Chitinase-Like Protein Brp-39/YKL-40 Modulates the Renal Response to Ischemic Injury and Predicts Delayed Allograft Function

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ABSTRACT
Kidney hypoperfusion during episodes of systemic hypotension or after surgical procurement for transplantation can lead to tubular cell death via necrosis and apoptosis, which trigger a series of responses that promote repair. The factors that contribute to the repair phase after kidney injury are not well understood. Using a urine proteomic screen in mice, we identified the macrophage-secreted chitinase-like protein Brp-39, the murine protein product of the chitinase 3-like 1 gene, as a critical component of this reparative response that serves to limit tubular cell apoptotic death via activation of Akt, improving animal survival after kidney ischemia/reperfusion. Examination of graded times of renal ischemia revealed a direct correlation between the degree of kidney injury and both Chi3l1/Brp-39 expression in the kidney and its levels in the urine. In samples collected from patients undergoing deceased-donor kidney transplantation, we found higher levels of the orthologous human protein, YKL-40, in urine and blood from allografts subjected to sufficient peri-transplant ischemia to cause delayed graft function than from allografts with slow or immediate graft function. Urinary levels of YKL-40 obtained within hours of transplant predicted the need for subsequent dialysis in these patients. In summary, these data suggest that Brp-39/YKL-40 is a sensor of the degree of injury, a critical mediator of the reparative response, and a possible biomarker to identify patients at greatest risk of sustained renal failure after transplantation.


Renal ischemia, occurring in clinical settings such as sepsis, cardiopulmonary bypass or kidney transplantation, can lead to AKI that in turn triggers complex pathophysiologic responses associated with increased morbidity, mortality, and hospital length of stay.1–4 Unfortunately, we have yet to establish successful therapeutic interventions that either limit initial kidney injury or accelerate the subsequent repair, with current treatment focused on mechanical replacement of kidney filtration function in anticipation of successful regeneration of the damaged nephrons via endogenous repair mechanisms. For this reason, morbidity and mortality rates in patients who develop AKI have remained stable or improved only slightly over the past several decades, whereas the incidence of AKI has steadily increased.5,6 Whereas most studies of AKI have focused on the initiation of injury and its associated risk-factors

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and outcomes, there are fewer studies that address the biology and clinical patterns of recovery. To identify factors that might promote the repair phase after kidney injury, we performed a proteomic analysis of mouse urine after ischemia/reperfusion (I/R) injury. Of the factors identified that were most highly upregulated in the urine during the time of kidney repair, several were fragments of the chitinase-like family of secreted proteins (CLPs).

CLPs are evolutionarily conserved 18 glycosyl hydrolase proteins that bind but do not cleave chitin. The best studied CLP is chitinase 3-like 1 (Chi3l1) that encodes the human protein YKL-40 and the mouse ortholog Brp-39. Recent studies from our laboratory and others have demonstrated that secreted YKL-40/Brp-39 are important regulators of innate and adaptive immunity, tissue injury, apoptosis, TGF-β1 elaboration, and parenchymal scarring. YKL-40 can be produced by a variety of cells including neutrophils, monocytes, macrophages, chondrocytes, synovial cells, smooth muscle cells, and endothelial and tumor cells and is readily detected in the blood of normal individuals. Elevated circulating levels of YKL-40 have been observed in patients with asthma, metastatic breast cancer, cardiovascular disease, type 2 diabetes, and hepatic fibrosis. In many of these disorders, YKL-40 correlates with disease activity and its expression is believed to reflect distinct pathways in disease pathogenesis. However, to date, the expression and biology of Brp-39/YKL-40 in the kidney, its roles in the pathogenesis of acute or other forms of kidney injury, and its utility as a biomarker for renal diseases have not been investigated.

