Injury and progressive loss of peritubular capillaries in the development of chronic allograft nephropathy

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Background. Chronic allograft nephropathy (CAN) remains the most important cause of late renal graft loss. However, the mechanism for graft dysfunction and the process of the development of CAN are not well understood. This study examined the role of microvascular injury in the development of CAN.

Methods. We studied renal biopsies obtained from grafts with CAN (N = 79) and pretransplant control kidneys (N = 20). Microvascular injury was examined morphologically, and was correlated with interstitial fibrosis, glomerular sclerosis, graft function, and the severity of CAN. The humoral and cellular immunity involved in CAN were examined by C4d, CD3, and TIA-1 staining.

Results. In all the cases of CAN, microvascular injury was evident with or without CD3-positive T cells, TIA-1-positive cytotoxic cells, and/or C4d+ complement deposition. Irrespective of chronic rejection (N = 14), C4d+ chronic humoral rejection (N = 6), or other CAN, the development process of CAN was characterized by injury and progressive loss of identifiable peritubular capillaries (PTCs) accompanied with the development of interstitial fibrosis. Injured PTCs were characterized morphologically by the process of angioregression with the presence of apoptotic cells, lamination of the basement membrane, and loss of PTCs. The low number of PTCs correlated significantly with the severity of CAN (r = −0.74, P < 0.001), the development of interstitial fibrosis (r = −0.75, P < 0.001), graft dysfunction (r = −0.69, P < 0.001), and also correlated weakly with proteinuria (r = −0.45, P < 0.05). In the glomeruli, capillary loss significantly correlated with the degree of glomerular sclerosis (r = −0.66, P < 0.001) and proteinuria (r = −0.65, P < 0.001), but did not correlate with the severity of CAN (r = −0.24, P > 0.05) or graft dysfunction (r = −0.32, P > 0.05).

Conclusion. CAN was characterized by progressive injury to the renal microvasculature and the development of renal scarring. In particular, injury, angioregression and progressive loss of the PTC network strongly contributed to the development of interstitial fibrosis and graft dysfunction in CAN, and might play a crucial role in the development of CAN.

As improvements are made in immunosuppressive therapy and early allograft survival, chronic allograft nephropathy (CAN) has become the dominant cause of late renal allograft failure [1]. There is general agreement that the pathogenic process of CAN is complex, and involves both immunologic and nonimmunologic factors [2–6]. A variety of alloantigen-dependent immune responses that contribute to the progression of CAN have been reported, including prior severe rejection, subclinical rejection, and chronic rejection [2–5]. Furthermore, several alloantigen-independent immune reactions and nonimmunologic factors, such as reperfusion-ischemic injury, chronic cytomegalovirus and polyomavirus infection, chronic toxic effects of calcineurin inhibitors, hypertension, hyperlipidemia, and chronic vesicoureteral reflux have also been reported, and are recognized as important causes of CAN [2–4, 6]. However, the process of the development of CAN has not been morphologically characterized. Moreover, the mechanism(s) for CAN remains poorly understood.

The results of recent studies suggest that the renal microvasculature plays a major role in maintaining hemodynamics and renal function, and that injury to the renal microvasculature is a crucial determinant of the progressive deterioration of renal function, as well as the progression of renal diseases [7–10]. Although CAN is usually characterized by a slow progressive graft dysfunction [4], few studies have characterized renal microvascular injury in the development of CAN. In the present study, we investigated the process of the development of CAN using human graft biopsies, with a special emphasis on renal microvascular injury. We also elucidated the mechanism for the progressive deterioration of renal function and the development of CAN.
METHODS
Renal biopsy

We selected 79 biopsy samples taken from grafts showing CAN. Renal biopsies were performed, with informed consent, at the time when unexplained deterioration of the graft function or nontransient proteinuria developed. We defined CAN as a state of impaired graft function lasting at least 6 months post-transplant, independent of acute rejection, overt drug toxicity, and recurrent or de novo specific disease entities. In addition, based on Banff’s classification [11], histopathologic examination of the biopsy material showed chronic interstitial fibrosis and tubular atrophy, independent of other changes. The histopathologic criteria for chronic rejection (CR) were either (I) glomerular basement membrane (GBM) duplication (chronic allograft glomerulopathy) in the absence of de novo or recurrent glomerulonephritis, or (2) arterial intimal fibrosis (chronic allograft arteriopathy) with intimal mononuclear cell infiltration [12]. The histopathologic criteria for chronic humoral rejection (CHR) fulfilled the histopathologic criteria for CR with diffuse complement split product C4d deposition along the peritubular capillaries (PTCs) [12, 13]. Renal biopsies taken before revascularization of the transplanted kidney (zero-hour biopsy), and samples showing no diagnostic abnormality were used as controls (N = 20). Graft function was assessed by measurement of the serum creatinine (Cr), blood urea nitrogen (BUN), and daily urinary protein levels (proteinuria). All patients were treated with a conventional immunosuppression regimen using prednisolone and cyclosporine A (CyA) or FK506 (tacrolimus) with strict blood level monitoring.

