Evidence of Tubular Hypoxia in the Early Phase in the Remnant Kidney Model

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Abstract. The remnant kidney model is a mainstay in the study of progressive renal disease. The earliest changes in this model result from glomerular hemodynamic alterations. Given that progressive renal disease is the result of subsequent interstitial damage initiated by undetermined pathogenic factors, the authors investigated the role of hypoxia as a pathogenic factor in tubulointerstitial damage after renal ablation in rats. Cortical tissue hypoxia in the early phase (4 and 7 d) in remnant kidney rats, sham-operated rats, and animals treated with the angiotensin II receptor blocker (ARB) olmesartan (10 mg/kg per d) was assessed by uptake of a hypoxic probe, pimonidazole, expression of HIF-1α, and by increased transcription of hypoxia-responsive genes. Physiologic perfusion status of the postglomerular peritubular capillary network was evaluated by lectin perfusion and Hoechst 33342 diffusion techniques. Results showed that the number of hypoxic tubules was markedly increased 4 and 7 d after nephron loss. These findings anticipated any histologic evidence of tubulointerstitial damage. The hypoxic state persisted until interstitial damage developed. These results were confirmed using HIF-1α immunoprecipitation and increase of hypoxia-responsive genes. Pathologic studies of the vasculature demonstrated significant functional changes that generated a hypoxic milieu. ARB treatment prevented vascular changes and ameliorated tubular hypoxia. These results suggest that the initial tubulointerstitial hypoxia in remnant kidney model plays a pathogenic role in the subsequent development of tubulointerstitial injury. The initial hypoxia in this model was dependent on activation of the renin-angiotensin system and hemodynamic alterations after nephron loss.

The remnant kidney is a representative model in the study of progressive renal disease. The earliest response in this model consists of intrarenal vasodilation with relatively poor dilation of efferent arterioles, which in turn generates glomerular hyperfiltration and hypertension (1). Glomerular hyperfiltration, which depends on activation of the renin-angiotensin system (RAS), eventually leads to glomerulosclerosis (1). However, the deterioration of renal function is well correlated with the progressive interstitial damage, which evolves in the late phase, rather than glomerular lesions (2–4), and the mechanisms that initiate(s) tubulointerstitial injury in the context of early hemodynamic alterations remain(s) to be elucidated.

Chronic tissue hypoxia is regarded as a contributing factor in the progression of renal failure (5). Loss of peritubular capillaries in the late phase of various disease models has been recently reported (6–11), giving indirect evidence of renal hypoxia. Hypoxia itself can initiate the inflammatory process and eventually lead to tissue fibrosis (12–17). In a remnant kidney model, Kang et al. (11) reported that the loss of peritubular capillaries occurred around the second week, at which time severe tubulointerstitial injury started to develop. Prevention of this loss by VEGF administration abolished subsequent tubulointerstitial damage. These data strongly support the notion that hypoxia might be a cause, rather than an epiphenomenon, of tubulointerstitial damage. However, evidence of hypoxia in the early phase of this representative model before the development of histologic damage remains to be clarified. We have investigated the hypothesis that hemodynamic alterations might lead to tubulointerstitial hypoxia in the very early phase in this model, which might in turn induce tubulointerstitial damage and lead to eventual kidney failure.

To prove this hypothesis, we designed the current study to look for evidence of hypoxia within the first week after renal mass reduction. At this time point, hemodynamic alterations are well established but tubulointerstitial damage is negligible (18). Here, findings showed that many tubules suffer from hypoxic conditions in the early phase of remnant kidney. Furthermore, hypoxia in the tubulointerstitial compartment preceded traditional evidence of tubulointerstitial damage and persisted until the damage developed. Investigations utilizing angiotensin II receptor blockage (ARB) suggested that the tubular hypoxia was dependent on the activation of RAS.
Materials and Methods

Animals
Male Sprague-Dawley rats weighing 260 to 280 g were purchased from Nippon Seibutsu Zairyo Center Co. (Saitama, Japan). They were housed in an animal room maintained at a constant temperature with a 12-h light-dark cycle with free access to water and standard diet. When urine was collected, rats were housed in metabolic cages under the same conditions. BP was measured under conscious conditions using the volume-oscillometric method (Ueda, Tokyo, Japan). All studies conformed to the principles of the Guide for Animal Experimentation at the University of Tokyo.

Antibodies
Monoclonal mouse anti-human α-smooth muscle actin (α-SMA) 1:1000 (Boehringer Mannheim, Mannheim, Germany), mouse antipimonidazole 1:200 (Chemicon, Temecula, CA), mouse anti-rat vimentin 1:1000 (Dako, Glostrup, Denmark), biotinylated anti-mouse IgG 1:400 (Vector Laboratories, Burlingame, CA), and alkaline phosphatase-conjugated anti-mouse IgG 1:1000 (Promega, Madison, WI) were used in immunohistochemical studies. Monoclonal mouse anti-human HIF-1α 1:1000 (Novus Biologic, Littleton, CO) was used in immunoprecipitation studies.

