Severe Renal Mass Reduction Impairs Recovery and Promotes Fibrosis after AKI

Aaron J. Polichnowski,* Rongpei Lan,† Hui Geng,† Karen A. Griffin,* Manjeri A. Venkatachalam,† and Anil K. Bidani*

*Division of Nephrology and Hypertension, Department of Medicine, Loyola University and Hines Veterans Affairs Hospital, Maywood, Illinois; and †Department of Pathology, University of Texas Health Science Center, San Antonio, Texas

ABSTRACT

Preexisting CKD may affect the severity of and/or recovery from AKI. We assessed the impact of prior graded normotensive renal mass reduction on ischemia-reperfusion–induced AKI. Rats underwent 40 minutes of ischemia 2 weeks after right uninephrectomy and surgical excision of both poles of the left kidney (75% reduction of renal mass), right uninephrectomy (50% reduction of renal mass), or sham reduction of renal mass. The severity of AKI was comparable among groups, which was reflected by similarly increased serum creatinine (SCr; approximately 4.5 mg/dl) at 2 days, tubule necrosis at 3 days, and vimentin-expressing regenerating tubules at 7 days postischemia-reperfusion. However, SCr remained elevated compared with preischemia-reperfusion values, and more tubules failed to differentiate during late recovery 4 weeks after ischemia-reperfusion in rats with 75% renal mass reduction relative to other groups. Tubules that failed to differentiate continued to produce vimentin, exhibited vicarious proliferative signaling, and expressed less vascular endothelial growth factor but more profibrotic peptides. The disproportionate failure of regenerating tubules to redifferentiate in rats with 75% renal mass reduction associated with more severe capillary rarefaction and greater tubulointerstitial fibrosis. Furthermore, initially normotensive rats with 75% renal mass reduction developed hypertension and proteinuria, 2–4 weeks postischemia-reperfusion. In summary, severe (>50%) renal mass reduction disproportionately compromised tubule repair, diminished capillary density, and promoted fibrosis with hypertension after ischemia-reperfusion–induced AKI in rats, suggesting that accelerated declines of renal function may occur after AKI in patients with preexisting CKD.


Clinical studies suggest that AKI worsens preexisting CKD and accelerates progression to end stage because of residual structural and functional deficits.1–7 CKD, per se, may increase the risk and severity of AKI and the likelihood of incomplete recovery from AKI.8 Thus, AKI and CKD reinforce each other to increase nephron loss and tubulointerstitial fibrosis (TIF).9 Nevertheless, causal relationships for both aspects of the AKI–CKD nexus10 (AKI resulting in CKD/ESRD and CKD, per se, predisposing to AKI) have been questioned.11,12 These concerns were recently reviewed.13 AKI–CKD relationships have also been questioned on the grounds that mechanisms for AKI–CKD interactions are ill-defined and controversial.14–16

We addressed these uncertainties by investigating the impact of normotensive renal mass reduction (RMR; 0%, 50%, and 75%) of 2-weeks duration on AKI induced by ischemia-reperfusion (I/R) in rats. We addressed three questions. (1) Does prior RMR increase AKI severity? (2) Does prior RMR impair recovery from AKI? (3) Does prior RMR predispose to the development of more severe TIF during

Received April 8, 2013. Accepted November 26, 2013.
A.J.P. and R.L. contributed equally to this work.
Published online ahead of print. Publication date available at www.jasn.org.
Correspondence: Dr. Anil K. Bidani, Loyola University Medical Center, 2160 South First Avenue, Maywood, IL 60153. Email: abidani@lumc.edu

Copyright © 2014 by the American Society of Nephrology
recovery from AKI? To achieve 75% RMR, we used the normo-tensive rat remnant kidney model produced by uninephrectomy and excision of approximately one half of the contralateral kid-ney.17 Normotensive 75% RMR, per se, causes minimal TIF even after 4 months,17–19 despite adaptations (e.g., glomerular hyperfiltration, hypertrophy, oxidative stress, etc.) that are postulated to cause fibrosis in hypertensive RMR models.20–22

RESULTS

The effects of AKI on body and kidney weights followed expected patterns (Table 1).

RMR Does Not Modify Acute Serum Creatinine Increases or Acute Tubular Necrosis after I/R

Two weeks of RMR resulted in modest serum creatinine (\(S_{\text{Cr}}\)) increases before I/R in proportion to lost renal mass (pre-I/R) (Figure 1). However, 48 hours post-I/R, \(S_{\text{Cr}}\) was comparably elevated (approximately 4.5 mg/dl) in all groups, suggesting similar AKI severity. Acute tubular necrosis 3 days after I/R was higher in the outer stripe of outer medulla versus cortex in all groups (\(n=5\) each) but not different between bilateral, uninephrectomy (UNX), and 3/4 nephrectomy (3/4 NX) groups (Figure 2).