Histopathologic and immunohistochemical examinations

Renal tissues were fixed in 10% buffered formalin, and embedded in paraffin for light microscopic examination. Serially cut (3-μm thick) paraffin-embedded tissue sections were stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), Masson trichrome (Masson), and periodic acid-methenamine (PAM) silver for histopathologic examination. The morphologic findings evaluated by light microscopy were also characterized by using electron microscopy. For electron microscopic examination, the kidney tissue was fixed in 2.5% glutaraldehyde solution in phosphate buffer (pH 7.4), and postfixed with 1% osmium tetroxide, dehydrated, and embedded in Epoct 812. Ultrathin sections were stained with uranyl acetate and lead citrate, and then examined with an electron microscope (model H7100; Hitachi Corp., Tokyo, Japan).

The following primary antibodies were used for immunohistochemistry. Monoclonal mouse antihuman CD34 antibody (NU-4A1; Nichrei, Tokyo, Japan), which reacted with the surface of all endothelial cells. This antibody has been used as a marker for endothelial cells [14, 15]. Polyclonal goat antihuman type I collagen antibody (Southern Biotechnology Associates, Birmingham, AL, USA), which can detect the accumulation of collagen in interstitial fibrosis. Monoclonal mouse antihuman type IV collagen antibody (JK-199; Shiseido, Tokyo, Japan), which can detect the accumulation of extracellular matrix in glomeruli. Monoclonal mouse antihuman alpha-smooth muscle actin (α-SMA) (1A4; Dako, Glostrup, Denmark), which has been used as a marker for myofibroblasts in the interstitium and activated mesangial cells in glomeruli [16, 17]. Polyclonal rabbit antihuman CD3 antibody (A0452; Dako), which can detect infiltrating T cells. Monoclonal mouse antihuman TIA-1 (GMP-17) antibody (Immunotech A Coulter, Marseille Cedex, France), which can detect infiltrating cytotoxic cells [18]. Monoclonal mouse antihuman C4d antibody (Quidel, San Diego, CA, USA), which can detect acute and chronic humoral rejection. The complement split product C4d, as a stable fragment of complement degradation activated by antigen-antibody complexes, has been considered an indicator of humoral activity in allografts [12, 13, 19, 20]. For immunohistochemical staining of CD34, type I collagen, type IV collagen, α-SMA, CD3, and TIA-1, 10% buffered formalin-fixed, paraffin-embedded tissue sections were used, and the specimens were stained by the standard avidin-biotin-peroxidase complex (ABC) technique. For type I collagen, type IV collagen, and CD3, tissue sections were incubated with 0.1% pepsin for 30 minutes and 0.1% proteinase for 5 minutes; 0.1% pepsin for 30 minutes and 0.1% proteinase for 5 minutes; and 0.1% pepsin for 45 minutes, respectively, before incubation with the primary antibody. To detect C4d deposits in the grafts, 4-μm thick frozen sections were stained by the standard indirect technique and observed with a fluorescence microscope. For all biopsies, negative controls were used in which the primary antibody was substituted with equivalent concentrations of an irrelevant antibody or omission of antibody. All control sections were negative.

In histologic sections, fragmented nuclear DNA associated with apoptosis was labeled by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling (TUNEL) method [21]. Briefly, after sections were deparaffinized and incubated with proteinase K, they were rinsed in TdT buffer, and then incubated with TdT 1:25 and biotinylated-dUTP 1:20 in TdT buffer for 60 minutes at 37°C. The biotinylated nuclei were detected with avidin-peroxidase and H2O2 containing 3,3'-diaminobenzidine tetrahydrochloride (DAB). Controls involved omission of the dUTP or TdT in TUNEL procedures.
Quantification of histopathologic findings