Disease Model and Experimental Protocol
Eight rats served as a sham-operated control (sham group), and 36 underwent 5/6 nephrectomy as described previously (19). In brief, after right subcapsular nephrectomy, infarction of approximately two thirds of the left kidney was accomplished by ligation of the posterior and one or two anterior extrarenal branches of the main left renal artery. Sixteen of these animals received an ARB, olmesartan (Pharmaceutical, Tokyo, Japan), at a dosage of 10 mg/kg per d (20) by gastric gavage (RK+ARB group). The remaining animals were not treated with this reagent (RK group, n = 20). Treatment was started the day after operation and continued until the day of sacrifice. Eight rats each from the RK and RK+ARB groups were sacrificed at 4 and 7 d (early phase) after the operation and designated as the 4d RK, 7d RK, 4d RK+ARB, and 7d RK+ARB groups, respectively. Sham animals were sacrificed on day 7. The four remaining rats in the RK group were killed at 2 wk to examine the persistence of findings (extended RK). Twenty-four–hour urine and tail vein blood were collected before sacrifice. To detect hypoxia, 60 mg/kg of hypoxic probe, pimonidazole (Chemicon), was injected intraperitonely 2 h before sacrifice (21–23). To demonstrate functioning vessels, 250 μg of biotinylated lectin (Lycopersicon esculentum Lectin, Vector Laboratories) was injected via the tail vein exactly 4 min before sacrifice (24). To study the tissue perfusion properties of each vessel, 15 mg/kg of bisbezamine (Hoechst 33342; Sigma Chemical, St. Louis, MO) was injected via the inferior vena cava just below the left renal vein. Exactly 30 sec after injection, the renal pedicle was clamped and the kidney was promptly removed (25). To maintain the most representative perfusion patterns of vessels, all materials were intentionally injected without the use of a pressure pump. Kidney tissues for analysis were carefully selected to exclude necrotic areas. One sample of kidney tissue was fixed in methyl Carnoy’s fixative and then embedded in paraffin. All specimens were stored in light-protected containers. A second piece of the kidney was dissected in ice-cold PBS, and the medulla was removed, which was then snap-frozen in liquid nitrogen before transfer to storage at −80°C until further analysis.

To validate our methodology of Hoechst dye perfusion, 12 additional rats were studied as described below in Nuclear Fluorescence Studies of Tissue Perfusion Properties of this section. Three of these animals underwent renal artery stenosis according to a model described previously (17), three underwent 6-wk 5/6 nephrectomy, three served as a sham-operated control, and three were examined without administration of the Hoechst dye.

Double and Triple Immunohistochemical Analysis
Immunohistochemical staining was performed as described previously (26). Avidin HRP was used to stain biotinylated lectin-perfused vessels. Chromogenic color was developed with 3,3'diaminobenzidine tetrahydrochloride (DAB). After staining the first antigen, the remaining peroxidase activity was extinguished with 3% H2O2 in methanol for 10 min. The remaining biotin was blocked by incubation with avidin solution (Vector Laboratories), after which the first antibodies for the second antigen were applied, followed by suitable secondary antibodies (biotinylated or alkaline phosphatase-conjugated). Color was developed with DAB plus 0.8% nickel or Vector Red (Vector Laboratories). When alkaline phosphatase reaction was used, endogenous alkaline phosphatase was blocked with levamisole solution (Vector Laboratories). The processes were repeated for the third antigen staining. Negative controls without first antibodies were carefully examined for each reaction. All histologic slides were examined by light microscopy using an Olympus BX51 (Tokyo, Japan), and pictures were taken with the Olympus DP12 system. In some experiments, nuclei were counter-stained with methyl green (Vector Laboratories).

Nuclear Fluorescence Studies of Hoechst Dye to Investigate Tissue Perfusion Properties
For indirect comparison of tissue perfusion properties of Hoechst dye, 4-μm sections of paraffin-embedded tissues were stepwise rehydrated in a light-protected container. Rehydrated tissues were mounted and immediately photographed at 600× magnification (Eclipse E600, Nikon, Tokyo, Japan; connected to a cool charge-couple device [CCD], Micromax, Princeton Instruments, Trenton, NJ, and Metamorph, Downingtown, PA) avoiding any part of the glomeruli. In this step, the exposure time was fixed at 50 msec and the CCD temperature was set below −10°C.

Semiquantitative Scoring System
All scoring was done in a blinded manner. Photographs were examined in gray scale. Tubulointerstitial injuries in the cortex were scored in a blinded manner on 20 randomly selected non-overlapping 200× fields per rat on periodic acid-Schiff (PAS) and Trichrome Masson staining sections as described previously (11). Semiquantitative scores for pimonidazole adduction, α-SMA expression, and tubular vimentin expression were evaluated from 20 nonoverlapping fields at 200× magnification from each rat according to the following scoring method: 0, no involvement; 1, involvement of <10% of the cortex; 2, involvement of 10% to 25% of the cortex; 3, involvement of 26% to 50% of the cortex; 4, involvement of 51% to 75% of the cortex; and 5, involvement of >75% of the cortex.

