Hypoperfusion of Peritubular Capillaries Induces Chronic Hypoxia before Progression of Tubulointerstitial Injury in a Progressive Model of Rat Glomerulonephritis

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Abstract. Chronic hypoxia likely plays a pivotal role in chronic renal disease, but the specifics of its involvement remain unclear. To elucidate how chronic hypoxia occurs and whether hypoxia participates in the progression of renal disease, the authors established an irreversible glomerulonephritis model induced by uninephrectomy and repeated anti-Thy-1 antibody injections. Glomerulosclerosis with microvascular obliteration was complete at 2 wk after antibody injection and was not restored until 11 wk. Tubulointerstitial injury was mild at 2 wk and was gradually exacerbated until 11 wk, a pattern that was in accordance with the loss of peritubular capillaries. Immunohistochemical analysis using pimonidazole revealed the augmentation of hypoxia in the cortex before the aggravation of tubulointerstitial injury and subsequent peritubular capillary loss. The preexistence of hypoxia implies that it had substantial participation in the progression of tubulointerstitial injury. To test whether blood flow was inhibited in diseased kidneys, capillaries with intact blood flow were identified by tail vein injection of biotinylated lectin specific to endothelial cells. The renal microvasculature was well recognized by lectin in the controls, whereas lectin binding to peritubular capillaries was strikingly decreased in diseased kidneys, suggesting a disturbance of blood flow. Intravital microscopy analysis confirmed that blood flow in peritubular capillaries was decreased by approximately 40% in the disease group compared with the controls. In conclusion, stagnation of blood flow in peritubular capillaries induced chronic hypoxia at an early stage in this model, which was followed by progressive tubulointerstitial injury and a loss of peritubular capillaries.

Considerable evidence from both clinical and experimental studies indicates that the progressive loss of renal function that occurs in various glomerular diseases correlates better with structural damage in the renal tubulointerstitium than with that in the glomeruli (1–3). Following from this, tubulointerstitial injury is considered a final common pathway to end-stage kidney disease. A number of findings have suggested a link between heavy proteinuria and subsequent tubulointerstitial injury; more recent reports, however, have illuminated the role of chronic hypoxia in the cortex before the aggravation of tubulointerstitial injury and subsequent peritubular capillary loss. The preexistence of hypoxia implies that it had substantial participation in the progression of tubulointerstitial injury. To test whether blood flow was inhibited in diseased kidneys, capillaries with intact blood flow were identified by tail vein injection of biotinylated lectin specific to endothelial cells. The renal microvasculature was well recognized by lectin in the controls, whereas lectin binding to peritubular capillaries was strikingly decreased in diseased kidneys, suggesting a disturbance of blood flow. Intravital microscopy analysis confirmed that blood flow in peritubular capillaries was decreased by approximately 40% in the disease group compared with the controls. In conclusion, stagnation of blood flow in peritubular capillaries induced chronic hypoxia at an early stage in this model, which was followed by progressive tubulointerstitial injury and a loss of peritubular capillaries.

Renal chronic hypoxia may occur as a consequence of impairment of blood flow or oxygen delivery (or both) to the tubulointerstitium. Recent studies have shown a correlation between a decline in the density of the renal microvasculature and the development of glomerular and tubulointerstitial injury in several kidney disease models (9–14) and progressive renal diseases in humans (15). These studies suggest that insufficient oxygenation resulting from peritubular capillary loss pivotal role in the pathogenesis of renal diseases, although the mechanism by which deterioration in renal function ensues from hypoxia remains unknown.

Glomerulosclerosis, a landmark feature of renal pathophysiology, is also thought to impair peritubular blood flow and thus oxygen supply. Anti-Thy-1 nephritis is a model of reversible mesangial proliferative glomerulonephritis (16). Previous reports showed exacerbation of this model by various boosters such as uninephrectomy or repeated injection of pathogenic antibodies to result in more severe damage and irreversible glomerular sclerosis (12,17,18). We induced an irreversible progressive model of anti-Thy1 nephritis by repeated anti-Thy-1 antibody injection after uninephrectomy in rats. This characteristic makes this model valuable in determining the relationship among impeded blood flow due to glomerulosclerosis, renal hypoxia, and progressive tubulointerstitial injury.
To confirm the existence of tubulointerstitial hypoxia in association with glomerular damage and to examine how hypoxia occurs, we clarified the mechanisms of hypoxia in uninephrectomized anti-Thy-1 nephritis. Furthermore, as a first step in addressing whether hypoxia accelerates renal disease, we also analyzed the sequence of events from tubular hypoxia in the early stage to severe tubulointerstitial injury in the late stage.