In this study, we demonstrate that Brp-39/YKL-40 serves a biologically relevant role in the ischémically injured kidney. Chi3l1 mRNA is upregulated in kidney macrophages in an injury-dependent fashion after renal I/R in the mouse, with increased Brp-39 protein levels in the urine. Similarly, these findings were translated in humans, in which we demonstrated that patients who have delayed graft function after kidney and Brp-39 protein in the urine, whereas 35 minutes of I/R, due to AKI rather than operative complications. Reduction of the warm ischemia time to 25 minutes resulted in improved survival, although Brp-39−/− mice continued to demonstrate increased mortality compared with controls (Figure 2A). Sham operation in Brp-39−/− mice did not result in any mortality, suggesting that the mice were dying due to AKI rather than operative complications. Reduction of the warm ischemia time to 25 minutes resulted in improved survival, although Brp-39−/− mice continued to demonstrate increased mortality compared with controls (Supplemental Figure 1). Serum analysis of mice subjected to 25 minutes of unilateral I/R with contralateral nephrectomy revealed that creatinine and BUN values peaked on day 1 in WT mice followed by improvement by day 3. In contrast, Brp-39−/− mice exhibited a progressive rise in BUN and creatinine on days 3–7 after injury and returning to baseline by day 10 when repair is essentially complete (Figure 1B). The time course of Brp-39 expression is nearly identical to that of macrophage infiltration in the ischemically injured kidney. FACS sorting of kidney cells isolated on day 3 after I/R or sham operation (control) revealed that CD45 negative cells (comprising the endogenous renal tubular, endothelial, and fibroblast cell populations), T cells (CD45+CD3ε+), and PMNs expressed very low levels of Chi3l1 mRNA at baseline with a modest increase after I/R, whereas intrarenal macrophages (CD45+F4/80+CD11clow) exhibited marked upregulation of the Chi3l1 message after injury (Figure 1C). The level of Chi3l1/Brp-39 expression was found to correlate with the severity of ischemic injury. Fifteen minutes of warm ischemia, which causes minimal tubular injury and little loss of GFR, resulted in a modest upregulation of Chi3l1 mRNA in the kidney and Brp-39 protein in the urine, whereas 35 minutes of I/R, which leads to severe tubular necrosis and significant mortality, induced a more substantial increase in both kidney mRNA expression and urinary protein levels (Figure 1, D and E).

**RESULTS**

**Brp-39 Is Induced after Ischemic Renal Injury**

Mice were subjected to bilateral renal I/R and urine was collected at 1 and 3 days after injury and compared with urine from sham-operated mice. Differential two-dimensional gel electrophoresis (DIGE) revealed 11 peptides upregulated >2 times on day 3 after injury (at the time of peak tubule cell reparative proliferation) compared with day 1 (time of peak injury) or sham. Of those identified by mass spectrometry, three were fragments of chitinase 3-like proteins, suggesting that CLPs are upregulated in response to AKI. Western blot analysis with α-Brp-39 confirmed high expression of this CLP in mouse urine by day 3 after I/R compared with baseline (Figure 1A). Quantitative RT-PCR of mRNA from mouse kidney outer medulla revealed nearly undetectable levels of Chi3l1 at baseline, with I/R inducing a >10-fold increase in gene expression peaking on days 3–7 after injury and returning to baseline by day 10 when repair is essentially complete (Figure 1B).

**Brp-39/Chi3L1 Is Required for Normal Renal Responses to I/R Injury In Vivo**

To determine the functional role of Brp-39 expression in AKI, wild-type (WT) and Brp-39−/− mice were subjected to 30 minutes of unilateral warm ischemia and simultaneous contralateral nephrectomy. Mice lacking Brp-39 exhibit a markedly increased mortality between 1 and 3 days after AKI compared with WT mice subjected to the same ischemia time (Figure 2A). Sham operation in Brp-39−/− mice did not result in any mortality, suggesting that the mice were dying due to AKI rather than operative complications. Reduction of the warm ischemia time to 25 minutes resulted in improved survival, although Brp-39−/− mice continued to demonstrate increased mortality compared with controls (Supplemental Figure 1). Serum analysis of mice subjected to 25 minutes of unilateral I/R with contralateral nephrectomy revealed that creatinine and BUN values peaked on day 1 in WT mice followed by improvement by day 3. In contrast, Brp-39−/− mice exhibited a progressive rise in BUN and creatinine on day 3 (Figure 2, B and C). Consistent with a failure in the normal onset of renal repair mechanisms between days 1 and 3, Brp-39−/− mice exhibited more tubular cell loss and cast formation on day 3 after injury than on day 1, with a
worse tissue injury score (Figure 2, D and E). Analysis of macrophages isolated from these kidneys revealed no statistical difference in total macrophage numbers or the proportion of Ly6C+ proinflammatory (M1) macrophages, but did demonstrate a greater percentage of MR+ reparative (M2) phenotypes on day 1 after injury compared with WT mice (Table 1).