In each kidney sample, 20 randomly selected interstitial fields (0.065 mm²), all glomerular cross-sections and all arterial cross-sections, were assessed at ×400 for the following parameters: (1) PTCs, the mean number of PTC lumina surrounded by CD34-positive cells, per interstitial field [22] (scores 0 to 3: score 0, no staining; score 1, focal staining; score 2, diffuse moderate staining; score 3, diffuse strong staining); (2) glomerular capillaries, the mean number of glomerular capillary lumina surrounded by CD34-positive cells, per glomerular cross-section; (3) glomerular sclerosis, the mean semiquantitative staining score of type I collagen, per glomerular cross-section [23] (scores 0 to 4: score 0, no localized increase of staining; score 1, up to 25% of the glomerular tuft showing focally increased staining; score 2, 25% to 50% of the glomerular tuft demonstrating a focally, strong staining; score 3, 50% to 75% of the glomerular tuft stained strongly in a focal manner; score 4, >75% of the glomerular tuft stained strongly). Glomerular cross-sections containing only a small portion of the glomerular tuft were excluded from the analysis, and more than 5 glomeruli were included in each biopsy sample in the present study. (4) Vascular occlusion, the mean semiquantitative score of vascular fibrous intimal thickening, per arterial cross-section [11] (scores 0 to 3: score 0, no chronic vascular changes; score 1, vascular narrowing of up to 25% luminal area by fibrointimal thickening of arteries; score 2, increased severity of changes described above with 26% to 50% narrowing of vascular luminal area; score 3, severe vascular changes with >50% narrowing of vascular luminal area). Histopathologic evaluations were performed by 2 investigators blinded to the clinical information. Intra and inter-observer reproducibility of the counts of PTCs and glomerular capillaries were assessed in selected high-power fields and selected glomeruli, respectively (magnification, ×400). Repeated counts of capillaries varied by less than 3% by 1 observer and 7% between 2 observers. Scores of interstitial fibrosis, glomerular sclerosis, and vascular occlusion did not vary by more than 3% by the single observer, or by more than 5% between 2 observers. Pearson correlations between the grading of the severity of CAN and serum Cr levels, daily proteinuria levels, or the duration after transplantation; serum Cr levels or daily proteinuria levels and the duration after transplantation; the score of vascular occlusion and the grading of the severity of CAN, serum Cr levels, or the duration after transplantation; the number of PTCs and the grading of severity of CAN, the score of interstitial fibrosis, serum Cr levels, daily proteinuria levels, or the duration after transplantation; the number of glomerular capillaries and the grading of the severity of CAN, the score of glomerular sclerosis, serum Cr levels, daily proteinuria levels, or the duration after transplantation; and the score of interstitial fibrosis or glomerular sclerosis and the duration after transplantation, were computed and analyzed.

RESULTS

Renal biopsies

Using Banff’s classification, the severity of CAN (N = 79) was divided into 3 grades: grade I (mild, N = 30, 38.0%); grade II (moderate, N = 27, 34.2%); and grade III (severe, N = 22, 27.8%) (Fig. 1A to C). Patients’ characteristics are summarized in Table 1. Importantly, the grading of the severity of CAN correlated significantly with renal dysfunction (r = 0.82, P < 0.001) (Fig. 2A). The levels of daily proteinuria were paralleled with the grading of the severity of CAN (r = 0.44, P < 0.05) (Fig. 2B). In the present study, 14 of 79 cases (17.8%) fulfilled the histopathologic criteria of CR (Table 1, Fig. 1D to F). In addition, prominent C4d deposits in the PTCs were detected by immunofluorescence in 6 of 14 cases (42.9%) that had been diagnosed as CR, and these cases were defined as CHR. The proteinuria was more prominent in CHR cases than in other CAN cases (Table 1, Fig. 2B). CR and CHR cases tended to progress into moderate to severe CAN within a relatively short period after transplantation, accompanied with the development of proteinuria and graft dysfunction (Fig. 2C to E). The arterial occlusive lesions were often minimal in cases of CAN without CR or CHR (Fig. 1G). The score of vascular occlusion did not correlated significantly with the severity of CAN (r = 0.34, P > 0.05), the graft dysfunction (r = 0.29, P > 0.05), or the duration after transplantation (r = 0.19, P > 0.05) (Fig. 3A to C). Typical chronic allograft vasculopathy with intimal fibrosis and mononuclear cell infiltration was detected mainly in CR and CHR cases (Figs. 1E and F, 3A and B).