Materials and Methods

Experimental Protocol

All experiments were conducted in accordance with the Guide for Animal Experimentation, Faculty of Medicine University of Tokyo, Japan. Six-week-old male SD rats (Nippon Seibutsu Zairyo Center Co., Ltd., Saitama, Japan) weighing 160 to 200 g received repeated intravenous injection of IgG (OX-7) mouse monoclonal anti-Thy-1 antibody (1.2 mg/kg body wt) or vehicle at 1 and 2 wk (weeks −1 and 0) after right nephrectomy (week −2). Rats were housed in metabolic cages for overnight collection of urine, and blood samples were obtained via the tail vein for determination of renal functions. A sham operation, consisting of laparotomy and manipulation of the right renal pedicle without nephrectomy, was performed as a control.

In the first set of experiments, we analyzed physiologic and histologic changes in nephrectomized anti-Thy-1 rats (NX Thy-1; n = 13), nephrectomized control rats (NX; n = 13), and sham-operated rats (sham; n = 11). For tissue analyses in the early phase of the disease, animals of each group were randomly selected (n = 6) and sacrificed at 2 wk after the final OX-7 injection (week 0). This time point was chosen because obvious glomerular damage and mild tubulointerstitial injury were observed in our preliminary studies. Two rats each from the NX and NX Thy-1 groups were sacrificed at week 1 to evaluate oxygenation of the kidney at an earlier time point, as described below. The remaining rats (n = 5) were sacrificed at week 11 to evaluate histologic changes in the late phase. No rats died during the experimental period. Physiologic and histologic data of the sham group were closely similar to those of the NX group (data not shown).

The second set of experiments investigated peritubular capillary blood flow in 10 NX Thy-1, 10 NX and five sham rats. Physiologic lectin perfusion was done by utilizing NX Thy-1, NX, and sham rats (n = 5 for each group). A separate set of experiments was performed to study NX Thy-1 and NX rats (n = 5 for each group) by intravital microscopy as described below. Physiologic and histologic data of animals of the second set were equivalent to those of the rats of the first.

Renal Histology Analysis

Tissues were fixed in methyl Carnoy’s solution and paraffin-embedded. Three-micrometer sections were stained with periodic acid-Schiff (PAS) and counterstained with hematoxylin. An indirect immunoperoxidase method was used to identify the following antigens: aminopeptidase P of microvascular endothelial cells with murine monoclonal IgG1 antibody JG-12 (19) (Bender MedSystems, San Bruno, CA); monocytes/macrophages with murine monoclonal IgG antibody ED-1 (Chemicon, Temecula, CA); vimentin with murine monoclonal IgG antibody V9 (Dako, Carpinteria, CA); and thiol-binding pimonidazole derivatives with murine monoclonal pimonidazole antibody (Chemicon).

Semiquantitative Analysis of Renal Histology Features

Quantification was performed in a blinded manner using 30 randomly selected glomeruli or more than 15 randomly selected fields of cortex per cross section. Glomerulosclerosis, defined as synchiae formation by PAS staining with focal or global obliteration of capillary loops, was graded as follows: 0, normal; 1, 0% to 25% of glomerular area affected; 2, 25% to 50% affected; 3, 50% to 75% affected; and 4, 75% to 100% affected (20). Tubulointerstitial injury was graded (0 to 5+) on the basis of the percentage of tubular cellularity, basement membrane thickening, cell infiltration, dilation, atrophy, sloughing, or interstitial widening as follows: 0, no change; 1, <10% tubulointerstitial injury; 2, 10% to 25% injury; 3, 25% to 50% injury; 4, 50% to 75% injury; and 5, 75% to 100% injury (21,22). Tubules that were vimentin-positive or were surrounded by vimentin-positive cells and ED-1–positive cells were counted in 20 randomly selected cortical fields with a ×20 objective.