Brp-39/Chi3L1 Activates Tubular Epithelial Cell Akt and Reduces Apoptosis

In light of the progressively worsening BUN values and tubular injury score seen in Brp-39-/-- mice after I/R, we hypothesized that secreted Brp-39 might normally function to suppress postinjury tubular cell apoptosis and/or activate subsequent regenerative responses in the ischemically injured kidney. Consistent with this, terminal deoxynucleotidyl transferase-mediated digoxigenin-deoxyuridine nick-end labeling (TUNEL) staining performed on day 3 after I/R revealed that Brp-39-/-- mice have significantly higher rates of tubular cell apoptosis in the outer medulla compared with WT animals (Figure 3, A and B), with a coincident reduction in reparative tubular cell proliferation (Figure 3, C and D).

In immune cells, Brp-39 has been shown to have an antiapoptotic effect via activation of intracellular signaling pathways downstream of the PI 3-kinase and mitogen-activated protein kinase (MAPK). To determine if Brp-39 can activate these anti-apoptotic pathways in renal epithelial cells, cultured mouse proximal tubular (MPT) cells were stimulated with recombinant Brp-39 followed by immunoblotting for the phosphorylated (activated) forms of Akt and Erk1/2. Akt was strongly activated at 60 and 120 minutes after Brp-39 addition, whereas Erk activation was more modest and not detected until the 2-hour time point.

To determine whether Brp-39 directly inhibits tubular cell apoptosis, primary cultures of renal tubular cells (PTECs) were
isolated for in vitro analysis. TUNEL staining of these cells revealed that freshly isolated PTECs exhibit a modest level of baseline apoptosis that is markedly increased by exposure to reactive oxygen species (ROS) via addition of H₂O₂ (Figure 3G). Pretreatment with recombinant Brp-39 decreased H₂O₂-induced PTEC apoptosis by nearly 50%. Inhibition of PI 3-kinase activation using LY294002 prevented the Brp-39–mediated antipapoptotic effect, demonstrating that activation of this pathway is critical for the protective effects of Brp-39 (Figure 3G).

Table 1. FACS profile of macrophages isolated from WT and Brp-39−/− kidneys

<table>
<thead>
<tr>
<th></th>
<th>Total Macrophage Number (Day 3)</th>
<th>CD45⁺F4/80⁺CD11c−Ly6c⁺ (% of Total CD45⁺F4/80⁺CD11c⁺)</th>
<th>CD45⁺F4/80⁺CD11c−MR⁺ (% of Total CD45⁺F4/80⁺CD11c⁺)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I/R Kidney (n=6)</td>
<td>Contralateral Kidney (n=6)</td>
<td>I/R Day 1 (n=3)</td>
</tr>
<tr>
<td>WT</td>
<td>204,579±81,998</td>
<td>95,014±33,268</td>
<td>12.5±2.20</td>
</tr>
<tr>
<td>Brp39−/−</td>
<td>387,494±175,607</td>
<td>125,594±24,606</td>
<td>17.4±4.03</td>
</tr>
</tbody>
</table>

CD45⁺F4/80⁺CD11c⁻Ly6c⁺ macrophages are viewed as proinflammatory (M1). CD45⁺F4/80⁺CD11c⁻MR⁺ macrophages are viewed as reparative (M2).

²P<0.01 for day 1 CD45⁺F4/80⁺CD11c⁻MR⁺ macrophages in Brp39−/− versus WT. All other comparisons were not significant.

