Urine NGAL and IL-18 are Predictive Biomarkers for Delayed Graft Function Following Kidney Transplantation

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Introduction

Acute renal failure (ARF) represents a common and potentially devastating problem in clinical medicine, with a persistently high mortality and morbidity (1–3). Renal ischemia-reperfusion injury is the leading cause of ARF in the native as well as the transplanted kidney. Although advances in basic research have identified successful interventions in animal models, the lack of early markers for human ARF has precluded initiation of therapy in a timely manner (1–4). In current clinical practice, ARF is typically diagnosed by measuring serum creatinine. Unfortunately, creatinine is an unreliable indicator during acute kidney injury, and does not reflect the degree of damage until a steady state has been reached, which may require several days (5).

Acute kidney injury due to ischemia-reperfusion occurs to some extent almost invariably in deceased donor renal allografts, and even in some live donor transplants, often resulting in varying degrees of early renal dysfunction (6). ARF due to delayed graft function (DGF) complicates 4–10% of live donor and 5–50% of deceased donor kidney transplants (7–9). In addition to the well-known complications of ARF and dialysis, DGF predisposes the graft to both acute and chronic rejection (10–12), is an independent risk factor for suboptimal graft function at one year post transplantation, and increases the risk of chronic allograft nephropathy and graft loss (13–20). A variety of clinical algorithms have been proposed for prediction of DGF based on preoperative risk factors (21,22), but no objective and validated tools are currently available for the early diagnosis of lesser degrees of acute kidney injury following kidney transplantation. Several clinical definitions of DGF that employ urine output, creatinine reduction ratios, or dialysis requirement have been reported in the literature (7–22). However, these clinical variables typically identify DGF only several days after kidney transplantation. Consequently, early therapeutic interventions that have ameliorated DGF in animal models have been ineffective in human studies (6), at least in part due to the paucity of early biomarkers for kidney injury.

We recently utilized a genome-wide interrogation strategy to identify kidney genes that are induced very early after ischemia in animal models, whose protein products might serve as novel biomarkers for the initiation phase of ARF. We identified neutrophil gelatinase-associated lipocalin
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NGAL (as one of the most dramatically up-regulated genes in the kidney after ischemia (23–25). NGAL protein was also markedly induced in kidney tubule cells early after ischemia in mouse models (24). Importantly, NGAL protein was easily detected in the urine very early after ischemic injury in animals, and was found to be a highly predictive biomarker of acute kidney injury in patients undergoing cardiac surgery (26). We therefore tested the hypothesis that urine NGAL represents a novel early biomarker of renal injury in another representative human population, namely patients undergoing kidney transplantation. Since our previous studies have shown that urine interleukin-18 (IL-18) levels are also increased in patients with established DGF (27), a secondary objective of this study was to compare the utility of NGAL and IL-18 measurements for the prediction of DGF.

Materials and Methods

Study design

This investigation was approved by the Institutional Review Boards of participating centers. Written informed consent was obtained from each patient or legal guardian before enrollment. Consecutive patients undergoing living related or deceased donor kidney transplantation at our centers were prospectively enrolled. The immunosuppressive regimen was similar in all patients, consisting of tacrolimus with prednisone and mycophenolate mofetil. Spot urine samples were collected within the first 24 hours (day 0) following transplantation. In the majority of patients, the urine sample was obtained soon after admission to the hospital unit, prior to administration of tacrolimus, and no patient received more than a single dose of tacrolimus prior to urine collection. Urine samples were centrifuged at 2000 g for 5 min, and the supernatants stored in aliquots at −80°C. Serum creatinine was measured at baseline, just before the kidney transplantation, and routinely monitored at least daily in the postoperative period. The primary outcome variable was the development of DGF, defined as the need for dialysis within the first week after transplantation. The decision to initiate dialysis originated from the primary transplant nephrologists and transplant surgeons, without any involvement from the study investigators. Other variables collected included age, gender, race, original kidney disease, cold ischemic time, urine output, serum creatinine, and urine creatinine.

ELISA for NGAL quantitation

The urine NGAL ELISA was performed as previously described (26). Briefly, microtiter plates pre-coated with a mouse monoclonal antibody raised against human NGAL (HYB211–05, AntibodyShop, Gentofte, Denmark) were blocked with buffer containing 1% BSA, coated with 100 μL of samples (urine or serum) or standards (NGAL concentrations ranging from 1–1000 ng/mL), and incubated with a biotinylated monoclonal antibody against human NGAL (HYB211–01B, AntibodyShop) followed by avidin-conjugated HRP (Dako, Carpinteria, CA, USA). TMB substrate (BD Biosciences, San Jose, CA, USA) was added for color development, which was read after 30 min at 450 nm with a microplate reader (Benchmark Plus, BioRad, Hercules, CA, USA). All measurements were made in triplicate. Pre-coated plates can be refrigerated and used for several days, and the entire ELISA procedure is typically completed in 4 h. The inter- and intra-assay coefficient variations were 5–10% for batched samples analyzed on the same day. The laboratory investigators were blinded to the sample sources and clinical outcomes until the end of the study. Urine creatinine was measured using a quantitative colorimetric assay kit (Sigma, St. Louis, MO, USA), and urine NGAL was expressed in ng/mg creatinine to standardize for changes in urine concentration.