Semi quantitative Analysis of Glomerular and Peritubular Capillary Loss

Glomerular or peritubular capillary loss was assessed by immunostaining for renal microvascular endothelium with JG-12 antibody. Loss of glomerular capillary loops was graded as follows: 0, no negative glomerular tuft staining for endothelium; 1%, 1% to 25% of glomerular tufts negative for endothelium; 2, 25% to 50% negative; 3, 50% to 75% negative; and 4, 75% to 100% negative (23). Peritubular capillary loss was analyzed using a previously reported rarefaction index of peritubular capillary sparseness, which was calculated as the percentage area with no capillaries identified with JG-12 antibody (9).

Briefly, this index was determined by counting the number of squares in 10 × 10 grids that did not contain JG-12–positive peritubular capillary staining in at least 10 non-overlapping sequential fields, at ×200 magnification. The minimum possible capillary rarefaction index is 0, i.e., every square in the grid contains a JG-12–positive peritubular capillary, whereas the maximal score is 100, i.e., JG-12–positive peritubular capillaries are absent from every square in the grid.

Assessment of Renal Hypoxia

Nitroimidazole compounds, which bind to thiols of cellular macromolecules at low oxygen concentrations, have been used to detect hypoxia (<10 mmHg) in a variety of tissues (24). In this study, pimonidazole (Chemicon) was used to detect renal hypoxia as previously reported (8,25,26). To investigate the existence of renal hypoxia, animals at week 2 (n = 3, 6, and 6 of sham, NX, and NX Thy-1 groups from the first set of experiments, respectively) were injected with pimonidazole (60 mg/kg) via the tail vein. Two hours after administration of pimonidazole, the kidneys were excised and fixed for subsequent histologic studies. Pimonidazole binding was detected by immunohistochemistry with a specific antibody as described above. For semiquantitative analysis of renal hypoxia, the number of pimonidazole-positive tubules per field was counted in more than ten randomly selected cortical areas per cross section. To verify the presence of renal hypoxia at an earlier time point, nephrectomized Thy-1 rats (n = 2) and nephrectomized control rats (n = 2) were injected at week 1 with pimonidazole, followed by sacrifice and tissue processing in the same manner as described above.

Evaluation of Microvasculature with Intact Circulation by Lectin Perfusion

To identify vessels with intact blood flow, renal vasculature was identified with lectin that binds uniformly to the luminal surface of endothelial cells as described previously (27). Animals at week 2 were intravenously injected with biotinylated Lycopersicon esculentum lectin (Vector Laboratories, Burlingame, CA) at a dose of 250 μg/
animal. After 2 min, the renal vasculature was perfused with phosphate-buffered saline to wash out residual nonbinding lectin for 3 min at a pressure of 120 mmHg via a blunt 22-gauge needle inserted into the abdominal aorta just below the renal artery. The left kidney was dissected, fixed, and paraffin-embedded for immunohistochemical analysis by indirect immunoperoxidase methods.

Measurement of BUN and Urinary Protein Excretion
BUN level was determined colorimetrically with a commercial kit that employed the urease-indophenol method (Wako Pure Chemical Industries, Tokyo, Japan). Urinary protein excretion was measured using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA).

Intravital Microscopy and Analysis of Peritubular Blood Flow
Animals at week 2 were anesthetized with ketamine hydrochloride (50 mg/kg). Peritubular capillaries were visualized with a pencil-lens probe, a charge-coupled videomicroscope device with a tip diameter of 1 mm, as described previously (28). The probe had a magnification of ×520, depth of field <60 μm, and spatial resolution of 0.86 μm, permitting identification of individual erythrocytes. Illumination was provided by a concentric set of optical fibers transmitting light from a xenon AC 100-V light source. Video signals were digitized with an analog-to-digital converter and fed into a digital videocassette recorder DVCAM (Sony, Tokyo, Japan) interfaced with a computer. Analysis was performed using the NIH Image program combined with Matlab or specifically written programs. Peritubular capillary blood flow was recorded with a pencil-lens microscope brought into direct contact with the decapsulated renal surface. Consecutive images of blood flow were collected at a rate of 30fps for 60 min. Images were analyzed using the freeze-frame mode. The velocity of red blood cells (RBC) in individual segments of the peritubular capillaries was analyzed using a specifically designed adaptation of a previously developed algorithm. Specifically, a line segment was set along a capillary bed in sequentially videotaped images, and a spatiotemporal image was constructed (the line-shift method), allowing us to discern differences in gray level during the passage of RBC. The angle of a line-shift striped pattern was estimated to compute the erythrocyte velocity vector.