Figure 2. Brp-39 is required for the normal repair phase after AKI. (A) Kaplan-Meier survival analysis of male WT or Brp-39−/− mice subjected to 30 minutes of unilateral I/R with contralateral nephrectomy and followed for 7 days. n=21 for WT and n=19 for Brp39−/−. P=0.001. (B and C) Creatinine and BUN values are obtained at the indicated times from mice subjected to 25 minutes I/R that survived until day 3. For creatinine, n=7 for each group. P=NS on day 1 and P<0.05 on day 3. For BUN, n=10 WT and n=11 Brp39−/− mice. P<0.05 for Brp39−/− versus control at 1 and 3 days. (E) Hematoxylin and eosin staining is shown from representative regions of the outer medulla of WT and Brp39−/− mice 3 days after 25 minutes of I/R. (F) Tissue injury scoring is performed on kidney sections from mice treated as in E and is reported as the percentage of tubules with cells exhibiting overt necrosis (n=3 on day 1, n=4 on day 3). **P<0.01 versus WT.

The first urine collected after transplantation (0 hours) demonstrated a markedly higher YKL-40 concentration in patients who subsequently exhibited DGF even though serum YKL-40 levels were indistinguishable at that point in all three groups (Figure 4, A and B), suggesting that YKL-40 is being expressed within the injured kidney (as it is in mice) rather than filtered from the serum. Urinary YKL-40 levels continued to rise in DGF patients for 18 hours after reperfusion, resulting in higher mean urine YKL-40 values in patients with DGF at all time points compared with SGF and IGF (Figure 4A). There

Urinary YKL-40 Levels Predict Severity of Post-Transplant AKI and Need for Dialysis after Kidney Transplantation

The observed correlation between the degree of ischemic renal injury and urinary Brp-39 levels in the mouse led us to consider the possibility that levels of urinary YKL-40, the protein produced by the human Chs31I gene, might be reflective of the degree of ischemic renal tubular injury in patients with AKI. To test this, we compared urinary and blood YKL-40 levels in patients who received deceased-donor kidney transplants and exhibited delayed graft function (DGF), which is indicative of severe ischemic injury, to levels in those patients who had slow graft function (SGF) and immediate graft function (IGF), which are indicative of less severe ischemic injury. Urine and blood samples were collected from 78 patients at early time points after transplantation, of which 26 had DGF, 28 had SGF, and 23 had IGF. Apart from a higher proportion of donations after cardiac death (DCD) in the DGF group, donor and recipient characteristics were similar between the three groups (Table 2). As would be expected, mean urine output on the first postoperative day (POD) was lower and mean discharge serum creatinine was higher in the DGF group.

**CLINICAL RESEARCH**
was no statistically significant difference in blood YKL-40 values between groups immediately after surgery, but values separated significantly for both first and second PODs (Figure 4B). Supplemental Table 1 lists mean and median values for both urine and blood YKL-40 measurements between groups at all time points.

Receiver-operating characteristic (ROC) curves indicated that urine YKL-40 predicted the development of DGF with moderately accurate areas under the curve (AUCs ± SEM) of 0.84 ± 0.06 and 0.88 ± 0.05 at 0 hours and the first POD, respectively (Figure 5). AUCs for blood YKL-40 at the same time points were 0.59 ± 0.08 and 0.76 ± 0.07. Consistent with this, normalization of urinary YKL-40 concentrations to urine creatinine did not significantly alter the discrimination observed between DGF patients and those not requiring dialysis. See Supplemental Table 2 for AUCs for predicting DGF with urine and blood YKL-40 at all time points and Supplemental Table 3 for the sensitivity and specificity of the biomarker at different cutoff values.

**DISCUSSION**

To further our understanding of the process of recovery from AKI, we used an unbiased approach with DIGE followed by mass spectrometry to discover proteins that are predominantly...
expressed in the urine during kidney repair. These studies revealed that levels of urinary CLP are markedly increased after kidney injury, correlating with upregulated renal expression of the mRNA for \textit{Chi3l1} that peaks during the time of kidney repair. In addition, the levels of renal \textit{Chi3l1} mRNA expression and urinary Brp-39 excretion directly correlate with the severity of kidney injury. Although multiple cell types are known to express this protein, including endothelial cells, inflammatory cells, and tubular cells (data not shown), the predominant source of \textit{Chi3l1} in the kidney after ischemic injury appears to be macrophages.