ELISA for IL-18 quantitation

The urine IL-18 ELISA was performed as previously described (27), using a human IL-18 ELISA kit (Medical and Biological Laboratories, Nagoya, Japan) that specifically detects the mature form of IL-18. All measurements were made in a blinded fashion. The inter- and intra-assay coefficient variations were 5–10%, corresponding to that reported by the kit manufacturer. The results were expressed in ng/mg creatinine to standardize for changes in urine concentration.

Results

Patient characteristics

A total of 53 patients were included in the study, whose demographic characteristics, diagnoses and outcome variables are shown in Tables 1–3. Urine samples obtained within the first 24 hours after transplantation were available from 23 patients with living related donor (LRD, Table 1), 20 subjects with deceased donor and prompt graft function (CAD, Table 2) and 10 individuals with deceased donor and delayed graft function (CAD DGF, Table 3). There were no differences between these three groups in gender, race, original kidney disease or serum creatinine on day 0. The LRD group tended to be slightly younger in age, but the ages of the CAD group with prompt graft function and DGF were similar. In patients with DGF, peak postoperative serum creatinine requiring dialysis typically occurred 2–4 days after transplant.
Urine NGAL and IL-18 measurements—ELISA

Urine NGAL levels were consistently low in healthy volunteers (0.2 ± 0.05 ng/mg creatinine, n = 10) and in hospitalized control subjects with normal kidney function (0.16 ± 0.05 ng/mg creatinine, n = 71). Median urinary NGAL values were significantly different in the three groups on day 0 (p < 0.0001, Figure 1). Similar results were noted for urinary IL-18 values, as previously published (27). Both urine
Table 3: Clinical characteristics of patients with deceased donor (CAD) kidney transplantation and delayed graft function (DGF)

<table>
<thead>
<tr>
<th>Pt ID</th>
<th>Orig dis</th>
<th>Donor</th>
<th>Age</th>
<th>Sex</th>
<th>Race</th>
<th>Function</th>
<th>S creat day 0</th>
<th>S creat day 1</th>
<th>S creat day 2</th>
<th>S creat day 3</th>
<th>S creat day 4</th>
<th>NGAL max</th>
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<td>FSGS</td>
<td>CAD</td>
<td>15</td>
<td>F</td>
<td>AA</td>
<td>DGF</td>
<td>7.2</td>
<td>8.5</td>
<td>12.4</td>
<td>10</td>
<td>8.5</td>
<td>1900</td>
</tr>
<tr>
<td>10754</td>
<td>GN</td>
<td>CAD</td>
<td>11</td>
<td>F</td>
<td>Hispanic</td>
<td>DGF</td>
<td>8.5</td>
<td>8.6</td>
<td>10</td>
<td>10.2</td>
<td>9.2</td>
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<td>Reflux</td>
<td>CAD</td>
<td>12</td>
<td>M</td>
<td>AA</td>
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<td>9.2</td>
<td>12.8</td>
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<td>14.2</td>
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<td>CAD</td>
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<td>M</td>
<td>Hispanic</td>
<td>DGF</td>
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<td>12.5</td>
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<td>13.5</td>
<td>13</td>
<td>5850</td>
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<td>F</td>
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<td>9</td>
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<td>DGF</td>
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<td>8</td>
<td>5.8</td>
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<td>3562</td>
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<td>AA</td>
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<tr>
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<td>Caucasian</td>
<td>DGF</td>
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<td>3.9</td>
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<td>2.6</td>
<td>1.8</td>
<td>17.6</td>
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Pt ID = patient identification; Orig dis = original disease; FSGS = focal segmental glomerulosclerosis; GN = glomerulonephritis; DM = diabetes mellitus; HTN = hypertension; ADPKD = autosomal dominant polycystic kidney disease; AA = African-American.

NGAL and IL-18 values on day 0 were highest in patients with deceased donor kidney transplants who subsequently developed DGF (Table 4).

NGAL and IL-18 for prediction of acute renal injury

Table 4 demonstrates baseline and clinical parameters in patients with deceased kidney transplantation, with either DGF or prompt graft function. Age, gender, race, cold ischemia time and urine output (< 1 L/day) were not significantly different in the two groups. Only 30% (six patients) displayed a fractional decrease in serum creatinine of > 30% in the group with prompt graft function. Median urinary NGAL and IL-18 values were significantly different in the two groups.