Statistical Analyses
All data are reported as mean ± SE. Statistical analyses were performed using the t test. Nonparametric data were analyzed with the Mann-Whitney test when appropriate. Differences with P values < 0.05 were considered significant.

Results
Renal Function of Uninephrectomized Anti-Thy-1 Nephritis Rats
Urinary protein excretion in the disease group at week 1 was significantly higher than that in the NX group and continued to increase gradually until the end of the study. BUN levels showed biphasic increases, as shown in Figure 1. * P < 0.05. **P < 0.01.

Development of Irreversible Glomerulosclerosis in Uninephrectomized Anti-Thy-1 Nephritis Rats Associated with Glomerular Capillary Destruction
Glomerulosclerosis occurred in the NX Thy-1 group at week 2, and the degree of the injury remained severe up to week 11, as confirmed by semiquantitative analysis (Figure 2 and Table 1). Almost all glomeruli were damaged to various degrees, with 50% of those investigated classified as grade 4 as early as at week 2 (grade 0, 2.9 ± 0.9%; grade 1, 10.0 ± 3.8%; grade 2, 12.8 ± 3.6%; grade 3, 24.3 ± 4.0%; grade 4, 50.0 ± 7.4%). Glomerular capillaries identified with JG-12 antibody were disrupted in association with glomerulosclerosis at both weeks 2 and 11 (Table 1).

Aggravation of Tubulointerstitial Injury from Week 2 until the End of the Study
Mild diffuse tubulointerstitial damage was observed in NX Thy-1 at week 2, and these lesions were aggravated at week 11 (Figure 3 and Table 1). Expression of vimentin as a marker of tubulointerstitial injury (22,29) was increased in diseased kidneys at weeks 2 and 11. Semiquantitative analysis showed that the tubulointerstitial injury in the NX Thy-1 group tended to progress from week 2 to week 11, although the difference did...
The number of infiltrating macrophages in the cortical tubulointerstitium was higher in the diseased group than in the controls. Macrophage infiltration in the cortex was significantly and gradually increased in the NX Thy-1 group from weeks 2 to 11 (Table 1).

Association of Peritubular Capillary Loss with Tubulointerstitial Injury

With regard to peritubular capillary density, an important factor in tubulointerstitial injury, the rarefaction index score was slightly but significantly higher in NX Thy-1 than NX group rats at 2 wk. Simultaneous scoring of tubulointerstitial injury and rarefaction index in individual subject fields at 2 wk showed a correlation between tubulointerstitial injury and damage to the microvasculature in the corresponding region. Cortical areas with no peritubular capillaries showed further expansion at week 11 compared with those at week 2 (Figure 5 and Table 1).

Exposure of Renal Tubules to a Hypoxic Environment before Progression of Tubulointerstitial Injury

To verify tubular hypoxia is present before the progression of tubulointerstitial injury, renal local oxygen tensions were evaluated at week 2 utilizing pimonidazole, which is incorporated into hypoxic cells and serves as a hypoxic marker. In the sham and NX groups, pimonidazole incorporation in tubular epithelial cells occurred mainly in the medulla and medullary rays, where borderline hypoxia is present even under physiologic conditions (30). Sham operation did not change the staining pattern of pimonidazole. In the NX Thy-1 group, in contrast, pimonidazole staining was widely distributed from the medulla to the outer cortex, and the number of tubules with pimonidazole uptake was higher than in the NX or sham animals (percentage of hypoxic tubules against total tubules per cortical field: 58.5 ± 5.45%, 26.2 ± 6.34%, and 18.0 ± 5.58%, respectively; NX Thy-1 versus NX; P < 0.01) (Figure 6). Additional experiments at week 1 showed that renal cortical hypoxia was already augmented at a very early time point (NX Thy-1, 43.3 ± 1.74; NX, 29.1 ± 0.04%).