It is known that severe ischemic kidney injury causes initial tubular cell necrosis with inflammatory cell infiltration, followed by a wave of tubular cell apoptosis that peaks at 24–48 hours after injury. This apoptotic response is driven in part by the local release of ROS and results in significantly worse tubular injury and kidney function, followed by a marked increase in proliferation of surviving tubular cells that functionally reconstitute the tubule.28–31 Using \textit{Brp-39} null mice, we discovered that the upregulation of \textit{Brp-39} in response to ischemic injury is critical in inhibiting tubular cell apoptosis \textit{in vivo}, and that this pathway serves to limit the severity of tubular injury and maintain sufficient kidney function to keep the animal alive and promote proliferation of viable tubular cells to effect subsequent kidney repair. Furthermore, \textit{in vitro} analysis of nonimmortalized cells demonstrates that \textit{Brp-39} acts directly on tubular cells to activate PI 3-K/Akt signaling and inhibit ROS-mediated apoptosis.

Compared with the relatively simple animal model of renal artery clamping followed by reperfusion, patients are often exposed to ischemic kidney injury in the setting of multiple insults, making it more difficult to identify specific pathophysiological events that are critical to the initial injury and subsequent repair. In those patients undergoing kidney transplantation,
these factors include donor characteristics such as advanced age, DCD, and cold ischemia time of the harvested organ, as well as recipient characteristics such as body habitus, warm ischemia time, and immunosuppressive regimens. By analyzing kidney biopsy specimens, Schwarz and coworkers found that tubular cells from patients with DGF had a significant increase in apoptotic responses with failure to upregulate typical antiapoptotic pathways such as Bcl-2 and Bcl-xL. Using a nonbiased gene expression profiling approach, Mas and colleagues found a strong correlation between activation of the inflammatory response, particularly innate immunity markers such as IFITM1, BCL3, and CD83, and the development of DGF in kidney transplant recipients.

Although it is clear that immune activation can lead to apoptosis in injured organs, our group and others have found that the innate immune response also plays a critical role in the reparative events after injury. For example, monocytes that enter the ischemically injured kidney adopt a proinflammatory expression profile in the first 24–48 hours, but then transition to an immune modulatory, proreparative phenotype during the following several days. In this study, we demonstrate that YKL-40, known to be secreted by neutrophils and monocytes/macrophages as part of the innate immune response to injury, is markedly elevated in the urine of patients with DGF compared with those with SGF or IGF, even though initial blood levels are indistinguishably high in all three groups. On the basis of studies in ischemically injured mice, we believe that the high urinary YKL-40 levels in patients with DGF may come in part from macrophages and other cells in the injured kidneys themselves and are indicative of greater tubular injury in those kidneys. Consistent with this, the group of patients who experienced DGF included a significantly higher number of recipients of kidneys from DCD donors and exhibited significantly lower urine outputs. It should be noted that filtered YKL-40 may also contribute to the high urinary levels seen in patients with DGF.

By combining studies of mice subjected to kidney I/R to identify the timing and importance of Brp-39 expression in the pathophysiology of I/R injury with studies in transplant recipients that establish a correlation between urinary YKL-40 and the severity of I/R injury during transplantation, we can propose several key considerations for further investigation of this pathway. First, urinary expression of YKL-40 may provide us with a preprocurement indicator of which kidneys will be the greatest risk for DGF. The fact that high levels of this protein are present in the urine immediately after transplantation suggests that many kidneys that exhibit DGF are likely to have suffered significant ischemic injury before or during organ procurement. I/R injury to such kidneys can be minimized via machine perfusion, with decreased risk for DGF and better 1-year allograft survival. The modality is expensive however, and could be more cost-effective if reserved for the subgroup of allografts identified as being at highest risk for DGF based on determination of donor biomarkers such as urinary YKL-40. In addition,
recent trials have demonstrated acceptable outcomes (with somewhat better long-term allograft function) for belatacept-based immunosuppressive regimens compared with calcineurin inhibitor-based therapy.\textsuperscript{37,38} Trials designed to evaluate the efficacy of these and other calcineurin-sparing regimens could be planned in recipients identified as high risk for DGF based on urinary YKL-40 levels before or immediately after transplant.