RMR by 3/4 NX Delays Functional Recovery and Predisposes to Severe TIF 4 Weeks after I/R

After acute increases, \(S_{\text{Cr}}\) declined in all groups by 1 week and then, declined to lower levels by 4 weeks; however, \(S_{\text{Cr}}\) remained higher in 3/4 NX I/R (\(n=12\)) rats at 1 and 4 weeks compared with values for pre-I/R and UNX I/R (\(n=12\)) or bilateral I/R (\(n=10\)) rats (Figure 1). Four weeks after I/R, all kidneys showed patchy TIF greater than the minimal fibrosis in 3/4 NX sham I/R kidneys (Figure 3A). However, corre-sponding to incomplete recovery of \(S_{\text{Cr}}\), TIF was more severe in 3/4 NX I/R rats than UNX and bilateral I/R rats, which did not differ between themselves (Figure 3B). Thus, 3/4 NX pre-disposed to greater TIF 4 weeks after I/R, despite similarly severe AKI in all groups. Dissociation between AKI severity and recovery is supported by the weak correlation between \(S_{\text{Cr}}\) at 48 hours post-I/R and TIF at 4 weeks post-I/R in the same animals (Supplemental Figure 1). In 5 of 12 3/4 NX I/R rats, TIF severity approached end stage.

TIF 4 Weeks after I/R in 3/4 NX RMR Rats Is Associated with Persistent Profibrotic Signaling in Greater Numbers of Regenerating Tubules That Fail to Redifferentiate

We and others have previously shown that TIF after AKI is associated with failed redifferentiation of regenerating tubule epithelium that continues to secrete profibrotic peptides.9,23–29 To investigate whether RMR deters regenerating proximal tu-bules (PTs) from differentiating during recovery from I/R, we performed immunohistochemistry (IHC) for vimentin. Absent in normal PTs, vimentin becomes expressed when surviving cells dedifferentiate and proliferate after AKI.30–34 As tubules redifferentiate, vimentin expression is suppressed.31 Therefore, vimentin expression in tubules late after injury (when recovery should be complete) indicates defective repair with abnormally dedifferentiated epithelium.26,27,31,33

Accordingly, 7 days post-I/R, tubules expressed vimentin in proportion to necrosis at 3 days (Figure 4A), with sharp transitions between epithelium without and with vimentin (Supplemental Figure 2A). As expected, 3/4 NX sham I/R kidneys showed staining only in glomeruli and interstitial fibroblasts. Vimentin-positive tubules were more numerous in the outer stripe of outer medulla than cortex, without differences between bilateral I/R (\(n=5\)), UNX I/R (\(n=5\)), and 3/4 NX I/R (\(n=5\)) groups 7 days post-I/R (Figure 4C), which parallels similar severities of acute injury.

As shown in Figure 4B, vimentin-positive tubules at 4 weeks post-I/R declined in all groups relative to 7 days post-I/R (Figure 4A). However, although kidneys in all I/R groups showed persistent vimentin expression in substantial numbers of tubules and fibroblasts at 4 weeks post-I/R, such tubules were significantly higher in 3/4 NX (\(n=12\)) versus bilateral (\(n=10\)) and UNX (\(n=12\)) I/R kidneys (Figure 4D). Most vimentin-positive tubules were undifferentiated and atrophic, with sharp transitions between cells without vimentin and vice

Table 1. Effect of I/R-induced AKI on body and kidney weights

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Weight (g)</th>
<th>Total Kidney Weight (g)</th>
<th>Kidney Weight/ Body Weight (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Final</td>
<td></td>
</tr>
<tr>
<td>Bilateral I/R ((n=10))</td>
<td>328±17</td>
<td>431±14*</td>
<td>6.3±0.2*</td>
</tr>
<tr>
<td>UNX I/R ((n=12))</td>
<td>338±4</td>
<td>431±5*</td>
<td>6.7±0.2*</td>
</tr>
<tr>
<td>3/4 NX I/R ((n=12))</td>
<td>309±11</td>
<td>365±14*</td>
<td>3.6±0.2</td>
</tr>
<tr>
<td>3/4 NX sham I/R ((n=5))</td>
<td>287±13</td>
<td>473±21*</td>
<td>2.9±0.2</td>
</tr>
</tbody>
</table>

Pre-I/R body weights obtained approximately 2 weeks after I/R were not different among groups. Increases in weight gain after 4 weeks were observed in all groups but to a significantly lesser extent in the 3/4 NX I/R rats. Absolute kidney weights exhibited the expected pattern with graded RMR. Compared with the 3/4 NX sham I/R rats, kidney weights tended to be higher in 3/4 NX I/R rats but did not reach statistical signifi-cance (\(P=0.06\)). However, when normalized to body weight, relative kidney weights were significantly greater in 3/4 NX I/R versus 3/4 NX sham I/R rats. Values are mean±SEM.

\*\(P<0.05\) versus respective baseline body weight.

\^\(P<0.05\) versus all other groups.
versa (Supplemental Figure 2B). Vimentin positivity at 4 weeks post-I/R was highly correlated ($r^2=0.80$, $P<0.001$) with corresponding TIF scores (Figure 4E).