Decrease in Lectin Binding to Peritubular Capillary Endothelial Cells in the Early Stage of Disease

While hypoxic tubules were located over a wide range in the cortex, peritubular capillary loss around damaged tubules was limited, as described above. This observation prompted us to test the possibility that a disturbance in blood flow in tubulointerstitial compartments might cause tubular hypoxia. For this purpose, we identified capillary endothelium by physiologic perfusion with biotinylated *lycopersicon esculentum* lectin, which is specific for N-acetyl-D-glucosamine oligomers of vessel luminal surfaces. Lectin bound to all endothelial cells of both glomerular and peritubular capillaries in the NX group in a more diffuse and intense manner than in the sham group. However, significantly less binding of lectin to capillaries was seen in the NX Thy-1 group than in either the NX and sham groups (rarefaction index score of lectin binding capillaries: sham, 16.4 ± 2.5%; NX, 6.8 ± 1.9%; NX Thy-1, 31.3 ± 3.2%; NX Thy-1 versus sham, P < 0.01). Furthermore, the number of endothelial cells identified by lectin perfusion was less than that identified by JG12 in the disease group at the same time point (rarefaction index score of lectin-binding capillaries: 31.3 ± 3.2%; that of staining with JG-12, 12.1 ± 0.40%; P < 0.01, NX Thy-1 group at week 2). Double staining with lectin and pimonidazole showed that low oxygenation co-localized with poor lectin perfusion in diseased kidneys (Figure 7).

Direct Demonstration of a Decline in Blood Flow in Peritubular Capillaries of Uninephrectomized Anti-Thy-1 Nephritis Rats by Intravital Microscopy

Our findings on the hypoperfusion of lectin in diseased kidneys suggested that blood flow stagnates in this renal disease model. To test this hypothesis, RBC velocity was directly measured by intravital videomicroscopy. RBC velocity in peritubular capillaries of the NX group averaged 0.476 mm/s, whereas that in the NX Thy-1 group decreased to 0.296 mm/s, 0.047 mm/s, whereas that in the NX Thy-1 group decreased to 0.296 mm/s (NX Thy-1 versus NX group, P < 0.05). In some capillaries, flow switched from orthograde to retrograde; in others, RBC displayed a pulsatile behavior with periodic stagnation followed by the resumption of flow in the orthograde direction. These findings are similar to those previously observed in rat renal ischemia reperfusion (28).

Discussion

In this study, we demonstrated that chronic hypoxia occurs even in the early stage of disease in the uninephrectomized anti-Thy-1 nephritis model with irreversible glomerulosclerosis. Blood flow in peritubular capillaries at this time was also shown to be decreased. In the late stage, deterioration of
tubulointerstitial damage proceeded and was associated with severe peritubular capillary loss.

Repeated injection of anti-Thy-1 antibody after uninephrectomy led to severe and irreversible glomerulosclerosis within 2 wk. In contrast, tubulointerstitial scarring with this treatment was mild at 2 wk and progressed chronically up to 11 wk, when severe tubulointerstitial injury was observed. Mild increases in some injury markers such as macrophage infiltration and peritubular capillary loss from week 2 to week 11 in the control group could be attributed to aging effects, as previously reported by Thomas et al. (31). BUN response was biphasic; we speculate that the first peak was due to the primary glomerular injury induced by the antibody injections and the second to the progression of tubulointerstitial disease. Similar findings were observed in a uninephrectomized anti-Thy1 model induced by a different anti-Thy1 monoclonal antibody, 1–22–3 (Sogabe H, Tomita M, Kawachi H and Shimizu F; personal communication).

In this study, we confirmed that hypoxia is present from week 1, at a time when the overall structure of tubules was still maintained. This finding implicates low oxygenation as an important mediator of the progression of tubulointerstitial injury. The changes showed a heterogeneous pattern of hypovascularity and tubulointerstitial injury, including extracellular matrix (ECM) accumulation, while a distribution of hypoxic tubules was ubiquitous. Because of the diffuse development of glomerulosclerosis in the early phase of this model, we hypothesized that the destruction of glomerular capillaries may disturb postglomerular microcirculation in peritubular capillaries.