The amount of postglomerular peritubular capillaries was evaluated using 10 randomly selected 400× pictures per rat (334.2 ± 55 lumina/animal) in a blinded manner with the Image J software (http://rsb.info.nih.gov/ij/).
Perfusion status with the nucleus-staining Hoechst dye was evaluated using Photoshop, version 7.0 (Adobe, San Jose, CA), as follows. Pictures were normalized by decreasing background signals of autofluorescence of tubular cell cytoplasm to undetectable levels (27,28). Nuclei were selected with the Wand tool of Photoshop, which automatically selects areas of nuclei. A mean log scale of luminosity of selected areas from each picture was then measured by the histogram function. Our preliminary studies of a renal artery stenosis model \((n = 3)\) showed almost undetectable parenchymal nuclear fluorescence. Mean nuclear luminosity of 6-wk remnant kidney animals \((n = 3)\) was 63 ± 8.6. Nuclear luminosity of sham-operated animals \((n = 3)\) by this method was 121.9 ± 4.3, and background luminosity of non-injected animals \((n = 3)\) was 0.34 ± 0.2.

Proliferation of endothelial cells was evaluated on double-staining of perfused lectin and PCNA by counting PCNA-positive nuclei per thousand visualized cortical capillary lumina from each rat.

**Immunoprecipitation of HIF-1α**

To confirm that remnant kidneys were hypoxic, 20 mg of protein of homogenized cortical tissues of each animal was subjected to protein A Sepharose immunoprecipitation (Amersham International, Buckingham, UK) according to the manufacturer’s protocol (29). The precipitated protein was separated by electrophoresis in 8% SDS-PAGE gel or 4 to 20% SDS-PAGE gradient gel (Daiichi Pure Chemical, Tokyo, Japan), followed by electrotransfer to PVDF membranes (Amersham International). Transfer membranes were blocked with 5% nonfat milk in TBS, 0.01% Tween-20, for 30 min at room temperature. The membranes were then blotted with anti-HIF1α antibody (Novus Biologic) at 1:100 at room temperature for 2 h. The bound antibodies were detected with alkaline phosphatase-conjugated secondary antibodies. 5-Bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablets (Sigma Fast; Sigma Chemical) were used as a chromogenic substrate.

**Total RNA Isolation and Real-Time RT-PCR**

Total RNA from cortical tissues from each rat was isolated with Isogen according to the manufacturer’s protocol (Wako Chemical, Osaka, Japan). cDNA synthesis was carried out with 2 µg of isolated RNA at 42°C for 1 h with an oligoDT Inpromptu-II kit (Promega). One microgram of cDNA was subjected to real-time PCR (iCycler iQ; Bio-Rad, Hercules, CA) utilizing Syber Green Supermix (Bio-Rad) with the correspondence primers as follows: β-actin: 5'-CTTTCTCAATAGAGGCTGCTG-3', 5'-TCATGAGGGTAGTCTGTCAGG-3'; erythropoietin (EPO): 5'-TACGTAACCTCAGTCCATCTGCTGTTCT-3', 5'-TGCAAGAAATTCGCGTCTGAGTTCC-3'; glucose transporter-1 (GLUT-1): 5'-CAGTTGGCGTAAAACCCCGTGTGTC-3', 5'-ATAGCGGCTGTTCCATGTGTTC-3'; and vascular endothelial growth factor (VEGF): 5'-TTACTGCTGTACCTCCAC-3', 5'-ACAGGACGCTTTGAAGATA-3' (30). The PCR was amplified for forty cycles. Each PCR product was subjected to melting curve analysis after PCR was completed. Threshold cycles for each primer were separately analyzed. The amount of target mRNA/β-actin mRNA were calculated according to the following equation: target mRNA/β-actin mRNA = 2^-ΔcT^-ΔcT where tc and bc are threshold cycles of the target gene and β-actin, respectively. Representative data are presented in terms of fold mRNA changes compared with sham.

**Statistical Analyses**

All numerical data are presented as mean ± SD. Distributions of some data are shown by histogram. One-way ANOVA followed by the least significant difference (LSD) method was used to determine differences among groups for all continuous parameters. The Mann-Whitney test was applied for noncontinuous parameters. Correlations between two parameters were analyzed with Pearson correlation test. The significance level was set at \(P < 0.05\) for all tests (SPSS for Windows 10.0).

**Results**

**Absence of Histologic Interstitial Injury in the Early Phase in the Remnant Kidney Model**

Remnant kidney animals (RK) showed a significant increase in urinary protein excretion, at 34.2 ± 8.7 and 51.5 ± 8.6 mg/d in the 4d RK and 7d RK rats, respectively, compared with 8.2 ± 0.5 mg/d in the control rats. ARB decreased urinary protein excretion to 20.3 ± 3.7 and 20.3 ± 4.5 mg/d in the 4d RK+ARB and 7d RK+ARB rats, respectively \((P < 0.05\) versus RK). BUN concentrations were 16.1 ± 1.2, 32.8 ± 9.1, 36.5 ± 11.7, 4. and 62.5 ± 6.7 mg/dl for sham, 4d RK, 7d RK, and Extended RK animals, respectively. BP was higher in RK animals than in the control. ARB treatment significantly decreased BP (Table 1).