We validated vimentin as a marker for failed differentiation, vicarious signaling, and profibrotic peptide expression. Serial sections showed increased phospho-c-Jun in the same vimentin-positive tubules (Figure 5A, Supplemental Figure 3A). c-Jun is phosphorylated by Jun N-terminal kinase that is activated within minutes of I/R and suppressed during recovery, unless tubule repair becomes compromised and TIF develops. Thus, vimentin-positive tubules associated with TIF 4 weeks after I/R pathologically retained signaling activity that is initiated earlier during proliferation. They also exhibited a lack of differentiation...
Figure 4. Greater failure of tubules to redifferentiate 4 weeks after I/R in 3/4 NX rats, as indicated by vimentin positivity. Tubular vimentin expression in (A and C) bilateral (n=5), UNX (n=5), and 3/4 NX (n=5) groups at 7 days and (B and D) bilateral (n=10), UNX (n=12), and 3/4 NX (n=12) groups at 4 weeks after I/R. Tubules regenerating after I/R injury express vimentin at 7 days (early after I/R) and thus, can be used as an index of the severity of AKI. As shown in A and C similar to the pattern of acute tubular necrosis, tubular vimentin expression was greater in OSOM versus cortex in all groups but without significant differences between groups. Vimentin staining is absent in tubules but normally expressed in glomeruli and interstitial fibroblasts in 3/4 NX sham I/R kidneys. Additional evidence supporting the use of vimentin as an index of regenerating tubules after AKI is illustrated in Supplemental Figure 2. As shown in B and D, 4 weeks after I/R, vimentin-positive tubules declined relative to (A and C) those tubules seen at 1 week in different groups of bilateral and UNX I/R rats, suggesting that significant numbers of tubules had redifferentiated. However, vimentin-positive tubules in the 3/4 NX I/R group remained elevated at 4 weeks versus bilateral and UNX I/R groups, paralleling the TIF grades (Figure 3). E shows the linear regression analysis between TIF and vimentin-positive tubules (average score from OSOM and cortex) at 4 weeks after I/R in the same bilateral (n=10), UNX (n=12), and 3/4 NX (n=12) I/R rats. A strong (r²=0.80) and significant (P<0.001) correlation between the extent of vimentin-expressing tubules and the level of TIF is evident. †P<0.05; ††P<0.01 versus respective cortex value. *P<0.05; **P<0.01 versus the respective 3/4 NX I/R group. Values are mean±SEM. Scale bar, 1 mm.
markers, which was reflected by the lack of phytohaemagglutinin-E–lectin–bound brush borders and the lack of Na⁺K⁺-ATPase staining in basolateral membranes (Figure 5B, Supplemental Figure 3B). The same tubules showed increased immunostaining for connective tissue growth factor (Figure 5B, Supplemental Figure 3B), PDGF-B, and TGFβ1 (not shown).

We performed additional quantitative validation of the lack of differentiation markers in vimentin-expressing tubules by grading PT expression of aquaporin 1 (AQP1), which is normally abundantly expressed in amplified brush border and basolateral membranes of PT. Tubules lost AQP1 more severely in 3/4 NX I/R kidneys than bilateral I/R and UNX I/R kidneys (Figures 6 and 7A, Supplemental Figure 4). Furthermore, we assessed phosphatase and tensin homolog (PTEN) expression in tubules (Figures 6 and 7B, Supplemental Figure 4). PTEN regulates PT differentiation and is lost from the undifferentiated vimentin-positive tubules during TIF development after I/R.26 The highly significant ($P<0.001$) inverse relationships and strong correlations between vimentin positivity and AQP1 ($r^2=0.92$) and between vimentin positivity and PTEN ($r^2=0.91$) are shown in Figure 7, C and D, respectively. Similar inverse relationships were also observed between AQP1 and TIF ($r^2=0.77, P<0.001$) (Supplemental Figure 5A) and PTEN and TIF ($r^2=0.75, P<0.001$) (Supplemental Figure 5B).

Increased TIF in 3/4 NX I/R Kidneys Is Accompanied by Markedly Decreased Expression of VEGF in Tubules and Increased Rarefaction of Peritubular Capillaries Compared with Bilateral I/R and UNX I/R Groups

Because diminished tubule vascular endothelial growth factor (VEGF) expression, microvascular rarefaction, and tissue hypoxia may promote TIF after I/R injury and fibrotic renal disease,36–45 we assessed VEGF and microvascular density (Figure 8, Supplemental Figure 6). IHC using an antibody recognizing all VEGF-A isoforms showed staining in tubules but not interstitial cells. Tubule VEGF was more severely

