Peritubular Capillary Regression during the Progression of Experimental Obstructive Nephropathy

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Abstract. Injury to the renal microvasculature may be a major factor contributing to the progression of renal disease. Although severe disruption of peritubular capillaries (PTC) could lead to marked tubulointerstitial scarring, elucidation of a process remains incomplete. This study investigated the morphologic changes in PTC and their likely regulation by vascular endothelial growth factor (VEGF) during the progression of tubulointerstitial injuries. Unilateral ureteral obstruction was induced in Wistar rats by ligation of the left ureter, and the kidneys were then collected at selected times. PTC lumina and the expression of VEGF and its receptor Flk-1 were immunohistologically detected. Morphologic changes in PTC endothelial cells were examined by using Ki67 staining, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling, and electron-microscopic studies. In the first week of the disease period, immunohistochemical labeling of tubular VEGF intensified, with accompanying deformation and dilatation of adjacent thrombomodulin (TM)-positive PTC lumina; an angiogenic response of endothelial cells was demonstrated with Ki67 and TM double-staining. During the subsequent 2 wk, tubular VEGF labeling decreased until it was virtually absent, an effect confirmed by Western blotting. Concomitantly, labeling of the VEGF receptor Flk-1 in PTC endothelial cells decreased and PTC lumina began to regress, demonstrating endothelial cell apoptosis (as detected in terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling and electron-microscopic studies). By the end of week 4, the numbers of TM-positive PTC lumina were significantly decreased in areas of marked tubulointerstitial scarring. These results suggest that PTC regression, involving an early, unsustained, angiogenic response followed by progressive endothelial cell apoptosis, could be a potential factor contributing to tubulointerstitial scarring in this unilateral ureteral obstruction model.

Tubulointerstitial injuries are regarded as major determinants of progressive renal disease, and accumulating evidence suggests that the severity of tubulointerstitial changes could be the best indicator of the progression of renal dysfunction, regardless of the original insult (1–3). In the theories introduced to explain such changes, injury to the peritubular capillary (PTC) network of the kidney is regarded as a key factor (4,5). Recently, our group (6) and Kang et al. (7,8) indicated that rarefaction of PTC is crucial for the progression of tubulointerstitial injury. However, detailed characterization of this process has not been performed.

Vascular endothelial growth factor (VEGF) is a potent endothelial cell mitogen that acts via specific receptors, i.e., VEGF receptor-1 (Flt-1) and VEGF receptor-2 (Flk-1), to promote angiogenesis and increase vascular permeability (9–12). Therefore, it has a potential role in a wide variety of situations, including liver fibrosis, tumor growth, and wound healing (13–15). In the kidney, VEGF is known to exist in all tubular epithelial cells and to induce nephrogenesis and vasculogenesis (16,17). VEGF is also considered to be indispensable for endothelial cell survival and repair during the course of renal disease (7,8,18). Although recent reports indicated that hypoxia affects the function and localization of VEGF in the tubulointerstitium (19,20), our understanding of the function of VEGF within the PTC network remains incomplete.

In this study, therefore, we characterized the morphologic changes that occur in PTC during chronic unilateral ureteral obstruction (UUO) in rats, which is a well-established model known to proceed to marked tubular atrophy and interstitial fibrosis (21–24). For better understanding of the underlying mechanism responsible for interstitial fibrosis and its relationship to the observed changes in the PTC network, VEGF expression was immunohistochemically assessed throughout the disease period and confirmed in Western blot analyses.