A second use of urinary YKL-40 may be as an indicator of the degree and duration of repair pathway activation that occurs after kidney transplantation. In our mouse model of moderate I/R injury, intrarenal \textit{Chi3l1} mRNA expression peaked on days 3–7 and returned to baseline by day 10, paralleling the course of successful kidney repair. In patients with IGF or SGF, urinary YKL-40 levels were consistently low, suggesting that ischemic injury of these kidneys was mild. In contrast, those with DGF exhibited high urinary YKL-40 levels at all three time points, suggesting more severe I/R injury and marked activation of this reparative pathway. Work in rodent models of kidney injury has shown that repair pathways, including the innate immune response, are critical in re-establishing normal tubular function.\textsuperscript{34,41,42} However, sustained activation of these same pathways in more severely injured kidneys can lead to maladaptive attempts at repair with fibrosis and nephron loss rather than tubule regeneration.\textsuperscript{34,41,42} In support of the possibility that this paradigm may hold true in transplant recipients, allografts that recover promptly have excellent short- and long-term outcomes compared with those with delayed recovery.\textsuperscript{43} In fact, the increasing use of expanded-criteria donor kidneys over the past decade has led to a greater risk for DGF and a concomitant plateau in long-term allograft survival despite a decline in acute rejection rates during this time.\textsuperscript{44,45} This suggests that the severe I/R injury that underlies DGF may permanently compromise allograft function and lead to prolonged attempts at unsuccessful repair. By providing a better understanding of the degree of injury and the extent and duration of the pathophysiologic response to that injury, biomarkers like YKL-40 have the potential to improve both early and late outcomes after kidney transplantation. As a comparison, within this cohort of transplant patients other kidney biomarkers have not performed as well as YKL-40. Plasma neutrophil gelatinase-associated lipocalin levels did not discriminate between DGF patients and those with good graft function.\textsuperscript{27} Similarly, urine biomarkers of AKI such as neutrophil gelatinase-associated lipocalin, IL-18, and cystatin C had lower accuracy than that observed with YKL-40 in this study.\textsuperscript{26,46}

Finally, serum YKL-40 levels may provide new insights into the systemic effects of CKD. A recent study demonstrated that normal participants have plasma YKL-40 levels of approximately 40 ng/ml,\textsuperscript{14} whereas patients who are at increased risk for cardiovascular disease have significantly higher levels that are believed to reflect the chronic activation of macrophages in atherosclerotic plaques.\textsuperscript{47,48} In our patient population, the average blood level of YKL-40 was >500 ng/ml in all three patient groups when measured immediately after transplantation.

Patients with SGF and IGF had a subsequent decline in blood YKL-40 levels, whereas those requiring dialysis did not, resulting in statistically significant differences on the first and second PODs. The well described correlation between the development of CKD and accelerated risk of cardiovascular complications suggests that CKD/ESRD patients may constantly exhibit high circulating levels of YKL-40 and that the rapid decline seen in those with IGF after transplantation represents early resolution of this inflammatory state.

Cumulatively, the data in this study demonstrate an unexpected role of chitinase-like proteins in ischemic organ injury and reinforce the concept that the innate immune response has evolved to identify and dispose of cells that are severely injured while promoting the survival and expansion of sub- stially injured cells to effect subsequent organ repair. The discovery of chitinase 3-like 1/I/Brp-39/YKL-40 as both a sensor of the degree of injury and a critical mediator of this reparative response provides us with a potentially powerful biomarker that can promote rapid identification of those patients at greatest risk to have sustained renal failure after transplantation. Although our mouse studies are encouraging, subsequent studies in carefully selected patient populations will be required to determine whether this factor can also predict the severity of injury in native kidneys and/or in other organ systems.

**CONCISE METHODS**

**I/R Surgery and Experimental Protocol**

All animal protocols were approved by the Yale University Animal Care and Use Committee. Male WT and \textit{Brp-39}−/− mice (on the C57Bl/6 background, previously described by Lee \textit{et al.}\textsuperscript{11}), aged 9–12 weeks, were anesthetized on a 37°C warming pad, the abdomen opened, and warm renal ischemia induced using a nontraumatic microaneurysm clip (FST Micro Clamps, Foster City, CA) on the renal pedicle for the indicated time. To induce ARF, the right kidney was surgically removed at the time of left kidney ischemia. During surgery, the mice were hydrated with 1 ml of normal saline intraperitoneally and injected with 100 \mu l of Buprenex to avoid postoperative pain. The animals were additionally given 0.5 ml normal saline subcutaneously on day 1, and blood, urine, and tissue samples were obtained at the indicated times after I/R.