Figure 5. Vimentin-positive tubules exhibit lack of differentiation markers, but persistent profibrotic signaling, 4 weeks after I/R. (A) Representative IHC images for vimentin (Vim; brown) and phospho-c-Jun (p-cJun; red) in serial sections from 3/4 NX kidneys 4 weeks after I/R or sham I/R surgery. Increased nuclear phospho-c-Jun was present in the same tubules that expressed vimentin in kidneys from 3/4 NX I/R rats, whereas 3/4 NX sham I/R kidneys showed only occasional phospho-c-Jun–positive nuclei. Transcription factor c-Jun is phosphorylated in tubules within minutes of I/R and suppressed during recovery. Abundance of tubules coexpressing vimentin and phospho-c-Jun at 4 weeks post-I/R, when such signaling should be abated, is indicative of a dedifferentiated, persistently signaling profibrotic phenotype consistent with impaired recovery from AKI. Similar patterns of tubular vimentin and phospho-c-Jun expression were observed in bilateral and UNX kidneys at 4 weeks after I/R, although to a lesser extent (Supplemental Figure 3A). B shows representative IHC images for vimentin (brown)–phytohaemagglutinin-E–lectin (red) co-staining, Na⁺K⁺-ATPase, and connective tissue growth factor (CTGF) in serial sections from 3/4 NX kidneys 4 weeks after I/R or sham I/R surgery. Numerous tubules in 3/4 NX I/R kidneys expressed vimentin, lacked the brush border and basolateral membrane differentiation markers PHAE-lectin and Na⁺K⁺-ATPase, respectively, and showed increased staining for profibrotic peptide CTGF. Kidneys of 3/4 NX sham I/R rats exhibited robust tubule PHAE-lectin and Na⁺K⁺-ATPase expression with minimal CTGF staining. The vimentin-positive tubules in the 3/4 NX I/R rats are consistent with the dedifferentiated, persistently signaling profibrotic phenotype associated with impaired recovery from AKI that we have previously described.26 Of note, a similar profibrotic, dedifferentiated tubule phenotype was observed in both bilateral and UNX groups at 4 weeks after I/R, although to a lesser extent compared with 3/4 NX I/R rats (Supplemental Figure 3B). Scale bar, 100 μm.
We surmise, as have others, that strong inverse correlations with corresponding TIF grades. Figure 7A) and JG12 (Supplemental Figure 7B) also exhibited with JG12 scores (Figure 9D). Scores for VEGF (Supplemental Figure 4). Scale bar, 100 μm.

documented, PTEN regulates PT differentiation, and such decreases in PTEN also indicate a heightened state of dedifferentiation in tubules 4 weeks after I/R, when tubules should have re-differentiated. Of note, a similar pattern of PTEN reduction was observed in bilateral and UNX kidneys 4 weeks after I/R, although to a lesser extent (Supplemental Figure 4). Similar to the pattern of AQP1 expression, PTEN levels in PTs were lower in 3/4 NX I/R versus 3/4 NX sham I/R kidneys. As we have previously described, PTEN regulates PT differentiation, and such decreases in PTEN also indicate a heightened state of dedifferentiation in tubules 4 weeks after I/R, when tubules should have re-differentiated. Of note, a similar pattern of PTEN reduction was observed in bilateral and UNX kidneys 4 weeks after I/R, although to a lesser extent (Supplemental Figure 4). Scale bar, 100 μm.

descended in 3/4 NX I/R kidneys (Figure 9A) together with markedly increased capillary rarefaction (reduced epithelium aminopeptidase P [JG12] staining) by quantitative grading compared with the other groups (Figure 9B). Scores for vimentin positivity exhibited a strong inverse correlation \( r^2=0.67, P<0.001 \) with tubular VEGF expression (Figure 9C). Scores for tubule VEGF were strongly correlated \( r^2=0.76, P<0.001 \) with JG12 scores (Figure 9D). Scores for VEGF (Supplemental Figure 7A) and JG12 (Supplemental Figure 7B) also exhibited strong inverse correlations with corresponding TIF grades.

Interestingly, interstitial fibrosis was of greater severity around atrophic tubules of small diameters than similarly atrophic but dilated tubules. We surmise, as have others, that dense fibrosis protects against tubule dilatation.27

**DISCUSSION**

Our data provide the first experimental verification of the emerging clinical concept that loss of renal mass by prior disease adversely affects the outcome of superimposed AKI. Substantially greater TIF was observed at 4 weeks after I/R injury in rats with 75% RMR compared with rats with no or 50% RMR. In general, TIF and nephron loss after AKI have been observed to parallel the severity of the initial AKI.2,9,28,46 However, our results show that it was not the case in the present studies. The extent of RMR did not affect the severity of the initial acute injury after I/R, which is reflected by lack of differences in \( S_{Cr}\), the histologic severity of tubular necrosis at 3 days after I/R, or vimentin positivity during early dedifferentiation and regeneration at 7 days after I/R. However, at 4 weeks after I/R, when tubules should recover by redifferentiation, far greater numbers of tubules in the 3/4 NX I/R group continued to express a dedifferentiated vimentin-positive phenotype. We reported previously that such vimentin-positive tubules associated with fibrosis are growth-arrested but vicariously continue to signal through proliferation-associated pathways, exhibit undifferentiated morphology by electron microscopy, and lack IHC markers for epithelial differentiation.25,26,47,48