Materials and Methods

Experimental Design

Male Wistar rats (190 to 200 g) were subjected to UUO. With anesthesia, the left ureter was ligated with silk sutures at two points and was cut between the ligatures. Five rats each were euthanized, to yield left ligated and right contralateral kidneys, on day 0 and 1, 2, 3, and 4 wk after ligation. Five sham-operated rats were also euthanized at each time point, to serve as control animals. Kidneys were decapsulated, weighed, and processed for histologic analyses.
Histologic Examinations

Kidneys were removed, fixed in 4% buffered paraformaldehyde, embedded in paraffin, sectioned (2.5 μm thick), and stained with periodic acid-Schiff and Masson trichrome stains. For identification of PTC endothelial cells, the tissue was labeled with polyclonal rabbit anti-rat thrombomodulin (TM) antibody (Ab) (courtesy of Dr. David Stern, Columbia University, New York, NY) (25,26). For detection of VEGF and Flk-1 expression, a mouse monoclonal Ab raised against human VEGF-121 (VEGF Ab-5, clone JH; Oncogene, Cambridge, MA) (17,19) and a mouse monoclonal anti-Flk-1 Ab (A-3; Santa Cruz Biotechnology, Santa Cruz, CA), respectively, were used. The anti-Flk-1 Ab was confirmed to detect PTC endothelial cells in control rat frozen sections and was further tested for specific reactions with cultured rat glomerular endothelial cells by using Western blotting (data not shown). Infiltrated macrophages and monocytes were labeled with anti-rat ED-1 Ab (BMA, Nagoya, Japan) (27,28).

For labeling with Ab, the tissue sections were first deparaffinized and treated for 30 min with 0.3% H2O2 in methanol. Sections to be assayed for TM, Flk-1, and ED-1 were then sequentially incubated with 0.1% pepsin for 30 min, 0.1% protease for 10 min, and 0.1% pepsin for 45 min, whereas sections to be assayed for VEGF were heated in a microwave oven for 10 min, in an alkaline buffer (10 mM Tris-HCl, pH 10.0), according to the method described by Kanellis et al. (19). All sections were then incubated for 60 min with anti-TM (1:400 dilution), anti-VEGF (1:50), anti-Flk-1 (1:100), or anti-ED-1 (1:100) Ab and for 60 min with biotinylated anti-rabbit IgG or goat anti-mouse IgG (1:100; DAKO, Carpinteria, CA), and labeling was observed by using H2O2-containing diaminobenzidine (DAB) buffer.

Proliferating endothelial cells were immunohistochemically identified by double-labeling with anti-TM and anti-proliferating cell nuclear antigen (Ki67) monoclonal Ab (MIB5, 1:50 dilution; Immunotech, France). In this case, labeling was observed by using a color modification method, in which DAB precipitated with NiCl changed from brown to black in color (29). Sections were incubated with anti-Ki67, followed by a peroxidase-conjugated goat anti-mouse IgG (DAKO), H2O2, and NiCl-containing DAB buffer (DAB substrate kit for peroxidase; Vector, Burlingame, CA). Sections were then incubated with anti-rat TM Ab and a peroxidase-conjugated swine anti-rabbit IgG (DAKO), followed by H2O2 in DAB buffer.

For confirmation of TM labeling of endothelial cells, several tissue samples collected at each time point were frozen in dry ice/acetone and stored at −75°C. Cryostat sections (4 μm) were labeled by using a mouse monoclonal anti-rat endothelial cell Ab (RECA-1; Serotec, Oxford, UK) (5,7,8,30). Sections were then incubated with Texas red-conjugated goat anti-mouse IgG Ab and were observed with a fluorescence microscope.

For electron microscopy, tissues were fixed with 2.5% glutaraldehyde in phosphate buffer (pH 7.4), postfixed with 1% osmium tetroxide, dehydrated, and embedded in Epon 812 (Okenshoji, Tokyo, Japan). Ultrathin sections were stained with uranyl acetate and lead citrate and were then examined by using a Hitachi H7100 electron microscope (Hitachi, Yokohama, Japan).