Capillary binding of lectin in the NX Thy-1 group was

**Table 1. Summary of semiquantitative analyses**

<table>
<thead>
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<th>Characteristic</th>
<th>Week</th>
<th>NX Thy-1</th>
<th>NX</th>
</tr>
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<tr>
<td>Glomerulosclerosis score (PAS)</td>
<td>2</td>
<td>2.73 ± 0.31b</td>
<td>0.09 ± 0.03</td>
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<td></td>
<td>11</td>
<td>3.00 ± 0.26b</td>
<td>0.10 ± 0.05</td>
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<tr>
<td>Glomerular capillary loss score (JG12)</td>
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<td>2.48 ± 0.20b</td>
<td>0.07 ± 0.03</td>
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<tr>
<td></td>
<td>11</td>
<td>2.30 ± 0.22b</td>
<td>0.04 ± 0.02</td>
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<tr>
<td>Tubulointerstitial injury score (PAS)</td>
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<td>1.62 ± 0.42b</td>
<td>0.09 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>3.10 ± 0.59b,c</td>
<td>0.42 ± 0.20</td>
</tr>
<tr>
<td>Rarefaction index score (%)</td>
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<td>12.10 ± 0.40b</td>
<td>5.45 ± 0.56</td>
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<tr>
<td></td>
<td>11</td>
<td>27.00 ± 3.54b,c</td>
<td>12.20 ± 0.51</td>
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<tr>
<td>Vimentin-positive tubules (number/field)</td>
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<td>6.69 ± 2.16c</td>
<td>0.19 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>21.30 ± 7.37a</td>
<td>2.94 ± 1.00</td>
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<tr>
<td>Monocytes/macrophages (number/field)</td>
<td>2</td>
<td>9.96 ± 1.70b</td>
<td>4.08 ± 1.03</td>
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<td></td>
<td>11</td>
<td>41.30 ± 10.63a,c</td>
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* P < 0.05 versus NX group.

b P < 0.01 versus NX group.

c P < 0.01 versus NX Thy-1 group at 2 wk.

**Figure 3.** Progressive tubulointerstitial injury. No injury was observed in the NX group at weeks 2 (A) or 11 (B). In the NX Thy-1 group, tubulointerstitial injury was mild at week 2 (C) and progressed at week 11 (D). Periodic acid-Schiff staining. Magnification, ×200.

**Figure 4.** Immunohistochemical analysis of vimentin, a marker of tubulointerstitial injury, at weeks 2 (A and C) and 11 (B and D) in the NX and NX Thy-1 groups, respectively. Vimentin-positive cells were clearly increased in diseased kidneys in comparison with control kidneys. Magnification, ×200.
that did not contain JG-12—much lower than that of JG-12. Furthermore, the number of lectin-positive capillaries was significantly decreased compared with the two control groups.

Changes in peritubular capillaries in uninephrectomized anti-Thy-1 nephritis. Peritubular capillaries in the NX group were preserved at weeks 2 (A) and 11 (B). In the NX Thy-1 group, glomerular capillary density started to decrease at week 2 (C) and the decline was obvious at week 11 (D). Endothelial cells were stained with JG-12 antibody. Magnification, ×200. Rarefaction index score was determined by counting the number of squares in 10 × 10 grids that did not contain JG-12-positive peritubular capillary staining. Simultaneous scoring of tubulointerstitial injury and rarefaction index per respective subject fields at week 2 (E).

Figure 5. Changes in peritubular capillaries in uninephrectomized anti-Thy-1 nephritis. Peritubular capillaries in the NX group were preserved at weeks 2 (A) and 11 (B). In the NX Thy-1 group, glomerular capillary density started to decrease at week 2 (C) and the decline was obvious at week 11 (D). Endothelial cells were stained with JG-12 antibody. Magnification, ×200. Rarefaction index score was determined by counting the number of squares in 10 × 10 grids that did not contain JG-12-positive peritubular capillary staining. Simultaneous scoring of tubulointerstitial injury and rarefaction index per respective subject fields at week 2 (E).

significantly decreased compared with the two control groups. Furthermore, the number of lectin-positive capillaries was much lower than that of JG-12–positive vessels even in the same kidneys of the NX Thy-1 group. Although we cannot exclude the possibility that changes in the endothelial glomerulocalyx lead to decreased lectin binding, the loss of binding in these capillaries was more likely due to a decrease in blood flow conveying lectin to the corresponding vessels, because the expression of N-acetyl-D-glucosamine oligomers specific for *lycopersicon esculentum* lectin is known to be relatively independent of pathologic conditions (32). In addition, our intravital videomicroscopy analysis also demonstrated the stagnation of blood flow in peritubular capillaries in diseased kidneys.