Examination of PAS and Trichrome Masson samples in the early phase of remnant kidneys, at 4 and 7 d after operation, showed hypertrophic glomeruli and tubules. No significant interstitial changes were seen at this time point (Figure 1, a and e, b and f). In contrast, rats sacrificed at 2 wk showed some atrophic tubular cells, thickening of tubular basement membrane, and mild interstitial expansion (data not shown). Interstitial damage scores did not differ between sham and early RK groups (Table 2). The extended RK group had a higher interstitial damage score, although damage in this group was not prominent even at this time point. In the ARB-treated group, glomerular hypertrophy was less prominent.

**Table 1. Basic biological data**

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>4d RK</th>
<th>4d RK + ARB</th>
<th>7d RK</th>
<th>7d RK + ARB</th>
<th>Extended RK</th>
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</thead>
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<tr>
<td>Systolic BP (mmHg)</td>
<td>118 ± 6.3</td>
<td>146 ± 5.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>113 ± 5.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>166 ± 9.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>128 ± 7.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>187 ± 4.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>79 ± 12.9</td>
<td>105 ± 10.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>80 ± 12.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>120 ± 3.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>90 ± 15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>127 ± 3.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Blood urea nitrogen (mg/dl)</td>
<td>16.1 ± 1.2</td>
<td>32.8 ± 9.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27.6 ± 10.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>36.5 ± 11.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31.7 ± 5.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>62.5 ± 6.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>24-h urine protein (mg)</td>
<td>8.2 ± 0.5</td>
<td>34.2 ± 8.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.3 ± 3.7&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>51.5 ± 8.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.3 ± 4.5&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>86.5 ± 8&lt;sup&gt;a&lt;/sup&gt;</td>
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</tbody>
</table>

<sup>a</sup> \(P < 0.05\) versus sham.

<sup>b</sup> \(P < 0.05\) versus matched time point RK (ANOVA).
Development of Tubular Hypoxia in the Early Phase in the Remnant Kidney Model

In the sham group, pimonidazole, which serves as an indicator of hypoxic cells (21–23), was detected in medullary tubules (Figure 2, a and d), which as previously reported are normally in a state of borderline hypoxia (22,23). The number of pimonidazole-positive tubules in the cortex was markedly increased at 4 and 7 d after nephron loss (Figure 2, b and e). Semiquantitative scores of cortical pimonidazole uptake were 2.6 ± 0.4 and 3.1 ± 0.3 for 4d RK and 7d RK, respectively, while that of the control was 0.6 ±

Table 2. Semiquantitative scores of histological analysis

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>4d RK</th>
<th>4d RK + ARB</th>
<th>7d RK</th>
<th>7d RK + ARB</th>
<th>Extended RK</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PAS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tubular atrophy</td>
<td>0.3 ± 0.1</td>
<td>0.3 ± 0.2</td>
<td>0.3 ± 0.2</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>0.9 ± 0.2a</td>
</tr>
<tr>
<td>Interstitial infiltration</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>1.0 ± 0.1a</td>
</tr>
<tr>
<td><strong>Trichrome Masson</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interstitial expansion</td>
<td>0.1 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>1.3 ± 0.2a</td>
</tr>
<tr>
<td>Pimonidazole adduction</td>
<td>0.6 ± 0.2</td>
<td>2.6 ± 0.4b</td>
<td>0.9 ± 0.3b</td>
<td>3.1 ± 0.3a</td>
<td>1.4 ± 0.6b</td>
<td>3.0 ± 0.2a</td>
</tr>
<tr>
<td>Vimentin</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.4 ± 0.1a</td>
</tr>
<tr>
<td>α-SMA</td>
<td>0 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.2 ± 0.1</td>
</tr>
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</table>

a P < 0.05 versus sham.
b P < 0.05 versus matched time point RK (Mann-Whitney U test).

