Peritubular Capillary Injury during the Progression of Experimental Glomerulonephritis in Rats

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Abstract. The functional and morphologic changes occurring in the peritubular capillaries (PTC) of the kidney during the progression of renal disease are not yet completely understood. In this study, the features of PTC disruption observed in a rat anti-glomerular basement membrane-induced glomerulonephritis (GN) model were characterized. Contributions to the progression of the disease made by other interstitial components, including ED-1-positive macrophages and CD3-positive T cells, were also investigated. Within 7 d of inducing GN, severe necrotizing glomerular injuries were observed. Thrombomodulin staining revealed that within 3 to 8 wk, there was a significant \((P < 0.001)\) decline in the number of PTC, accompanied by a marked accumulation of macrophages, T cells, and fibrotic material. By the end of this period, most PTC were severely damaged or lost, and tubulointerstitial scarring was noted in the affected areas. Furthermore, PTC endothelial cell apoptosis occurred concomitantly, as shown by application of terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling methods and electron microscopy. It was presumed that the PTC injury was mediated possibly by the infiltrating macrophages and T cells, which, together with destruction of the PTC structure, correlated significantly with the impairment of renal function. These findings suggest that PTC disruption and the subsequent regression of the capillary network may contribute to the development of the tubulointerstitial injury largely responsible for the renal dysfunction in progressive GN.

Since Henle et al. first reported that tubulointerstitial fibrosis rather than glomerular changes is responsible for renal dysfunction (1), there have been many studies stressing the importance of interstitial injury in the progression of renal disease (2–4). A variety of factors contributing to the progression of tubulointerstitial lesions have recently been reported, including macrophages that play a pivotal role in the tubulointerstitial lesions (5–8). T cell-mediated immune responses have also been demonstrated and are now recognized to be a principal cause of interstitial injury (5,9–12). Thus, it seems that the mechanisms of tubulointerstitial injury are gradually becoming clear.

Among the various components making up the tubulointerstitial regions of the kidney, the peritubular capillaries (PTC), which are a network of interstitial vessels, are thought to play a major role in maintaining renal function and hemodynamics. In fact, several studies of human biopsy specimens have shown that PTC may be all or partially lost from cortical areas exhibiting tubulointerstitial injury. Moreover, the degree of loss is strongly related to the progression of the disease (13–15). Despite these observations, the role of PTC has tended to be deemphasized in recent studies of renal disease. In particular, no studies of the time course of progressive glomerulonephritis (GN) have yet been carried out to resolve the role played by PTC damage in the impairment of renal function.

In the present study, we characterized the PTC disruption occurring in the anti-glomerular basement membrane (GBM) GN model of Wistar-Kyoto rats that proceeds to end-stage kidney disease, exhibiting severe necrotizing proliferative GN and marked interstitial fibrosis (16). Because visualization of PTC is difficult by routine light microscopy, PTC structures were identified using an antibody (Ab) raised against thrombomodulin (TM), a known endothelial cell marker (17,18). TM labeling on endothelial cells was confirmed by using a second endothelial cell marker, anti-rat endothelial cell Ab, RECA-1 (19). Furthermore, apoptotic PTC endothelial cells were recognized by the characteristic nuclear DNA fragmentation using terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL). Macrophages and T cells were identified using anti-ED-1 and anti-CD3 Ab, respectively. Finally, the relationship between PTC disruption and renal function during the progression of GN was analyzed.

Materials and Methods

Experimental Design

Progressive GN was induced in groups of male Wistar-Kyoto rats (100 g body wt; Charles River Japan, Kanagawa, Japan) with a single intravenous injection of 50 \(\mu\)g IgG/100 g body wt of rabbit anti-rat GBM Ab (courtesy of Dr. Yasuhiro Natori, International Medical Center of Japan, Tokyo) (18). Five rats each were sacrificed on days 3 and 7, and then 2, 3, 4, 6, and 8 wk after administration of the anti-GBM Ab. Five un.injected rats each were sacrificed on day 0 and after 4 and 8 wk, respectively, served as controls.
Histologic Examination

Kidneys were removed, fixed in 4% buffered paraformaldehyde, embedded in paraffin sections (2.5 μm thick), and stained with periodic acid-Schiff (PAS) and periodic acid-methenamine silver for histologic examination. To detect the endothelial cells of the glomerular and peritubular capillaries, the tissue was labeled with polyclonal rabbit anti-rat TM Ab (courtesy of Dr. Stern, Columbia University) (17), previously biotinylated according to the method of Shimizu et al. (18). To detect macrophages, pan T cells, and type IV collagen, tissues were respectively labeled with anti-rat ED-1 Ab (20,21), anti-human CD3 Ab (Dako, Glostrup, Denmark), and anti-human type IV collagen Ab (Southern Biotechnology Associates, Birmingham, AL). The anti-CD3 Ab was confirmed to react with rat pan T cells using rat spleen.