Identification of Apoptosis

Apoptotic cells were identified on the basis of the presence of fragmented nuclear DNA in histologic sections labeled by using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling (TUNEL) method (31). Deparaffinized sections (2.5 μm thick) were incubated with proteinase K (20 μg/ml) for 15 min at room temperature. After blocking of endogenous peroxidase via immersion in 2% H2O2 in distilled water, sections were rinsed in TdT buffer (30 mM Tris-HCl, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride) and then incubated for 60 min at 37°C with TdT (1:100) and biotinylated dUTP (1:200) in TdT buffer. The biotinylated nuclei were observed by using avidin-peroxidase and H2O2- and NiCl-containing DAB buffer.

Apoptotic endothelial cells were identified by double-labeling with the TUNEL method and anti-TM Ab. Sections were initially labeled by using the TUNEL protocol described above, after which they were blocked for 20 min each with 0.1% avidin D (Vector) and 0.01% biotin (Sigma Chemical Co., St. Louis, MO) in phosphate-buffered saline (32). The sections were then incubated with rabbit anti-rat TM Ab and avidin-biotin-peroxidase complex and were observed with H2O2/DAB. Negative control samples were produced by omitting dUTP or TdT from the TUNEL protocol, by substituting normal rabbit IgG for anti-TM Ab, and by pretreating preparations with anti-TM Ab before TUNEL.

Evaluation of PTC and Tubulointerstitial Injury

In each sample, 40 randomly selected fields were examined under ×400 magnification, for assessment of (1) PTC changes (i.e., the numbers of TM-positive lumina, TM- and Ki67-positive cells, and TM- and TUNEL-positive endothelial cells), (2) the total number of macrophages or monocytes (i.e., the number of ED-1-positive cells), (3) the degree of interstitial fibrosis (i.e., the semiquantitative score for interstitial fibrosis), (4) the degree of VEGF expression (i.e., the staining area and semiquantitative staining score), and (5) the degree of Flk-1 expression (i.e., the staining area). For exclusion of the effects of tubular dilation and atrophy on measured values, the number of tubules in each field was also assessed and the PTC changes and ED-1-positive cell numbers were expressed per 100 tubules. The degree of interstitial injury was assessed in Masson-stained sections (5), as follows: grade 0, normal tubules and no fibrosis; grade 1, slightly increased interstitial fibrosis, with almost normal tubules; grade 2, more severe interstitial fibrosis, with some atrophic tubules; grade 3, marked interstitial fibrosis, with atrophic tubules. VEGF expression was also graded semiquantitatively (33), as follows: grade 0, no staining; grade 1, focal staining; grade 2, diffuse mild or moderate staining; grade 3, diffuse strong staining.

For quantification of VEGF and Flk-1 expression, areas with positive staining were measured by using computer image analysis (Optimas 6.2, Cybernetics, Des Moines, IA) (7). In each biopsy, the VEGF-positive areas in tubular cells and the Flk-1-positive areas in PTC endothelial cells were identified and expressed as percentages of all kidney areas.

Western Blot Analysis of VEGF

For assessment of alterations in VEGF expression during the course of the disease, Western blotting was performed with polyclonal rabbit anti-VEGF Ab (147; Santa Cruz Biotechnology). Whole kidneys were homogenized in lysis buffer (150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 40 mM Tris-HCl, pH 7.4). After centrifugation at 15,000 × g for 30 min at 4°C, the supernatant was collected and used for analysis. Samples containing 10 μg of protein per lane were separated on 10% acrylamide gels by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After electrophoresis, the separated proteins were transferred to a Hybond-P nitrocellulose membrane (Amersham Life Science, Buckinghamshire, UK) and incubated with anti-VEGF Ab (1:2000). Bound Ab was detected by using peroxidase-
conjugated anti-rabbit IgG Ab (1:1000; Jackson ImmunoResearch, West Grove, PA) and an enhanced chemiluminescence detection system (ECL Western blotting detection reagents; Amersham). The membranes were then washed and exposed to films.

**Statistical Analyses**

All values were expressed as means ± SD. Comparisons at each time point were made by using Mann-Whitney tests. The relationships between variables were assessed by Pearson correlation analysis.