Figure 1. Histologic and immunohistochemical analysis of remnant kidneys in the early phase. Periodic acid-Schiff (PAS) and Trichrome Masson staining of normal kidneys (sham group) showed no evidence of tubulointerstitial damage (a and b). PAS and Trichrome Masson staining of the early phase of remnant kidneys (7d RK) (e and f) also showed no interstitial damage. Double-staining with perfused lectin (brown) and vimentin (black) showed that expression of vimentin in tubules could not be detected in either sham (c) or 7d RK (g). Immunohistochemical studies of α-smooth muscle actin (α-SMA) showed lack of interstitial myofibroblasts in sham (d) and 7d RK (h).
Increased pimonidazole uptake by remnant kidneys in the early phase. Double immunohistochemical staining with perfused lectin (brown) and a hypoxic probe, pimonidazole (gray) (×200). (a) No pimonidazole uptake was observed in the renal cortex in the sham group, while tubules in the deep medulla were pimonidazole-positive. (b) In the early phase after nephron loss (4 and 7 d), many tubules in the cortex showed pimonidazole uptake, indicating that these tubules were in a hypoxic condition. Treatment with an ARB, olmesartan, decreased pimonidazole uptake in the cortex of remnant kidneys at both time points (4 and 7 d), indicating that hypoxia of the tubulointerstitium may be mediated at least in part by activation of RAS. (c) In the extended phase (2 wk) of RK, rats showed sustained adduction of pimonidazole. (d) A high-magnification picture (×400) of sham animals showed the normal structure of perfused postglomerular capillary networks surrounded by tubules negative for pimonidazole. (e) In contrast, a high-magnification picture of the RK group showed pimonidazole adduction, indicating localized hypoxia.
Pimonidazole staining intensity was still high in the cortex in the extended phase at 2 wk after the renal mass reduction (Figure 2c). ARB treatment decreased the pimonidazole-positive score to 0.9 ± 0.3 and 1.4 ± 0.6 at 4 and 7 d, respectively (Figure 2b).

To confirm the development of hypoxia in the early phase, we investigated expression levels of HIF-1α protein (Figure 3a). No expression of HIF-1α was detectable in the cortex in the sham group. In contrast, increased HIF-1α expression in the cortex was observed in 7d RK animals, which was consistent with the results of the pimonidazole uptake studies. RT-PCR analysis of a series of hypoxia-responsive genes also supported the presence of hypoxia in the kidney in the early phase. EPO, GLUT-1, and VEGF were markedly increased at this time point in the remnant kidneys, and ARB attenuated these changes (Figure 3b).

**Tubulointerstitial Damage after Tubular Hypoxia**

To evaluate tubulointerstitial injury in detail, vimentin was employed as markers of tubular damage. α-SMA was employed to detect interstitial myofibroblasts, which are associated with interstitial damage and fibrosis. No difference between the sham and early remnant groups was seen (Figure 1, c, d, g, and h). In contrast, an increase in the number of vimentin-positive tubular cells as well as in α-SMA expression was seen in the extended phase (Table 2). These findings indicated that hypoxia during the early study period (within the first week) was unlikely to have been the result of tubulointerstitial damage.

**Association of Hypoxia with Decreased Perfusion of the Peritubular Capillary Beds in the Early Phase**

Because injected lectin binds to perfused endothelial cells under physiologic conditions, this method allows evaluation of both the amount of and morphologic changes in functioning postglomerular peritubular capillaries. The number of peritubular capillaries per 100 tubules and rarefaction index in 4d RK and 7d RK did not differ from that in the sham rats (Table 3). In contrast, the number of peritubular capillaries in the ex-

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**Figure 3.** Upregulation of hypoxia-responsive proteins in remnant kidneys. (a) Immunoprecipitation of HIF-1α using cortical tissues of sham and remnant kidney rats. HIF-1α was undetectable in sham kidneys, but was increased in remnant kidneys. (b) Transcription of hypoxia-responsive genes. Quantitative RT-PCR of the renal cortex of sham and remnant kidneys showed that all genes, including EPO, GLUT-1, and VEGF, were increased in remnant kidneys. Olmesartan-treated animals showed a decrease in all hypoxia-responsive genes. A, *P* < 0.05 *versus* sham; B, *P* < 0.05 *versus* RK.
tended RK clearly decreased, to 137.8 ± 12.5 per 100 tubules, with rarefaction of 12 ± 3.7% (P < 0.05 versus sham). On this basis, hypoxia in the early phase could not be explained by any structural loss of peritubular capillary beds, in contrast to the extended phase, when histologic loss of peritubular capillaries was established. Notwithstanding this, significant morphologic changes in peritubular capillaries in the early stage were observed. In normal kidneys, peritubular capillaries are elliptical, circular, or triangular in cross-section, which preserves the patency of the lumina (Figure 4a). In contrast, peritubular capillaries in remnant kidney animals showed a distorted outline with relatively narrow capillary lumina (Figure 4b). Analysis of cross-sectional capillaries showed that the mean of capillary lumina area in remnant kidney animals was significantly lower than that of the control rats (Table 3). The histogram demonstrated that remnant kidney animals harbored abundant narrow capillaries (Figure 4d). Double immunostaining analysis using pimonidazole and lectin showed co-localization of narrow capillaries and hypoxic tubules (Figure 2e). This result was supported by correlation analysis (Figure 4e). Furthermore, ARB treatment preserved the structural integrity of postglomerular capillaries as well as luminal patency (Figure 4c and Table 3). These data suggested that the abnormality in peritubular capillaries might have been mediated at least in part by the activation of RAS.