Specifically, labeling was accomplished by deparaffinizing the tissue sections and treating first for 30 min with 0.3% H₂O₂ in methanol, and then incubating for 60 min with either avidin-biotinylated anti-TM Ab (1:400 dilution), anti-ED-1 Ab (1:100), anti-CD3 Ab (1:100), or anti-type IV collagen Ab (1:200). The TM-labeled tissue sections were then incubated for 60 min with avidin-biotin peroxidase complex (Dako) and visualized using 3,3′-diaminobenzidine (DAB) in 0.05 mol/L Tris buffer. The ED-1-, CD3-, and type IV collagen-labeled sections were incubated for 60 min with peroxidase-conjugated goat anti-mouse IgG or mouse anti-goat IgG (1:100; Dako) and visualized using H₂O₂ containing DAB buffer.

To confirm TM labeling on endothelial cells, some tissue samples were frozen on dry ice-acetone and stored at −75°C. Cryostat sections (4 μm) were later labeled using the mouse monoclonal anti-rat endothelial cell Ab, RECA-1 (Serotec Ltd., Oxford, United Kingdom). Sections were incubated with rhodamine-labeled goat anti-mouse IgG antibody and were observed with a fluorescence microscope.

For electron microscopy, tissues were fixed in 2.5% glutaraldehyde in phosphate buffer, pH 7.4, post-fixed with 1% osmium tetroxide, dehydrated, and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate, and then examined with a Hitachi H7100 electron microscope.

Identification of Apoptosis

Apoptotic cells were identified based on the presence of fragmented nuclear DNA in histologic sections labeled using the TUNEL method (22). Deparaffinized 2.5-μm-thick sections were incubated with proteinase K (100/ml) for 15 min at room temperature. After blocking endogenous peroxidase by immersion in 2% H₂O₂ in dis-
tilled water, sections were rinsed in TdT buffer (30 mmol/L Tris/HCl buffer, pH 7.2, 140 mmol/L sodium cacodylate, 1 mmol/L 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 visualized using avidin-peroxidase and H2O2- and NiCl-containing DAB. Apoptotic endothelial cells were identified by double labeling using TUNEL and anti-TM Ab. Initially, sections were labeled using the TUNEL protocol described above, after which the sections were blocked for 20 min each in 0.1% avidin (AVIDIN D, Vector Laboratories, Burlingame, CA) and 0.01% biotin (d-BIOTIN; Sigma Chemical Co., St. Louis, MO) in phosphate-buffered saline (23). The sections were then incubated with biotinylated rabbit anti-rat TM Ab and avidin-biotin peroxidase complex and visualized with H2O2-DAB. Negative controls were produced by omitting dUTP or TdT from the TUNEL protocol, by substitution of biotinylated anti-TM with biotinylated normal rabbit IgG and by pretreating preparations with anti-TM Ab before TUNEL.

Quantification of Glomerular Alteration

For each kidney sample, more than 30 glomerular cross sections were examined for the following: (1) glomerular capillary regression (i.e., the number of glomerular capillary lumina surrounded by TM-positive cells); (2) total number of macrophages (i.e., the number of ED-1-positive cells); (3) total number of pan T cells (i.e., the number of CD3-positive cells); and (4) glomerulosclerosis progression (i.e., the area positive for type IV collagen). In each case, quantities were expressed per glomerular cross section. The type IV collagen-positive area was measured by means of a Luzex IIIU digital image analyzer (Nireco, Tokyo, Japan) and expressed as percentage of total cross sectional area.

Quantification of PTC and Interstitial Alteration

In each sample, 40 randomly selected fields (0.065 mm²) were examined for the following under ×400 magnification: (1) PTC regression (i.e., the number of TM-positive lumina); (2) total number of macrophages (i.e., the number of ED-1-positive cells); (3) total number of pan T cells (i.e., the number of CD 3-positive cells); and (4) degree of interstitial injury (i.e., the area of the cortical interstitium). Numbers of PTC, macrophages, and pan T cells were expressed per mm². The area of the cortical interstitium was measured by means of a Luzex IIIU digital image processor analyzer and expressed as percentage of total cortical area.