**Results**

**Natural Course of the UUO Model**

On day 0, all tubules and the interstitium of the ligated kidneys were intact, and no infiltrating cells were observed (Figure 1A). By the end of the first week, dilation of tubules began to appear in some regions (Figure 1B), with mononuclear cell infiltration of the interstitium, mild edema, and fibrosis. Also during this period, there was a significant increase in the ligated kidney/body weight ratio (Table 1). During the next 2 wk, tubular deformation, including dilation and atrophy, became more severe. Inflammatory cell infiltration became conspicuous, reaching a maximum in both the cortex and the medulla by the end of week 3. Intense interstitial fibrosis was also observed, mainly around the affected tubules (Figure 1C). By the end of the observation period (week 4), inflammatory cell infiltration decreased, although the interstitial area was notably widened and dense fibrosis was present (Figure 1D).

**Table 1.** Characteristics of rats with UUO and control rats

<table>
<thead>
<tr>
<th>Time (wk)</th>
<th>Body Weight (g)</th>
<th>Kidney/Body Weight Ratio</th>
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<tbody>
<tr>
<td></td>
<td>UUO</td>
<td>Control</td>
</tr>
<tr>
<td>0</td>
<td>189.6 ± 6.4</td>
<td>189.6 ± 6.4</td>
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<tr>
<td>1</td>
<td>217.0 ± 6.4</td>
<td>245.5 ± 4.1</td>
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<tr>
<td>2</td>
<td>254.8 ± 5.0</td>
<td>302.0 ± 1.9</td>
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</tr>
<tr>
<td>4</td>
<td>306.0 ± 11</td>
<td>322.6 ± 1.9</td>
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^a UUO, unilateral ureteral obstruction.
^b P < 0.01, compared with 1 wk, right (contralateral) kidney.
^c P < 0.01, compared with 1 wk, left (control) kidney.
^d P < 0.01, compared with 4 wk, right (contralateral) kidney.
^e P < 0.05, compared with 4 wk, left (control) kidney.
throughout the kidney (Figure 1D). Most tubules appeared markedly atrophic, having lost their original integrity, but some remained extremely dilated. The ligated kidney/body weight ratio was significantly decreased at this later stage (Table 1).

**Changes in PTC Morphologic Features and VEGF Expression**

On day 0, no significant differences were noted between ligated and sham-operated kidneys with respect to the appear-

![Figure 2. Labeling of thrombomodulin (TM)-positive peritubular capillary (PTC) lumina (A, C, E, and G) and vascular endothelial growth factor (VEGF) (B, D, F, and H) in ligated kidneys from rats with UUO. (A and B) In control kidneys, TM-positive PTC lumina and VEGF in tubules are preserved. (C and D) One week after disease induction, some PTC lumina are mildly dilated, and VEGF labeling has become locally intense because of redistribution to the basolateral aspect of the tubular cells (arrowheads). (E and F) After 2 wk, many PTC lumina are misshapen; narrowed lumina are particularly apparent in fibrotic lesions (arrows). With few exceptions (asterisks), VEGF labeling is decreased in most tubules. (G and H) After 4 wk, PTC lumina are absent from scarring lesions (arrowheads), and VEGF labeling is absent from most tubules. Magnification, ×200 in A to H.](image)
ance and number of rat TM-positive PTC (Figure 2A). All tubules appeared normal, and diffuse expression of VEGF was noted throughout (Figure 2B). By the end of week 1, some of the PTC lumina were enlarged or misshapen, with mild expansion of the fibrotic interstitial areas of the ligated kidneys (Figure 2C). A corresponding intensification of VEGF labeling at the basolateral aspect of the tubules was noted, mainly in the outer medulla (Figure 2D), and double-labeling with anti-proliferating cell nuclear antigen (Ki67) and anti-TM Ab revealed that some endothelial cells were in a proliferative state (Figure 3, A and B). During the course of weeks 2 and 3, PTC deformation (e.g., narrowing and dilation) within the fibrotic areas became more pronounced, and the number of TM-positive PTC lumina retaining their original shape decreased significantly (P < 0.001) (Figures 2E and 4, A and B). Although some focal labeling remained in some regions, the level of VEGF expression was decreased in most tubules, and the total staining areas revealed significant depletion of VEGF in the kidney as a whole (Figures 2F and 5, A and B). Most of the infiltrating cells in the areas in which VEGF expression was reduced were identified as ED-1-positive macrophages or monocytes (Figures 6 and 7, A and B). The numbers of Ki 67- and TM-positive proliferative endothelial cells decreased from that time point onward and eventually decreased to below the control group levels (Figure 4, C and D).