We now provide quantitative data to support the strong inverse relationships between vimentin positivity and expression of the differentiation marker (AQP1) or the differentiation determinant (PTEN). Like in our previous reports,25,26,47,48 the current data also indicate that tubules with the failed differentiation phenotype produce profibrotic peptides and give rise to surrounding fibrosis. Accordingly, scores for vimentin-positive tubules and TIF were strongly correlated 4 weeks after I/R in all groups, indicating a similar pathogenesis that is quantitatively more severe in 3/4 NX I/R versus other I/R kidneys. Collectively, these data support the concept developed earlier that failed differentiation of tubule epithelium after AKI with persistent proliferative signaling and production of profibrotic peptides is increased in 3/4 NX I/R kidneys (Figure 9A) together with markedly increased capillary rarefaction (reduced epithelium aminopeptidase P [JG12] staining) by quantitative grading compared with the other groups (Figure 9B). Scores for vimentin positivity exhibited a strong inverse correlation \( r^2=0.67, P<0.001 \) with tubular VEGF expression (Figure 9C). Scores for tubule VEGF were strongly correlated \( r^2=0.76, P<0.001 \) with JG12 scores (Figure 9D). Scores for VEGF (Supplemental Figure 7A) and JG12 (Supplemental Figure 7B) also exhibited strong inverse correlations with corresponding TIF grades.

Interestingly, interstitial fibrosis was of greater severity around atrophic tubules of small diameters than similarly atrophic but dilated tubules. We surmise, as have others, that dense fibrosis protects against tubule dilatation.27

**DISCUSSION**

Our data provide the first experimental verification of the emerging clinical concept that loss of renal mass by prior disease adversely affects the outcome of superimposed AKI. Substantially greater TIF was observed at 4 weeks after I/R injury in rats with 75% RMR compared with rats with no or 50% RMR. In general, TIF and nephron loss after AKI have been observed to parallel the severity of the initial AKI.2,9,28,46 However, our results show that it was not the case in the present studies. The extent of RMR did not affect the severity of the initial acute injury after I/R, which is reflected by lack of differences in \( S_{Cr}\), the histologic severity of tubular necrosis at 3 days after I/R, or vimentin positivity during early dedifferentiation and regeneration at 7 days after I/R. However, at 4 weeks after I/R, when tubules should recover by redifferentiation, far greater numbers of tubules in the 3/4 NX I/R group continued to express a dedifferentiated vimentin-positive phenotype. We reported previously that such vimentin-positive tubules associated with fibrosis are growth-arrested but vicariously continue to signal through proliferation-associated pathways, exhibit undifferentiated morphology by electron microscopy, and lack IHC markers for epithelial differentiation.25,26,47,48

We now provide quantitative data to support the strong inverse relationships between vimentin positivity and expression of the differentiation marker (AQP1) or the differentiation determinant (PTEN). Like in our previous reports,25,26,47,48 the current data also indicate that tubules with the failed differentiation phenotype produce profibrotic peptides and give rise to surrounding fibrosis. Accordingly, scores for vimentin-positive tubules and TIF were strongly correlated 4 weeks after I/R in all groups, indicating a similar pathogenesis that is quantitatively more severe in 3/4 NX I/R versus other I/R kidneys. Collectively, these data support the concept developed earlier that failed differentiation of tubule epithelium after AKI with persistent proliferative signaling and production of profibrotic peptides is increased in 3/4 NX I/R kidneys (Figure 9A) together with markedly increased capillary rarefaction (reduced epithelium aminopeptidase P [JG12] staining) by quantitative grading compared with the other groups (Figure 9B). Scores for vimentin positivity exhibited a strong inverse correlation \( r^2=0.67, P<0.001 \) with tubular VEGF expression (Figure 9C). Scores for tubule VEGF were strongly correlated \( r^2=0.76, P<0.001 \) with JG12 scores (Figure 9D). Scores for VEGF (Supplemental Figure 7A) and JG12 (Supplemental Figure 7B) also exhibited strong inverse correlations with corresponding TIF grades.

Interestingly, interstitial fibrosis was of greater severity around atrophic tubules of small diameters than similarly atrophic but dilated tubules. We surmise, as have others, that dense fibrosis protects against tubule dilatation.27

**DISCUSSION**

Our data provide the first experimental verification of the emerging clinical concept that loss of renal mass by prior disease adversely affects the outcome of superimposed AKI. Substantially greater TIF was observed at 4 weeks after I/R injury in rats with 75% RMR compared with rats with no or 50% RMR. In general, TIF and nephron loss after AKI have been observed to parallel the severity of the initial AKI.2,9,28,46 However, our results show that it was not the case in the present studies. The extent of RMR did not affect the severity of the initial acute injury after I/R, which is reflected by lack of differences in \( S_{Cr}\), the histologic severity of tubular necrosis at 3 days after I/R, or vimentin positivity during early dedifferentiation and regeneration at 7 days after I/R. However, at 4 weeks after I/R, when tubules should recover by redifferentiation, far greater numbers of tubules in the 3/4 NX I/R group continued to express a dedifferentiated vimentin-positive phenotype. We reported previously that such vimentin-positive tubules associated with fibrosis are growth-arrested but vicariously continue to signal through proliferation-associated pathways, exhibit undifferentiated morphology by electron microscopy, and lack IHC markers for epithelial differentiation.25,26,47,48