**Histology and Immunohistochemistry**

Kidneys were fixed in 4% paraformaldehyde and embedded in paraffin. For histologic evaluation of renal injury, sections were stained with hematoxylin and eosin and scored by the renal pathologist (G.M.), masked to the identity of the study animal. Multiple sections of renal tissue areas were evaluated for tubular necrosis, with or without regenerative features, and were scored using a square grid technique. Small squares of a 10×10 integrated grid, falling on tubules with morphologic features of overt necrosis (sloughing of cells, brush border loss, blebbing of cytoplasm), were counted in both cortex and outer medulla. Ten independent fields were counted per kidney (1000 squares per kidney), and the percentage of lesion area was calculated as the percentage of total
squares counted. To minimize the effect of injury variability on histologic evaluation, kidney sections were scored from those animals that had BUN levels closest to the average BUN of all animals in that group. For detection of proliferating cells, deparaffinized kidney sections were boiled in Retrieval A buffer (BD Pharmaning, San Jose, CA), incubated overnight with rabbit anti–Ki-67 (1:50; Clone Sp6; Thermo Fisher Scientific, Pittsburgh, PA), and visualized using Alexa 488 secondary antibody ( Molecular Probes, Eugene, OR). For detection of apoptotic cells, TUNEL-positive nuclei were visualized using the In Situ Cell Death Detection Kit (Roche Diagnostics, Indianapolis, IN) as per the manufacturer’s instructions. After labeling with TUNEL or Ki-67, tissue sections and cells were mounted in Vectashield 4',6-diamidino-2-phenylindole (DAPI) and viewed with a Leica fluorescence microscope equipped with a ×40-Planapochromat objective and selective filters for fluorescein isothiocyanate, DAPI, and Texas red. Quantification of cells expressing the specified marker was performed in a blinded fashion by counting positive tubular cells/total tubular cells (identified as DAPI+ nuclei) in 10 randomly chosen ×400 fields from the outer medulla.

FACS Analysis of Kidney Cell Populations
Kidneys were harvested, minced, and a single cell suspension made by incubating with Liberase and DNAse-1 (Roche Diagnostics) and filtered with a 40-μm cell strainer. For FACS, cells were stained with the following antibodies: anti-F4/80 FITC-conjugated (eBiosciences, San Diego, CA); anti-CD45 PERCP conjugated, anti-Ly6C, and anti-CD11c (BD Biosciences, San Jose, CA); and anti-mannose receptor (AbD Serotec, Raleigh, NC) with the background set using the appropriate isotype controls. For quantification of total macrophage numbers per kidney, cell suspensions were mixed with a known amount of 5.1-μm AccuCount particle beads (Spherotech, Lake Forest, IL) before aliquot and staining. For flow sorting, cells were stained with the following antibodies: anti-F4/80 (eBiosciences), and anti-CD45, anti-Ly6g, anti-CD3e, and anti-CD11c (BD Biosciences). Kidney cells (CD45+), T cells (CD45+CD3e+), PMNs (CD45+Ly6g+), and macrophages (CD45+CD8+CD11c+) were then sorted and collected by BD FACS Aria (BD Biosciences) with cutoff settings using the appropriate isotype controls.

Cell Culture Experiments
Isolation of MPT epithelial cells (PTECs) was done following a modified protocol by Schafer et al. Kidneys were harvested after cardiac perfusion with 0.025% collagenase (Worthington, Lakewood, NJ) in M199 Hank’s solution (Lonza, Walkersville, MD). Renal cortex was isolated, minced, and then incubated at 37°C in collagenase solution aerated with 5% CO₂ for 40 minutes. Tissue and cells were resuspended in renal epithelial basal medium (Lonza) containing 2.5% FBS and penicillin/streptomycin, followed by passage through a 40-μm cell strainer. Cells were then plated and grown to confluence at 37°C maintained in 5% CO₂ before incubation with recombinant Chi3L1/Brp-39 (R&D Systems, Minneapolis, MN) and/or H₁0₂ (GIBCO, Language, OK). MPT cells were grown in DMEM/F12 media (GIBCO) containing 10% serum and penicillin/streptomycin. For cell signaling experiments cells were serum starved for 12 hours before incubation with recombinant Brp-39 (R&D Systems) and protein extraction. For in vitro induction of apoptosis, cells were incubated in 0.25mM H₂O₂ for 6 hours ± LY294002 (50 μM) (Promega, Madison, WI).