By the end of week 4, TM-positive PTC lumina were absent in some interstitial regions, having been displaced by fibrotic elements, and most remaining PTC lumina appeared compressed, disintegrated, or dilated (Figure 2G). The reductions in TM-positive PTC lumina in the fibrotic regions were clearly apparent and highly significant, compared with week 4 control samples (P < 0.001) (Figure 4, A and B). VEGF expression was absent throughout the kidney at that time, although expression persisted in a few epithelial cells present in dilated tubules (Figure 2H).

In double-labeling assays using TUNEL and anti-TM Ab, apoptotic cells were identified among the PTC endothelial cells from week 2 onward (Figures 3C and 4, E and F). Electron-microscopic analysis confirmed the presence of condensed chromatin, which is characteristic of apoptosis, in the nuclei of the affected cells (Figure 3D).

Labeling with RECA-1 in Frozen Sections
Labeling with a second endothelial cell marker, i.e., a mouse monoclonal anti-rat endothelial cell Ab (RECA-1), demonstrated the same patterns of distribution as did the anti-TM Ab at each time point, confirming that the loss of PTC labeling was not attributable merely to loss of PTC endothelial cell antigen secondary to the disease process (Figure 8).

Flk-1 Expression
The VEGF receptor Flk-1 was readily detected in all glomeruli and PTC endothelial cells from control animals (Figure 9A). During weeks 1 and 2, specific areas of enhanced Flk-1 labeling were noted in some dilated PTC endothelial cells, and the total staining areas
demonstrated an upward trend in the whole kidney (Figures 5, C and D, and 9B). However, most of the other PTC in fibrotic interstitium only weakly expressed Flk-1. By the end of week 4, Flk-1 labeling was apparently depleted in most PTC within the scarred areas, and total expression was observed to be reduced in the whole kidney (Figures 5, C and D, and 9C). Expression of the other VEGF receptor, Flt-1, seemed absent in PTC endothelial cells throughout the course of the disease (data not shown), consistent with recent reports that it is rarely expressed in the kidney and it is not responsible for VEGF action (34–36).

**Western Blotting**

Downregulation of VEGF in ligated kidneys was confirmed by immunoblotting. Expression of VEGF was pre-
served throughout the observation period in the contralateral kidneys, whereas VEGF expression declined beginning at week 3 in the ligated kidneys (Figure 10), thus verifying the immunohistochemical findings.

**Correlations of Morphologic Changes in PTC with VEGF Expression and Interstitial Fibrosis**

At week 4, there was a negative correlation between the number of TM- and TUNEL-positive cells and the VEGF expression levels. This finding suggests a role for VEGF in regulating the morphologic changes in PTC during the early stages of UUO.

*Figure 5.* Quantification of VEGF and Flk-1 expression in the cortex (A and C) and medulla (B and D) of ligated (●), contralateral (○), and control (□) kidneys. Shown are the VEGF staining areas in tubules (A and B) and the Flk-1 staining areas in PTC endothelial cells (C and D), as detected by computer image analysis. *P < 0.05, **P < 0.001, compared with control kidneys.