We now provide quantitative data to support the strong inverse relationships between vimentin positivity and expression of the differentiation marker (AQP1) or the differentiation determinant (PTEN). Like in our previous reports,25,26,47,48 the current data also indicate that tubules with the failed differentiation phenotype produce profibrotic peptides and give rise to surrounding fibrosis. Accordingly, scores for vimentin-positive tubules and TIF were strongly correlated 4 weeks after I/R in all groups, indicating a similar pathogenesis that is quantitatively more severe in 3/4 NX I/R versus other I/R kidneys. Collectively, these data support the concept developed earlier that failed differentiation of tubule epithelium after AKI with persistent proliferative signaling and production of profibrotic peptides is
likely to be related to the development of fibrosis around tubules.9 Furthermore, these data suggest that severe RMR impairs the orderly recovery of a greater proportion of tubules regenerating after I/R by compromising their redifferentiation, thereby promoting TIF. Such a mechanism could explain the consistently observed impairment or nonrecovery of kidney function in outcome analysis studies of human CKD with additional superimposed AKI.

The mechanisms responsible for the enhanced failure of regenerating tubules to recover and redifferentiate after AKI in rats with severe RMR remain to be definitively established. Although increased tubule metabolic workload, hypertrophy, and/or altered oxygen metabolism associated with severe RMR21,40,49 may be insufficient by themselves to produce progressive TIF in the absence of glomerular hypertension,17–19 they may, nevertheless, be able to compromise tubule recovery after AKI. In this context, our finding of severely reduced tubular VEGF expression after I/R in 3/4 NX rats suggests a potential pathway. Although pericytes as well as tubules produce VEGF and may regulate capillary integrity,42 overwhelming evidence to date suggests that tubules are important sources of VEGF and that the angiogenic effects of tubular VEGF are believed to be critical for the preservation and maintenance of peritubular vasculature.36–39,41,42,45,50,51 Therefore, the loss of tubular VEGF as a component of the failed differentiation phenotype may be responsible for the severely reduced peritubular capillaries in the 3/4 NX I/R rats. The strong inverse correlations between vimentin positivity and VEGF expression and in turn, between VEGF and capillary density support such inferences. These observations are consistent with previous studies suggesting that compromised VEGF signaling and diminished microvascular density may contribute to fibrotic kidney disease.36–38,40–45,50 It is, therefore, reasonable to hypothesize that early loss of capillary density could lead to local tissue hypoxia that could prevent injured tubules from redifferentiation. A progressively worsening vicious cycle of tubule damage and capillary rarefaction with tissue hypoxia in such microenvironments may increase the likelihood that tubules do not recover.9

Most prior investigations of the relationships between severe RMR and AKI were limited to the examination of acute injury, usually shorter than 40 minutes in duration, 24 hours after I/R.14,16 In the only study where the impact of RMR on subsequent AKI was studied over longer periods, subtotal RMR by UNX+ablation/infarction 10 weeks before I/R decreased AKI severity compared with I/R of both intact kidneys, but AKI after I/R in UNX kidneys was more severe.15 However, there were no differences in repair, which was essentially complete by 10 days in all groups. Several explanations exist for the discrepancy between this study15 and our study, and they include the relatively modest AKI severity (peak SCr of only approximately 1.5 mg/dl), the use of an RMR model known to cause hypertension, the use of nephrotoxic,52,53 the use of renoprotective isoanesthetics,54,55 and the question of whether core body temperature, a modulator of AKI severity,28 was maintained at 37°C in the study by Vercauteren et al.15

Although our studies did not address CKD progression after I/R, the observed adverse effects of severe RMR on recovery after AKI may, nevertheless, provide clues to potential mechanisms of accelerated long-term CKD progression after AKI. One AKI episode, even with residual fibrosis, intuitively seems insufficient to initiate progression without prior CKD/RMR. Without additional injury events, sites of damage become scars, and surviving nephrons would maintain function if present in

Figure 7. Strong correlations are observed between tubular vimentin positivity and loss of AQP1 and PTEN 4 weeks after I/R. Semiquantitative analysis of (A) AQP1 and (B) PTEN in PTs from bilateral (n=10), UNX (n=12), and 3/4 NX (n=12) rats 4 weeks after I/R. Significant reductions in AQP1 were seen in PTs from 3/4 NX I/R versus other I/R kidneys. A similar tendency for decreased PTEN levels in PTs from 3/4 NX I/R kidneys was also noted. AQP1 and PTEN were abundantly expressed in bilateral, UNX, and 3/4 NX shams I/R kidneys (all scores of five; data not shown). The use of vimentin, AQP1, and PTEN IHC analyses as evidence of failed redifferentiation in PTs after I/R injury is shown by the very strong correlations between (C) AQP1–vimentin (r2=0.92, P<0.001) and (D) PTEN–vimentin (r2=0.91, P<0.001). **P<0.01 versus respective 3/4 NX I/R group. Values are mean±SEM.
adequate numbers. Consistent with such concepts, progressive CKD was shown in bilateral AKI models after repeated (not single) injury episodes.65,66 However, with severe parenchymal loss by prior CKD or surgical RMR in our model, a single episode of AKI may be sufficient to result in severe TIF. In this context, the development of hypertension in previously normotensive UNX and 3/4 NX rats after AKI is relevant. Hypertension developed gradually 2–4 weeks after I/R and was restricted to rats that had not only additional parenchymal loss by TIF but also, severe losses of capillary density. These considerations make it likely that hypertension developed secondarily to severe microcirculatory deficits through mechanisms similar to those mechanisms reported for salt-sensitive hypertension occurring late in the bilateral I/R model.58,59 These studies found that losses of capillary density caused by I/R were prevented by the administration of VEGF-121, an early intervention that also preempted the salt-sensitive hypertension and fibrosis that developed secondarily to later administration of high salt.60