Renal Function Studies

The urine of all animals was obtained during 12-h overnight collections using metabolic cages, after which the animals were killed. Blood samples were obtained from control and GN rats at the time of death. Blood urea nitrogen and serum creatinine levels were measured using the modified Folin-Wu and the urease-ultraviolet methods.
respectively. The protein excreted into the urine was quantified using the pyrogallol red-molybdenum method.

**Statistical Analyses**

All values were expressed as the mean ± SD or SEM. Comparisons were made using the Mann–Whitney test for the following: (1) values on day 3 and after week 1 versus the day 0 controls; (2) values after week 2, 3, or 4 versus the week 4 controls; and (3) values after week 6 or 8 versus the week 8 controls. Correlations between groups were calculated using Pearson’s test.

**Results**

**Glomerular Alteration in Progressive GN**

By 3 to 7 d after injection of the anti-GBM Ab, necrotizing and mesangiolytic lesions of glomeruli were observed along with massive exudative changes (Figure 1B). The number of...
TM-positive glomerular capillary lumina was reduced due to destruction of the capillary network (Figure 2A), accompanied by a notable macrophage infiltration (Figure 2C).

Necrotizing GN persisted from day 7 to week 4. During this period, the destruction of capillary network progressed with accumulation of mesangial matrix. The number of macrophages per glomerular cross section decreased as inflammation declined, whereas changes in the number of

Figure 4. CD3-positive T cells in the interstitium at day 7 (A) and week 3 (B). (A) CD3-positive T cells primarily appear in the deeper cortex. (B) They disperse dominantly in the interstitium, while only a few are seen within the glomerulus. Magnification, ×200.

Figure 5. Correlation between the number of TM-positive endothelial cells (A), TM and TUNEL-positive cells (B), ED-1-positive macrophages (C), CD3-positive T cells (D), and type IV collagen-positive area (E) per glomerular cross section during the course of the experiment. ● and ○, number in experimental and control groups, respectively. Values are expressed as mean ± SD in A and C through E, and as mean ± SEM in B. *P < 0.05; **P < 0.001 versus control.
glomerular pan T cells were very mild during the course of the disease (Figure 2D).

From week 4, destruction of the capillary network became evident, glomerular inflammation almost disappeared, and macrophages were rarely observed. The accumulation of mesangial matrix continued to progress (Figure 2E), however, resulting in global glomerulosclerosis by week 8 (Figure 1D).

PTC and Interstitial Alteration in Progressive GN

Although most glomeruli exhibited notable changes shortly after induction of GN, changes of the interstitium were still not apparent on day 3, and no significant changes in the appearance or number of TM-positive PTC lumina were seen (Figure 3A). By day 7, a small number of macrophages (Figure 3B) and pan T cells appeared in the interstitium. The macrophages were particularly numerous around Bowman’s capsule, whereas the T cells were mainly seen in the interstitium of the deeper cortex (Figure 4A).

During the period between day 7 and week 4, TM-positive PTC lumina appeared compressed and misshapen due to mild expansion of the interstitium caused by edema and fibrosis (Figure 3C), and there was a marked influx of macrophages into the corresponding interstitium (Figure 3D). Dilation of PTC lumina was noted in other regions. Increased numbers of macrophages and T cells were dispersed diffusely throughout most of the cortical interstitium. By week 3, T cell numbers had reached a maximum (Figures 4B and 5D), while macrophage infiltration was maximal by week 4 (Figure 5C).

From week 4, the number of PTC lumina retaining their original shapes was significantly decreased ($P < 0.001$), and most of the interstitium was expanded due to accumulation of fibrotic materials compared to week 4 controls ($P < 0.001$). By week 8, increases in areas of the interstitium (27.2 ± 4.2% of the cortical interstitium) and reductions in TM-positive PTC lumina were clearly apparent and highly significant compared to week 8 controls (Figure 5, A and E) ($P < 0.001$, respectively). In some interstitial regions, TM-positive PTC lumina were absent, having been displaced by fibrotic material, and most remaining PTC lumina were compressed, disintegrated, or dilated (Figure 3E). Dilation of tubules was also noted in the vicinity of injured PTC lumina. Macrophage infiltration was diminishing in corresponding interstitial areas (Figure 3F), but in other cortical regions, inflammatory cell numbers were unchanged. Figure 6 shows that there is statistically significant correlation between the number of TM-positive PTC lumina and the area of the cortical interstitium at week 8 ($r = 0.79$, $P < 0.001$). To confirm the findings of TM, PTC lumina were stained with a second marker of endothelial cell, RECA-1, using frozen sections in which antigens were preserved better than in paraffin sections. Immunostaining showed similar patterns, with a loss of PTC staining in areas of tubulointerstitial scarring at the later stage of the disease (Figure 7).