*Figure 6.* Labeling of VEGF (A) and ED-1 (B) in serial sections from ligated kidneys at week 2. A decrease in VEGF staining is noted in some tubules (asterisks) surrounded by infiltrated mononuclear cells, whereas other tubules exhibit relative preservation of VEGF expression (arrowheads). Most of the infiltrated cells are identified as ED-1-positive (B). Original magnification, ×200 in A and B.
labeling score \((r = 0.66, P < 0.05)\) (Figure 11A). The total number of TM-positive PTC lumina demonstrated a positive correlation with the VEGF labeling score \((r = 0.78, P < 0.001)\) (Figure 11B) but a negative correlation with the fibrosis score \((r = 0.92, P < 0.001)\) (Figure 11C).

**Discussion**

This work describes the morphologic changes that occur in PTC and the corresponding changes in VEGF expression with UUO. Although earlier studies indicated PTC changes in marked scarred lesions after UUO (37,38), we have demonstrated that PTC regression occurs and might contribute to the progressive tubulointerstitial fibrosis. Moreover, we demonstrated that endothelial cell apoptosis, which might be induced by VEGF depletion, is considerably involved in that process.

By the end of week 1, overall VEGF expression was somewhat suppressed in the kidney as a whole; nevertheless, localized regions of enhanced VEGF labeling were noted, because of VEGF
report that demonstrated that the medulla was more vulnerable than the cortex to ischemic injury, leading to loss of VEGF expression (44).

In addition to the aforementioned theory regarding VEGF depletion, a recent study demonstrated that macrophage-derived cytokines such as interleukin-1α, interleukin-6, and tumor necrosis factor-α downregulated VEGF expression in vitro, which led to PTC regression in a 5/6 nephrectomy model (7). In our study, prominent macrophage infiltration was also observed in the affected areas of the tubulointerstitium, indicating that VEGF expression could be reduced by those cells.

Accompanying the depletion of VEGF in the affected areas were marked occlusion and stenosis of PTC lumina, with subsequent development of interstitial fibrosis. Within the same time period, the angiogenic response in PTC endothelial cells ceased to be below baseline levels and the number of apoptotic cells began to increase, followed by prominent regression of the PTC network toward the end of the disease period. Furthermore, the depletion of VEGF demonstrated a negative correlation with the number of apoptotic endothelial cells. Because VEGF functions to prevent endothelial cell apoptosis (45), the absence of VEGF likely contributed to the subsequent capillary regression (46,47). We therefore think that PTC endothelial cell apoptosis, triggered by depletion of VEGF in adjacent tubular cells, was a major contributor to PTC regression in our model.

Others have already indicated that the primary mechanism of UUO is related to tubular injury and subsequent activation of vasoactive factors initiating fibrosis (23,24). In our model also, the fibrotic changes somewhat preceded PTC regression in the early stages of the disease; however, PTC loss was eventually correlated with the degree of tubulointerstitial injury. This finding suggests that PTC regression may be an amplifier, as opposed to an initiator, of the tubulointerstitial lesions.

We observed that the loss of PTC was most prominent in scarred areas and that there was a negative correlation between the total number of TM-positive PTC lumina and the degree of interstitial fibrosis. These findings strongly support the idea that renal ischemia caused by vascular obliteration is a major contributor to renal scarring (48). We previously reported that PTC disruption can lead to tubulointerstitial scarring in the anti-glomerular basement membrane glomerulonephritis...
model (6), but we could not rule out the possibility that the disruption was specifically caused by glomerular damage. In this study, however, we have successfully demonstrated that PTC regression associated with local VEGF depletion is correlated with progressive tubulointerstitial scarring.

In summary, we have demonstrated that PTC regression occurs in a later stage of the UUO model and is correlated with the severe tubulointerstitial scarring. An impaired angiogenic response, followed by endothelial cell apoptosis, was documented and might be related to the downregulation of tubular VEGF expression. We conclude that PTC regression might act to amplify tubulointerstitial disease progression.

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References