Decreased Tissue Perfusion in Remnant Kidneys Was Observed by Administration of Hoechst 33342

When injected, the nuclear dye Hoechst 33342 rapidly diffuses from capillaries and is taken up by nuclei of the surrounding tissues. The nuclear fluorescence signals of Hoechst 33342 in a short perfusion time thus indirectly represent tissue perfusion states, which are determined by a number of factors, including capillary density, capillary permeability, and total blood flow of capillary networks. Uptake of Hoechst 33342 in glomeruli appeared to be higher than that of tubules in both nephrectomized and sham rats. The remnant kidney groups showed less and weaker nuclear fluorescence signals in the tubulointerstitial area compared with the control animals, further suggesting the poor tissue perfusion in this area (Figure 5, a and b; Table 3). Again, ARB treatment restored fluorescence signals of nuclei in tubular cells (Figure 5c; Table 3), indicating the role of RAS activation in the poor perfusion of peritubular capillaries.

Endothelial Cell Proliferation Occurred in Hypoxic Areas

An increase was seen in the number of PCNA-positive endothelial cells at day 7 after nephron loss (Figure 6, b and f). In contrast, PCNA-positive endothelial cells were hardly detected in the control (61.7 ± 14.7 versus 3 ± 2 per 1000 visualized capillary lumina; P < 0.05) (Figure 6, a and f). The number of PCNA-positive endothelial cells in the extended phase was slightly decreased to 51.5 ± 7.7 per 1000 visualized capillary lumina, although this value was still higher than that in the sham-operated animals (Figure 6f). ARB significantly decreased PCNA-positive endothelial cells at Day 7 of nephron ablation to 18.0 ± 3 per 1000 visualized capillary lumina (P < 0.05 versus 7d RK) (Figure 6, c and f). Triple immunostaining with lectin, pimonidazole, and PCNA indicated that endothelial proliferation occurred in the hypoxic area (Figure 6, d, e, and g). These findings suggested that endothelial proliferation may be one path to neoangiogenesis as a response to hypoxic stimuli.

Discussion

This current study demonstrates for the first time that perfusion is decreased in peritubular capillaries in the early phase of remnant kidney models, and that this decrease is associated with hypoxia in the tubulointerstitium. The hypoxic milieu in the tubulointerstitium precedes any pathologic changes in the corresponding region, suggesting a pathogenic role of ischemia at early time points.

We intended to perform this study at the early phase after nephron loss at an equivalent time point to that used in the landmark study of Hostetter and Brenner (1), when tubulointerstitial injury is negligible despite significant changes in renal hemodynamics. On this basis, our results were accompanied by glomerular hyperfiltration but were unlikely to be the result of tubulointerstitial damage. Increased cellular adduction of pimonidazole in remnant kidney tubules suggested that these tubular cells were hypoxic. The mechanism of cellular adduction of pimonidazole specifically depends on a hypoxia-induced redox state, and previous studies have demonstrated a good correlation between adduction and tissue O2 tension (31). However, to exclude the possibility that hypertrophic tubules might adduct pimonidazole without hypoxia via unidentified mechanisms, we measured HIF-1α expression.

HIF-1α is the master hypoxia response regulator, the amount of which is determined by oxygen-dependent degradation (32). Rosenberger et al. (33) found no detectable expression of HIF-1α in the normal kidney cortex. Here, we demonstrated an increased amount of this protein in the renal cortex in remnant kidneys, confirming that the remnant kidney milieu was indeed hypoxic. We also measured changes in mRNA levels of hypoxia-responsive genes, including EPO, GLUT-1, and VEGF. All these genes contain hypoxia-response elements (HRE) in their cis-regulatory promoter regions (30) and increase their transcription in the presence of hypoxia. Although the possibility of non-hypoxic transcriptional regulation of HIF-1 cannot be excluded (34), the upregulation of HIF and these hypoxia-responsive genes, together with the results using a hypoxia marker and the evaluation of tissue perfusion status, all point to the establishment of local hypoxia in our studies.

Previous studies suggest that kidney injury in the early phase is dependent on hemodynamic changes, whereas tubulointerstitial damage in the late phase is mediated by massive proteinuria (35). Development of tubulointerstitial hypoxia in the very early phase suggests its possible pathogenic role in tubulointerstitial injury at this stage. Basile et al. (22) reported that kidneys did not fully recover after severe ischemic acute renal failure and that this lack of recovery was associated with the loss of peritubular capillaries and an increase in tubular pimonidazole adduction. Suga et al. (23) also showed increased
Figure 4. Change in pattern of postglomerular peritubular capillaries in remnant kidneys. Immunohistochemical studies were conducted on binding of injected lectin to endothelial cells of functioning capillaries. (a) In sham-operated animals, peritubular capillaries were arranged in a regular pattern with patent capillary lumina. (b) In early remnant kidneys (RK) (representative picture from 4d RK), most capillary lumina were distorted and had relatively narrow lumens. (c) ARB treatment restored the normal capillary morphology (representative picture from 7d RK+ARB). (d) Histogram of luminal area demonstrated that remnant kidneys harbored more narrow peritubular capillaries. Treatment with ARB prevented this change. (e) Correlation between mean capillary luminal area and pimonidazole score in each animal showed that narrowing of capillaries correlated with hypoxia.
adduction of pimonidazole in a hypokalemic nephropathy model with tubulointerstitial injury. Using BOLD MRI, Ries et al. (36) reported hypoxia in the renal cortex of diabetic rats in the early hyperfiltration phase. Recent studies by Johnson et al. (37) demonstrated that chronic systemic hypoxia causes renal interstitial damage. Previous work by Truong group (38) and our group (17) on kidneys subject to renal artery stenosis also supported a role of hypoxia in tubulointerstitial injury. In contrast to these previous and our present findings, Priyadarshi et al. (39) performed direct measurement of tissue oxygen tensions by microelectrode in the late phase of a remnant kidney model and reported a relative increase in tissue oxygen tension. The reason for this discrepancy is unclear, but the microelectrode method may have detected regional hyperoxia. An alternative explanation may be the difference in study’s time point.