In any event, regardless of its precise pathogenesis, the development of hypertension in the RMR setting is likely to have significant long-term effects. Hypertension adversely affects the progression of established CKD.17–19,61,62 Enhanced glomerular BP transmission of elevated systemic BP caused by impairment in renal autoregulation after RMR results in progressive proteinuria, glomerulosclerosis, and incremental loss of additional nephrons.61,62 Indeed, AKI leads to CKD progression, with increasing proteinuria and glomerulosclerosis, in rats with preexistent 50% RMR (i.e., UNX).37,63–65 Rapid development of proteinuria and glomerular pathology in the most severely hypertensive 3/4 NX I/R rats in our study is consistent with such interpretations. In this context, bilateral and UNX I/R kidneys showed similar numbers of tubules with failed differentiation and similar TIF scores when considered as fractions of available renal mass, but UNX I/R rats (not bilateral I/R rats) developed proteinuria, glomerulosclerosis, TIF, and renal dysfunction after many months.37,64 Therefore, it seems possible that the long-term consequences of AKI in RMR settings depend on not only the extent of tubules lost by failed differentiation and TIF but also, the hemodynamic effects of RMR additionally imposed by AKI (hypertension and impaired autoregulation), the severity of which would be governed by the amounts of functional renal mass remaining after AKI.

In summary, our studies show striking adverse effects of severe RMR on the ability of kidney tubules to recover and redifferentiate after AKI. The defective tubules that are produced have profibrotic properties that are associated with TIF. Failure of recovering tubules to differentiate during recovery from AKI with the associated TIF and reduced peritubule capillary density may also be an antecedent cause for the development of hypertension in previously normotensive settings of CKD. Of particular relevance, the current findings support a recent population-based cohort study implicating an association between poor recovery from AKI and increased risk of CKD progression.66

CONCISE METHODS

CKD and AKI Models
Male Sprague–Dawley rats (Charles River) were obtained at 6–8 weeks of age and provided a standard rat chow (1.0% NaCl; Purina) and drinking water ad libitum. The animals were cared for in accordance with National Institutes of Health and institutional guidelines, and studies were approved by the Institutional Animal Care and Use Committee of Loyola University and Hines Veterans Administration Hospital. Mice were prepared for BP radiotelemetry (sampled every 10 minutes [24 h/d]).17 During the same surgery, kidneys were left intact in one group of rats (bilateral; n=20), whereas the remaining rats underwent normotensive RMR by right UNX (n=22) or right UNX+surgical excision of both poles of the left kidney (3/4 NX; n=27).17 After 14–18 days of recovery and compensatory renal adaptations,67 body weight, BP, SCr, and 24-hour protein excretion were measured. After this baseline assessment, rats were anesthetized (50 mg/kg sodium pentobarbital), placed on a servo-controlled heated surgical table to maintain body temperature at 37°C, and subjected to a 40-minute I/R through a clamp placed on the left (UNX I/R and
from AKI. In addition to continuous BP measurement, proteinuria (sulfosalicylic acid method) and $S_{Cr}$ (Quantichrom Creatinine Assay Kit; BioAssay Systems, Hayward, CA) were monitored at various time points over the 4-week period in this group of animals. Kidneys were perfusion-fixed and harvested from rats at the end of the study. The extent of renal injury was assessed by the semiquantitation of TIF (vide infra).

**Morphology and IHC**

At all time points after I/R, rat kidneys were perfusion-fixed with periodic acid-lysine-paraformaldehyde, dehydrated in graded ethyl alcohol, and processed into paraffin as previously described. 26,48 Deparaffinized sections were stained with Hematoxylin and Eosin, periodic acid–Schiff and Hematoxylin, and Masson’s Trichrome stain. Sections stained with periodic acid–Schiff and Hematoxylin were used to score tubular necrosis in kidneys harvested 3 days after I/R. Acute tubular necrosis was assessed semiquantitatively on a scale from zero to five, with zero indicating no evidence of tubular necrosis. Scores=1–5 represented visual assessment of the percent distribution of necrotic cells in tubules present in an entire cross-section in a quintile distribution. The blinded analysis was done by one of the investigators (M.A.V.). Sections stained with Masson’s Trichrome were similarly used to allocate TIF grades (scored zero to five, with zero being no evidence of TIF) on kidneys harvested 4 weeks after I/R. Percent distribution of areas involved by TIF, as described above for the distribution for necrotic cells, was visually assessed (by M.A.V.). It has previously been shown that visual estimation of the percentage of kidney tissue involved by fibrosis on Masson’s Trichrome–stained slides correlates well with morphometric techniques. 26 For IHC, deparaffinized sections were heated to 99–100°C in 1 mM Tris–EDTA (pH 8.0) for 20–30 minutes, and endogenous peroxidase activity was neutralized with 3% H$\textsubscript{2}$O$_{2}$ in water. Sections were blocked with 2.5% horse serum and incubated overnight with primary antibodies at 4°C. Primary antibodies and reagents were vimentin (clone V9; Thermo-Fisher, Waltham, MA); phospho-c-Jun, c-Jun (60A8; Ser63 54B3), and PTEN (clone 138G6; Cell Signaling, Danvers, MA); $\text{Na}^{+}$, K$^{+}$-ATPase α-subunit (DSHB, Iowa City, IA); PDGF-B LS-C112205 (LSBio, Seattle, WA); connective tissue growth factor, sc-14939, VEGF, Clone VG1, sc-53462, and AQP1 (B-11) sc-25287 (Santa Cruz Biotechnology, Santa Cruz, CA); TGFβ1 AHP1734 (AbD Serotec, Raleigh, NC); phytohaemagglutinin-E–AP lectin conjugate (EY Laboratories, Inc., San Mateo, CA); ImmPRESS horseradish peroxidase polymer-conjugated secondary antibodies and ImmPACT DAB reagent (Vector Labs, Burlingame, CA); and MACH 2 mouse and rabbit AP-Polymer