The mechanism of the impairment of peritubular capillary blood flow may be multifactorial. For example, narrowing of glomerular capillaries as a result of morbid mesangial matrix accumulation due to sclerosis could decrease perfusion in postglomerular capillaries. In addition to anatomical damage in glomerular tufts, imbalance of vasoactive substances in the kidney may reduce peritubular capillary blood flow. We recently observed that inappropriate activation of the renin-angiotensin system reduced circulation in peritubular capillaries in the early phase of a remnant kidney model (33). Furthermore, Oyanagi-Tanaka et al. (34) reported that glomerular injury in anti-Thy1 nephritis was associated with suppression of red blood cell velocity in glomerular capillaries, which may also have contributed to the stagnation of peritubular capillary blood flow in our model.

A number of studies have shown that hypoxia leads to pro-fibrogenic responses in tubular epithelial cells and renal fibroblasts (4,35). We previously demonstrated that hypoxia promoted epithelial-mesenchymal transdifferentiation of tubular cells (36). These phenotypic changes of resident cells in the tubulointerstitium might disturb the homeostasis of ECM, and subsequent accumulation of ECM may block oxygen diffusion and exaggerate hypoxia. We have also shown that hypoxia increases apoptotic cell death of proximal epithelial cells (37) as well as endothelial cells (38). We speculate that the tubular hypoxia found in the early stage of this study may play a crucial role in the progression of interstitial injury via these multiple pathways. In addition, the notable decline in peritubular capillary density in the late stage likely renders both the lesion itself and the surrounding area hypoxic, instituting a vicious cycle to end-stage renal failure.

What is the clinical relevance of these findings? Recent research has focused on the development of novel therapeutic approaches to tubulointerstitial hypoxia. Administration of VEGF was effective in renal disease models associated with the loss of peritubular capillaries (10,39). Induction of hypoxia-activated genes as a treatment modality is another current topic in kidney research, and Eckardt and colleagues have demonstrated the usefulness of this strategy (40,41). We also demonstrated that cobalt renders the kidneys resistant to hypoxia by activating hypoxia-induced genes (42). Our findings in the current study suggest that, once available for clinical use, these therapeutic modalities will be effective even in the early phase of kidney disease.

In summary, we have provided a direct demonstration that tubular hypoxia is present in the early stage of disease in a progressive glomerulonephritis model. Our findings confirm that hypoxia is attributable not only to hypovascularity associated with tubulointerstitial injury but also to a decrease in blood flow due to glomerular injury. To our knowledge, this is the first study to demonstrate that destruction of the glomerular capillaries leads to hypoperfusion of peritubular capillaries and the corresponding interstitial area, and that this precedes the development of tubulointerstitial scarring. These results suggest that renal chronic hypoxia is not merely a result of tubulointerstitial injury but plays a pivotal role in the progression of renal disease.

**Acknowledgments**

We acknowledge research grants from the Japanese Ministry of Health, Labor and Welfare, KIRIN brewery pharmaceutical research laboratory (Japan), and NOVARTIS Foundation (Japan) for the Promotion of Science. We thank Dr. Takamoto Ohse, Dr. Krissanapong
Figure 6. Immunohistochemical analysis of pimonidazole, a hypoxic marker, at week 2 in the sham (A), NX (B), and NX Thy-1 (C) groups. Pimonidazole accumulation was intense and ubiquitous in the cortex of NX Thy-1 rats compared with both control groups. Magnification, ×200.

Figure 7. Determination of hypoperfused areas by physiologic lectin perfusion. In the NX group, glomerular and peritubular capillaries were stained with lectin (brown), which was associated with less pimonidazole staining (blue) (A). In contrast, lectin binding to capillaries was strikingly decreased in the NX Thy-1 group, in which the number of hypoxic tubules was increased (B). Magnification, ×200.

Manotham (University of Tokyo), Dr. Kiyoshi Kurokawa (Tokai University), and Dr. Takeyoshi Yamashita (Kirin Brewery Co., Ltd) for many helpful advices and generous supports.

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