A univariate analysis of our data revealed that the following outcomes were not predictive of DGF: age, gender, race and serum creatinine on day 0. By multivariate logistic
Table 5: Multivariate analysis for predicting DGF after deceased donor transplantation

<table>
<thead>
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<th>Adjusted OR</th>
<th>Confidence interval</th>
<th>p-value</th>
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<tr>
<td>Fractional s creat decrease &lt;30%</td>
<td>1.3</td>
<td>0.06–27</td>
<td>0.8</td>
</tr>
<tr>
<td>Urine output &lt;1 L</td>
<td>11.7</td>
<td>0.1–913</td>
<td>0.2</td>
</tr>
<tr>
<td>Cold ischemia time (h)</td>
<td>0.9</td>
<td>0.6–1.1</td>
<td>0.4</td>
</tr>
<tr>
<td>Urine NGAL, 100 ng/mg</td>
<td>1.2</td>
<td>1.0–1.5</td>
<td>0.01</td>
</tr>
</tbody>
</table>

regression analysis, urine NGAL on day 0 predicted DGF in the early posttransplant period, after adjusting for effects of fractional decrease in serum creatinine, urine output and cold ischemic time (p = 0.01). Every 100 ng/mg increase in urine NGAL was associated with an increased odds of DGF by 20%, after adjusting for other variables (Table 5).

Since dialysis requirement represents a delayed definition of DGF, we also studied the trends in serum creatinine during the first three postoperative days. Lower urine NGAL values on day 0 predicted a steeper postoperative decline in serum creatinine on a multivariate generalized linear model with repeated measures testing (p < 0.01), after adjusting for age, gender, urine output and cold ischemia time. A similar finding was noted for urine IL-18, as previously reported (27). An ROC curve was constructed to determine the discriminatory power of urine NGAL measurements on day 0 for the early prediction of DGF. The area under the curve was 0.9 (CI 0.71–1.0) at day 0 post transplant and the performance of NGAL with respect to derived sensitivities, specificities and predictive values at different cut-off levels are shown in Figure 2. A cut-off of 1000 ng/mg creatinine yielded the optimal sensitivity and specificity at day 0 post transplant. For urine IL-18, the area under the curve for predicting DGF was also 0.9 (CI 0.81–0.98). The sample size was insufficient to conduct a multivariate analysis of a combination of the two biomarkers.

Discussion

Our major findings are that urinary NGAL and IL-18 measured on Day 0 of deceased donor kidney transplantation predict DGF earlier than other clinical definitions that are currently in use. There has been an active quest for urinary biomarkers that predict DGF. We have previously demonstrated that urinary levels of IL-18 increase within 24 h in patients with deceased donor transplants who subsequently developed DGF (27). The present study confirms these previous observations in a larger cohort of patients. Similarly, elevated urinary IL-6, IL-8 and actin levels have been demonstrated on day 0 in nine recipients of a deceased donor renal allograft who displayed sustained acute renal failure (defined as a creatinine clearance of less than 25 mL/min on postoperative day 7 (29). The present study adds urinary NGAL to the short list of promising biomarkers for allograft dysfunction that can be noninvasively assayed for during the early posttransplant period.

The potential clinical utility of our findings lies in the fact that several modalities of therapy have succeeded in animal models of ischemia-reperfusion injury, but have generally been ineffective in human studies of delayed graft function (6). This may be, at least in part, due to the lack of reliable methodologies to distinguish between initial degrees of kidney injury. It is likely that the availability of early
predictive biomarkers such as urine NGAL will allow for a more accurate stratification of acute kidney injury and for the rational selection of patients who might benefit most from a variety of interventions. Based on this report, it is envisioned that urinary NGAL and IL-18 determination may be relevant to patients receiving deceased donor transplants. The commercial availability of anti-NGAL antibodies (26), and the high likelihood of transplant centers being able to perform timely urine ELISA testing within 4 h of sample acquisition as reported herein, also render the clinical use of urinary NGAL determination as a viable predictive tool. Indeed, an entire NGAL ELISA kit with pre-coated plates and a 4-h protocol for the determination of urine NGAL is now commercially available (www.AntibodyShop.com). As detailed in the ‘Methods’ section, this also holds true for urine IL-18 measurements.

The molecular mechanisms underlying the induction of urinary NGAL in CAD transplant recipients who develop DGF remain unknown, but recent studies have provided clues toward understanding the role of NGAL in the kidney. In animal models of ischemic kidney injury, NGAL protein expression was detected predominantly in PCNA-positive proximal tubule epithelial cells that were undergoing proliferation and regeneration, suggesting a role in the repair process (24,25). In addition, recent studies have identified NGAL as an iron transporting protein, and indicate that exogenously administered NGAL ameliorates renal injury in animal models of ischemia reperfusion by tilting the balance of tubule cell fate toward proliferation and survival (30). Thus, NGAL has rapidly emerged from the initial discovery phase to potentially occupying center stage in the acute kidney injury arena, not only as a novel early biomarker but also as an innovative therapy.

There are important limitations to this study. First, it represents a relatively small number of patients. It is acknowledged that our results, although of clear clinical and statistical significance, will need to be validated in a larger population. Second, the possible confounding effects of pharmacologic therapy (such as calcineurin inhibitors) on NGAL excretion are unknown. Third, the potential value of serum NGAL was not examined in this study. Fourth, it is likely that not any one biomarker but a collection of strategically selected proteins may provide the sensitivity and specificity required for the prediction of DGF. The present study identifies urinary NGAL and IL-18 as prime candidates for inclusion in such a panel.

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