The finding of tubular hypoxia in the early phase stimulated us to investigate the responsible mechanisms. Intravenous in-

<table>
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<th></th>
<th>Sham</th>
<th>4d RK</th>
<th>4d RK + ARB</th>
<th>7d RK</th>
<th>7d RK + ARB</th>
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<tbody>
<tr>
<td>Capillary lumina/100 tubules</td>
<td>187.3 ± 8.9</td>
<td>180.9 ± 16.9</td>
<td>181.9 ± 11.1</td>
<td>180.2 ± 12.5</td>
<td>188.8 ± 15.9</td>
</tr>
<tr>
<td>Capillary rarefaction index (%)</td>
<td>1.1 ± 0.8</td>
<td>2.3 ± 1.5</td>
<td>1.1 ± 1.1</td>
<td>2.8 ± 1.2</td>
<td>2.8 ± 1.8</td>
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<tr>
<td>Luminal area (microns²)</td>
<td>84.2 ± 0.6</td>
<td>63.3 ± 7.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>73.7 ± 7.6&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>57.7 ± 8.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>73.5 ± 5.7&lt;sup&gt;a,b&lt;/sup&gt;</td>
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<tr>
<td>Nuclear fluorescent signal density</td>
<td>121.9 ± 4.3</td>
<td>88.8 ± 3.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>106.5 ± 8.9&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>82.5 ± 5.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>104.3 ± 10.5&lt;sup&gt;a,b&lt;/sup&gt;</td>
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<sup>a</sup> P < 0.05 versus sham.
<sup>b</sup> P < 0.05 versus matched time point RK (ANOVA).

Figure 5. Tissue perfusion studies with Hoechst 33342 dye. Tissue perfusion status was assessed by injection of Hoechst 33342 dye exactly 30 s before removal of the kidney. In sham animals, nuclear fluorescence was intense in every area (a), whereas nuclear fluorescence signals in the RK group were distinctly decreased in the tubulointerstitial area (b). Treatment with ARB increased nuclear fluorescence (c), indicating that ARB restored net perfusion in remnant kidneys. Semiquantitative measurement of nuclear luminosity supported these results (Table 3).
Injection of biotinylated lectin under physiologic conditions, which allows evaluation of the amount and structure of functioning capillaries (24), showed no changes in the number of capillaries per tubule or vascular rarefaction at our early time points, but it did reveal striking morphologic changes in the capillary network pattern. Many peritubular capillaries in remnant kidney animals were distorted and collapsed, showing a streak-like appearance. Quantitative analysis showed that remnant kidney harbored a large number of narrow peritubular capillaries. Furthermore, correlation analysis showed that the degree of capillary narrowing was related to that of pimonidazole adduction. Of interest, the narrowing of peritubular capillaries had been previously reported in several human diseases (6,40). It should be emphasized that the flow in individual capillary beds is dynamic but under the tight regulation of an active feedback system and that the diameter of a vessel is the primary determinant of its flow (41); we therefore speculate that these peritubular capillary changes may be related to poor tissue perfusion, leading to hypoxia. A second experiment provided indirect support for this speculation; on measurement of tissue perfusion by injection of nuclear dye, a well-established method in oncology research (25,27,28,42), remnant kidney animals showed a decrease in tubulointerstitial nuclear fluorescence signals.

Neoangiogenesis is a common response to chronic hypoxia. Kang et al. (11) and Pillebout et al. (43) reported transient endothelial proliferation, which may be considered as a component of neoangiogenesis, in a remnant kidney model while Ohashi et al. (7) observed this phenomenon in a unilateral ureteral obstruction (UUO) model. A recent study by Rosen-
berger et al. (44) suggested that hypoxia might stimulate peri-
tubular capillary endothelial proliferation. Our results also re-
vealed that endothelial proliferation co-localized with areas of 
hypoxia, suggesting that endothelial proliferation (in this 
model) might be another response to hypoxia.

