may be the full-length protein and the 50- and 25-kDa bands are proteolytic fragments. However, this possibility cannot be confirmed at this point. Consistent with the excretion of netrin-1 in urine, the posttransplant kidney showed an increased expression of netrin-1 in tubular epithelial cells. However, the significance of this increase in terms of kidney function is not clear.

Early diagnosis of many forms of AKI may be a key for the prevention of morbidity and mortality associated with acute renal failure. Recent studies have found that urinary NGAL increased 2 h after cardiac surgery and can accurately predict AKI in children (12). However, it was not useful in predicting AKI in adult ICU patients with multifactorial AKI (12). It has been reported that urinary IL-18 increased at 4–6 h after cardiac surgery (19) and also increased 24 h before rise in serum creatine in ICU patients with AKI. Both urinary NGAL and IL-18 could also partially predict the outcome of AKI (16). Urinary sodium/hydrogen exchanger isoform 3 isolated by ultracentrifugation could distinguish acute tubule necrosis from other forms of AKI (4). 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 netrin-1 might be useful in human AKI. However, additional studies will be needed to

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**Fig. 7.** Netrin-1 in urine from normal healthy volunteers and patients with acute renal failure (ARF). Urine and blood samples were collected on the day of the ARF diagnosis. Urine netrin-1 (A) and serum creatinine (B) were quantitated as described in MATERIALS AND METHODS. C: Western blot analysis of netrin-1 in human urine. ND, not determined.
determine whether urinary netrin-1 can predict, diagnose, or gauge the severity or prognosis in high-risk patients and with AKI of various etiology.

GRANTS

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