**Experimental Groups**

Different groups of rats were studied at three time points after I/R. To investigate whether underlying RMR alters the severity of AKI, one group of bilateral I/R ($n=5$), one group of UNX I/R ($n=5$), and one group of 3/4 NX I/R ($n=5$) rats were euthanized at 3 days post-I/R for the assessment of tubular necrosis. Separate groups of bilateral I/R ($n=5$), UNX I/R ($n=5$), and 3/4 NX I/R ($n=5$) rats were euthanized at 7 days post-I/R for the assessment of regenerating vimentin-positive tubules as an additional index of AKI severity. Finally, a third group of bilateral I/R ($n=10$), a third group of UNX I/R ($n=12$), a third group of 3/4 NX I/R ($n=12$), and a group of 3/4 NX sham I/R ($n=5$) rats were followed for 4 weeks after I/R to assess the impact of RMR on recovery

**Figure 9.** Strong correlative interrelationships were observed between vimentin positivity, loss of tubular VEGF, and loss of peritubular capillaries 4 weeks after I/R. Semiquantitative analysis of (A) VEGF and (B) capillary density (JG12 IHC) from bilateral, UNX and 3/4 NX sham I/R kidneys indicated abundant expression (all scores of five; data not shown). There was a strong inverse correlation between scores for (C) VEGF and vimentin ($r^{2}=0.67$, $P<0.001$) and a positive correlation between scores for (D) VEGF and JG12 ($r^{2}=0.76$, $P<0.001$). **P**<0.01 versus respective 3/4 NX I/R group. Values are mean±SEM.
Detection systems and Warp Red Chromogen (Biocare Medical, Concord, CA). Epithelium aminopeptidase P (rat) monoclonal antibody (JG12) was a gift from Donschko Kerjaschki. Incubation with primary antibody was followed by exposure to ImmPRESS horseradish peroxidase polymer–conjugated secondary antibodies (Vector Labs) as previously described.26 Tubules in kidneys processed by IHC were graded semiquantitatively on a scale from zero to five, with zero indicating the absence of specific antigens within tubules. Percentile distribution of tubule mass with the antigen in entire cross-sections was assessed by M.A.V. as described above for necrosis and TIF.

**Statistical Analyses**

Results are mean ± SEM. Statistical comparisons between groups were performed using one-way ANOVA, repeated measures ANOVA, and paired and unpaired t tests as appropriate. A Newman–Keuls post hoc test was used for multiple comparisons when appropriate. All semiquantitative data were log-transformed [log(1+x)] before statistical analysis. Linear regression analysis was used to calculate the slope of the relationship between variables. A P<0.05 value was considered significant.

**ACKNOWLEDGMENTS**

We acknowledge the technical support of Theresa Herbst and Hector Licea-Vargas and the secretarial support of Martha Prado.

This work was supported by Office of Research and Development of the Department of Veterans Affairs Career Development Award 1K2BX001285 (to A.J.P.), an Office of Research and Development of the Department of Veterans Affairs Merit Award (to K.A.G.), and National Institute of Diabetes and Digestive and Kidney Diseases Grants DK61653 (to K.A.G.) and DK40426 (to A.K.B.).

Some of these results were presented in abstract form at the American Society of Nephrology Annual Meeting held November 8–13, 2011, in Philadelphia, PA.

**DISCLOSURES**

None.

**REFERENCES**


47. Geng HL, Lan R, Singhka PK, Saikumar P, Weinberg JM, Venkatachalam MA: Lysophosphatidic acid (LPA) transactivates epidermal growth factor receptors (EGFR) via LPAR1/Gaiα signaling to potentiate LPA2/Gqα/Avb6 integrin dependent TGFβ signaling and increase the production of PDGF-B and CTGF by proximal tubule (PT) cells
BASIC RESEARCH


This article contains supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2013040359/-/DCSupplemental.