Activation of RAS is the earliest humoral response after 
nephron loss (45), and several clinical studies have shown that 
angiotensin-converting enzyme inhibitors (ACEI) and ARB 
effectively attenuate the progression of kidney disease (46,47).
Our results revealed that ARB attenuated pimonidazole adduc-
tion in tubular cells and preserved peritubular capillary net-
work structure. The expression of hypoxia-responsive genes 
was also decreased in ARB-treated animals, suggesting that 
tubulointerstitial hypoxia in remnant kidneys is mediated by 
the activation of RAS, possibly via their vasconstrictive ef-
fects on efferent arterioles. This speculation is supported by 
recent findings by Norman et al. (48), who showed that treat-
ment with ACEI and ARB increased cortical tissue \( \text{O}_{2} \) tension 
and proposed that this might be another renoprotective role of 
these drugs. In addition, Fujimoto et al. (49) reported that 
chronic angiotensin infusion resulted in a decrease in peri-
tubular blood flow, which was accompanied by adduction of 
pimonidazole. Taken together, these and the present results 
emphasize the role of RAS activation in the disturbance of 
tubulointerstitial microcirculation and subsequent hypoxia.
Moreover, they support the notion that improvement of cortical 
tissue oxygenation might be another renoprotective property of 
ARB and ACEI.

In conclusion, we have demonstrated that the hypoxic milieu 
in the tubulointerstitium precedes any pathologic change in 
the corresponding region, suggesting a pathogenic role of ischemia 
at early time points. Our results suggested that perfusion was 
decreased in peritubular capillaries in association with hypoxia 
in the tubulointerstitium in the early phase of remnant kidney 
models, and these changes may be mediated by the activation of 
RAS.

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References

1. Hostetter TH, Olson JL, Rennke HG, Venkatachalam MA, Bren-
nier BM: Hyperfiltration in remnant nephrons: a potential adverse 
2. Nath KA: Tubulointerstitial changes as a major determinant in 
3. Remuzzi G, Bertani T: Pathophysiology of progressive nephrop-
5. Fine LG, Bandyopadhyay D, Norman JT: Is there a common 
mechanism for the progression of different types of renal disease 
other than proteinuria? Towards the unifying theme of chronic 
postglomerular capillaries in the pathogenesis of chronic renal 
Sugisaki Y, Yamanaka N: Peritubular capillary regression during 
the progression of experimental obstructive nephropathy. J Am 
during the progression of experimental glomerulonephritis in 
kawa K, Yanagisawa M, Fujita T, Johnson RJ: ET(B) receptor 
protects the tubulointerstitium in experimental thrombotic mi-
JA, Gordon KL, Oyama TT, Hughes J, Hugo C, Kerjaschki D, 
Schreiner GF, Johnson RJ: Impaired angiogenesis in the aging 
kidney: vascular endothelial growth factor and throm-
2001
11. Kang DH, Joly AH, Oh SW, Hugo C, Kerjaschki D, Gordon KL, 
Mazzalli M, Jefferson JA, Hughes J, Madsen KM, Schreiner GF, 
Johnson RJ: Impaired angiogenesis in the remnant kidney model: 
I. Potential role of vascular endothelial growth factor and throm-
12. Norman JT, Clark IM, Garcia P: Hypoxia promotes fibrogenesis 
13. Sodhi CP, Phadke SA, Batlle D, Sahai A: Hypoxia and high 
glucose cause exaggerated mesangial cell growth and collagen 
synthesis: Role of osteopontin. Am J Physiol Renal Physiol 280: 
F667–F674, 2001
14. Orphanides C, Fine LG, Norman JT: Hypoxia stimulates prox-
imal tubular cell matrix production via TGF-β1 independent 
15. Tanaka T, Hanafusa N, Ingelfinger JR, Ohse T, Fujita T, Nang-
akau M: Hypoxia induces apoptosis in SV40-immortalized rat 
proximal tubular cells through the mitochondrial pathways, de-
void of HIF1-mediated upregulation of Bax. Biochem Biophys 
Nangaku M: Hypoxia-induced apoptosis in cultured glomerular 
endothelial cells: Involvement of mitochondrial pathways. Kid-
T, Kurokawa T, Fujita T, Ingelfinger JR, Nangaku M: Transdif-
ferentiation of cultured tubular cell induced by hypoxia. Kidney 
Int 65: 871–880, 2004
18. Johnson TS, Griffin M, Thomas GL, Skill J, Cox A, Yang B, 
Nicholas B, Birckbichler PJ, Muchaneta-Kubara C, El Nahas 
AM: The role of transglutaminase in the rat subtotal nephrec-
19. Kliem V, Johnson RJ, Alpers CE, Yoshimura A, Couser WG, 
Koch KM, Fleoge J: Mechanisms involved in the pathogenesis of 
tubulointerstitial fibrosis in 5/6-nephrectomized rats. Kidney Int 
49: 666–678, 1996
Fujita T: Imbalance of T-cell subsets in angiotensin II-infused