Immunoblot Analyses
Equal amounts of protein (30 μg) or mouse urine (40 μl) were loaded and electrophoresis was performed in a 10% polyacrylamide separating gel/5% stacking gel. Proteins were transferred to polyvinylidene difluoride membrane, and blocked with 5% milk in Tris-buffered saline with Tween 20 for 1 hour. The membrane was incubated overnight at 4°C with the following primary antibodies: anti-phospho-Akt (S473; Cell Signaling, Danvers, MA), anti-phospho-p44/42 MAPK (Cell Signaling), anti-Brp-39 (generated as previously described by Lee et al.11), and anti-Chi3L1 (R&D Systems, Minneapolis, MN). Blots were washed in 0.1% Tris-buffered saline with Tween 20 and incubated with secondary antibody for 35 minutes at room temperature. After washing, the second antibody was visualized by chemiluminescence reagents. Total Akt and total MAPK expression was determined using anti-Akt and anti-44/42 MAPK (Cell Signaling) as the loading control.

Analyses of mRNA/Real-Time PCR
RNA was extracted with a RNeasy Mini kit (Qiagen) and reverse transcribed. Gene expression analysis was determined by quantitative real-time PCR using an iCycler iQ (Bio-Rad) and normalized to hypoxanthine-guanine phosphoribosyltransferase (HPRT). Either a specific TaqMan Gene Expression Assay (Mm01545399_m1, Mm00801477_m1; Applied Biosystems, Foster City, CA) or the following primers were used with Cyber Green: Chi3L1 (forward: CAAGGAACTGAATGGGAAT; reverse: GGCTCCCAACGTTATCATGT) or HPRT (forward: CAGTACAGCCCCAAAATGGT; reverse: CAAGGCGCATATCCAAACACA). Data are expressed using the comparative threshold cycle (dCt) method and mRNA ratios are given by 2-dCt.

Study Patients, Data Collection, and Outcomes
This study was approved by the institutional review boards of all participating transplantation centers. We recruited patients aged at least 18 years who were dialysis dependent and admitted to receive deceased-donor kidney transplants. We excluded patients who did not give informed, written consent or had primary nonfunction of the graft due to surgical complications. For further details, see our previous publications from this cohort.26,27,46

We collected baseline donor, recipient, and transplant characteristics consistent with the variables reported to the United Network for Organ Sharing. The need for dialysis, which was determined by the clinicians caring for participants at each institution without a standardized study protocol, was recorded by prospective chart review. We defined DGF as at least one dialysis session within 7 days after transplant. In those without dialysis, SGF was defined as a creatinine reduction ratio (difference between the initial serum creatinine within an hour of transplant and the serum creatinine on day 7 divided by the initial serum creatinine) <0.7, and IGF was defined as a creatinine reduction ratio ≥0.7.52

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Sample Selection
We collected 10 ml of urine and 6 ml of blood upon arrival to the postanesthesia care unit (time 0), typically within an hour of transplant. We also collected urine samples at 6, 12, and 18 hours after surgery, and on the first and second postoperative mornings (POD1 and POD2). Samples were centrifuged at 5000 g for 10 minutes to remove cellular debris and supernatants aliquoted into 1-ml samples for urine and 0.5-ml samples for blood. Samples were barcode labeled and stored at −80°C. YKL-40 levels were determined by ELISA as previously described.15

Statistical Analyses
Analyses were two-tailed with a significance level of 0.05. We used chi-squared or Fisher’s exact tests to compare categorical variables, ANOVA to compare mean values for continuous variables, and Kruskal–Wallis tests to compare medians values between those with DGF, SGF, and IGF. We performed ROC curve analysis to compare the accuracy of urinary and blood YKL-40 at each time point for predicting DGF. The value for YKL-40 that gave the largest sum of sensitivity and specificity was chosen as the optimal cutoff. We used SAS 9.2 software for Windows (SAS Institute, Cary, NC) for all analyses.

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DISCLOSURES
None.

REFERENCES

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