Apoptotic cells were seen in PTC between weeks 3 and 8. Most of the apoptotic cells were identified as endothelial cells...
by positive anti-TM labeling and TUNEL (Figures 5B and 8). A few apoptotic cells within PTC were not labeled by anti-TM Ab; these cells were considered infiltrating mononuclear cells. Electron microscopic analysis revealed that at this stage, the basement membrane of PTC was partially disrupted and fragmented, desquamated endothelial cells were present within PTC lumina, and PTC lumina were barely detectable due to their complete loss of original shape (Figure 9, A through E). At the same time, the interstitium was widened and filled with infiltrating cells and fibrotic material (Figure 9F).

Renal Function Study
Blood urea nitrogen and serum creatinine levels significantly increased after 7 d and 2 wk, respectively (Figure 10, A and B). They both gradually increased for the entire experiment resulting in chronic renal failure. Most of the animals became proteinuric at day 3, and urinary protein levels continued to increase until the end of the disease (Figure 10C).

Relationship between Glomerular and Interstitial Disruption and Renal Function
Tables 1 and 2 show relations between glomerular and interstitial alteration, and renal function during the disease. It is notable that the PTC number shows significant correlation with all three indicators of renal function tested.

Discussion
In the present study, we characterized features of the PTC disruption during the progression of GN that have perhaps not attracted adequate attention given the large number of studies on tubulointerstitial injury. In addition, we attempted to elucidate the contribution made by PTC disruption to the development of renal disease and to the impairment of renal function.

The PTC network is directly originated from efferent arterioles of the glomeruli. It could be postulated, therefore, that if glomeruli are injured substantially, as in our GN model, the resultant reduction in blood flow from the glomeruli to the PTC would be expected to lead to disruption or regression of the PTC. Our findings cannot be explained solely by this sequence of events, however. Beginning relatively early in the progression of the GN, the numbers of TM-positive PTC lumina began to decrease in many areas of the interstitium, and some apoptotic endothelial cells were identified. In addition, ultrastructural analysis revealed that as PTC lumina became misshapen and basement membranes partially disintegrated, PTC endothelial cells became activated or degenerated and subsequently detached. At a later stage, a number of PTC apparently disappeared, replaced by fibrotic elements and numerous infiltrating inflammatory cells. Although recent studies primarily describe PTC injury as obliteration of its lumen (14,15), our findings show that PTC destruction during the course of progressive GN is better characterized by endothelial cell injury and loss of structure.

A notable finding of this study is that PTC endothelial cells undergo apoptosis. Although the significance remains controversial, vascular endothelial cell apoptosis is known to occur after cell injury or activation (24). Furthermore, macrophage-dependent vascular endothelial cell apoptosis may trigger capillary regression by blocking blood flow at the site of apoptosis, in turn triggering apoptosis of the remaining endothelial cells and resulting in regression of the entire capillary (25). In our study, accumulation of ED-1-positive macrophages was predominantly noted in areas where PTC injury was evident, suggesting the involvement of macrophages in PTC endothelial cell injury as well as in the subsequent disintegration of the entire capillary structure.

CD3-positive pan T cells first appeared in the interstitium on day 7 and reached a peak by week 3, whereas accumulation of macrophages was not maximal until week 4. This findings suggest that macrophage accumulation was mediated by T cells, consistent with recent reports (26,27). Cytotoxicity mediated by T cells may occur within the glomerulus of the anti-GBM-induced GN model, where T cells functioned as natural killer cells without expression of pan T cell ligands (16). In our model, most of interstitial T cells were CD3-positive, indicating that they were not cytotoxic natural killer cells. Therefore, we presume that interstitial T cells function to mediate interstitial inflammation rather than induce direct cytotoxicity.

We showed previously that glomerular capillary regression may lead to the development of glomerulosclerosis (18). Here, we attempted to determine the extent to which PTC regression

Figure 8. Apoptotic PTC endothelial cells double-labeled with both TUNEL (black) and anti-TM antibody (brown) at week 3 (A) and week 8 (B). A double-labeled apoptotic cell (arrow) is seen within a capillary lumen. Magnification, ×600.
contributes to tubulointerstitial scarring. Although precise details of that process have not been elucidated, tubulointerstitial scarring has been regarded as a major determinant in the progression of renal diseases (28). We found that marked interstitial fibrosis, tubular atrophy, and consequent tubulointerstitial scarring were colocalized within areas of PTC injury. Furthermore, statistical analysis revealed that there is a significant correlation between the degree of PTC injury and that of interstitial injury. Because PTC are essential for maintaining proper renal hemodynamics and for supplying oxygen to the

Figure 9. Morphologic alteration in injured PTC endothelial cells and lumina 6 wk (A through D) and 8 wk (E and F) after induction of disease. (A) An endothelial cell and basement membrane are partially disrupted (arrow). Apoptotic bodies that have been ingested by a mononuclear cell in the lumen are seen in the lumen (asterisk). Magnification, ×4000. (B) An endothelial cell (asterisk) is activated and swollen, and its fenestration cannot be recognized. Magnification, ×6000. (C and D) Typical apoptotic endothelial cells characterized by condensation of nuclear chromatin (C, asterisk) and crescentic condensed nucleus and nuclear fragments (D, asterisk). Magnification, ×7000. (E) Basement membrane of PTC is partially destroyed (arrow) and an endothelial cell is desquamated (asterisk). Magnification, ×3000. (F) Interstitium is widened and filled with infiltrating cells and fibrotic material. PTC lumina can rarely be identified. Magnification, ×1000.
entire kidney, it is possible that PTC injury causes localized hypoxia, leading to regression and scarring of the tubulointerstitium. Therefore, we conclude that PTC regression may contribute to the development of the tubulointerstitial scarring in progressive GN.

PTC disruption and the subsequent tubulointerstitial injury appear to promote the continued progression of the tubulointerstitial injury. Accordingly, the appearance of macrophages in the interstitium correlated with PTC disruption and impaired renal function. We suggest that the present study reaffirms the importance of the PTC itself and its involvement with other interstitial components in determining the level of renal functionality. However, the relationship between tubulointerstitial injury and progressive loss of glomerular function has not been fully investigated. It has been hypothesized that when resident glomerular cells are injured, as in GN, they secrete cytokines that activate interstitial cells and induce inflammatory cell infiltration (29). Fine et al. proposed that injured or occluded PTC might increase glomerular capillary pressure and thus lead to glomerulosclerosis (30). Our findings indicate that the progression of glomerulosclerosis coincides with that of the PTC and interstitial injury, suggesting that there may be cross-talk between the glomerulus and the interstitium during disease development. Further investigations to clarify this issue are now in progress in our laboratory.

In summary, we demonstrated that the injury and loss of PTC occur in tubulointerstitial scarring in a progressive GN model. Such PTC injury is characterized by endothelial cell

Table 1. Correlation between glomerular alterations and renal function

<table>
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<tr>
<th>Variable</th>
<th>TM + Capillary Lumina</th>
<th>TM and TUNEL + Cell</th>
<th>ED-1 + Cell</th>
<th>CD3 + Cell</th>
<th>Type IV Collagen + Area</th>
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<tr>
<td>BUN</td>
<td>NS</td>
<td>0.99^b</td>
<td>NS</td>
<td>NS</td>
<td>0.99^b</td>
</tr>
<tr>
<td>Cr</td>
<td>NS</td>
<td>0.96^b</td>
<td>NS</td>
<td>NS</td>
<td>0.98^b</td>
</tr>
<tr>
<td>UP</td>
<td>−0.79^c</td>
<td>0.95^b</td>
<td>NS</td>
<td>NS</td>
<td>0.96^b</td>
</tr>
</tbody>
</table>

^a Data are expressed as r values. TM, thrombomodulin; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling; BUN, blood urea nitrogen; Cr, serum creatinine; UP, urinary protein.

^b P < 0.01.

^c P < 0.05.

^d P < 0.01.

Table 2. Correlation between interstitial alterations and renal function

<table>
<thead>
<tr>
<th>Variable</th>
<th>TM + PTC Lumina</th>
<th>TM and TUNEL + PTC</th>
<th>ED-1 + Cell</th>
<th>CD3 + Cell</th>
<th>Area of Interstitial</th>
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<tr>
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<tr>
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<td>NS</td>
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<td>0.95^d</td>
<td>0.87^b</td>
<td>0.99^d</td>
</tr>
</tbody>
</table>

^a Data are expressed as r values. PTC, peritubular capillaries. Other abbreviations as in Table 1.

^b P < 0.01.

^c P < 0.05.

^d P < 0.001.
apoptosis and the subsequent destruction of the capillary structures. This process can be mediated by infiltrating macrophages, and the involvement of T cells is also suggested. The progression of PTC disruption coincides with the alteration of other interstitial components, most of which strongly correlate with the impairment of renal function. We conclude that PTC disruption and the subsequent regression of the capillary network contribute to the development of the tubulointerstitial injury largely responsible for the renal dysfunction occurring in progressive GN.

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