Microvascular injury in CAN

In all cases of CAN, various numbers of cellular infiltrates (CD3-positive T cells and TIA-1-positive cytotoxic cells) were seen in the renal microvasculature (PTCs and glomerular capillaries) (Fig. 4A to C). The degree of infiltrating CD3-positive T cells and TIA-1-positive cytotoxic cells in the microvasculature seemed to be more prominent in CR and CHR cases (data not shown). In addition, in CHR cases, prominent C4d deposits were detected in not only PTCs but also glomerular capillaries (Fig. 4D). Regardless of the cases with prominent CD3-positive and TIA-1-positive cell infiltration, CR, C4d-positive CHR, or other CAN, microvascular (PTC and glomerular capillary) injury was evident in all cases of CAN. A few apoptotic dead cells were evident in the PTCs and
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Fig. 1. Morphologic changes through the progression of CAN severity (A to E: PAM stain; A to C, ×200; D, E, G, ×400; F, PAS stain, ×200). In a representative case of mild CAN, the interstitium is in the process of fibrosis and eventual focal expansion (A). The glomerulus and small artery are almost fully intact. In a representative case of moderate CAN, interstitial fibrosis and expansion present with tubular atrophy (B). The glomerulus shows mild allograft glomerulopathy with thickening of GBM and mild expansion of the mesangial area. The small arteries show mild intimal thickening. In a representative case of severe CAN, note the prominence of interstitial fibrosis with tubular atrophy (C). One glomerulus shows nonspecific segmental glomerular sclerosis, and the other two show global sclerosis (arrow). The small arteries show mild intimal thickening and chronic cyclosporine A–associated vasculopathy. Chronic allograft glomerulopathy with GBM duplication is prominent in cases with CR and CHR (D). Chronic allograft vasculopathy of the small artery (E) and the medium-size artery (F) with arterial intimal fibrosis and intimal mononuclear cell infiltration is detected mainly in CR and CHR cases. Evidence of tubulointerstitial fibrosis, although arterial occlusive lesions are not prominent, in CAN cases without CR and CHR (G).

Table 1. Patients’ characteristics and summary of microvascular injury in chronic allograft nephropathy

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Age years</th>
<th>Duration to biopsy years</th>
<th>Serum Cr mg/dL</th>
<th>Proteinuria g/day</th>
<th>PTCs capillaries/field</th>
<th>Interstitial fibrosis (score: 0–3)</th>
<th>Glomerular capillaries capillaries/glomerulus</th>
<th>Glomerular sclerosis (score: 0–4)</th>
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<tr>
<td>CAN</td>
<td>79</td>
<td>43.4 ± 9.3</td>
<td>6.8 ± 4.4</td>
<td>3.1 ± 1.2</td>
<td>1.4 ± 0.9</td>
<td>19.9 ± 9.6</td>
<td>1.7 ± 0.7</td>
<td>54.2 ± 16.5</td>
<td>1.0 ± 0.7</td>
</tr>
<tr>
<td>Grade I (mild)</td>
<td>30</td>
<td>43.8 ± 9.5</td>
<td>6.5 ± 5.5</td>
<td>2.0 ± 0.4</td>
<td>0.9 ± 0.7</td>
<td>28.7 ± 8.7</td>
<td>0.9 ± 0.2</td>
<td>62.8 ± 15.2</td>
<td>0.9 ± 0.6</td>
</tr>
<tr>
<td>Grade II (moderate)</td>
<td>27</td>
<td>45.1 ± 10</td>
<td>5.9 ± 3.9</td>
<td>3.2 ± 0.7</td>
<td>1.6 ± 0.8</td>
<td>17.0 ± 5.0</td>
<td>1.8 ± 0.3</td>
<td>51.4 ± 13.0</td>
<td>0.8 ± 0.6</td>
</tr>
<tr>
<td>Grade III (severe)</td>
<td>22</td>
<td>41.1 ± 8.1</td>
<td>8.1 ± 2.8</td>
<td>4.4 ± 0.9</td>
<td>2.0 ± 1.1</td>
<td>11.4 ± 5.6</td>
<td>2.5 ± 0.2</td>
<td>46.0 ± 17.2</td>
<td>1.2 ± 0.7</td>
</tr>
<tr>
<td>CR</td>
<td>14</td>
<td>43.5 ± 10</td>
<td>5.9 ± 3.0</td>
<td>3.9 ± 1.0</td>
<td>2.2 ± 0.7</td>
<td>13.5 ± 5.0</td>
<td>2.1 ± 0.5</td>
<td>35.4 ± 10.1</td>
<td>2.4 ± 0.7</td>
</tr>
<tr>
<td>CHR</td>
<td>6</td>
<td>42.8 ± 12</td>
<td>5.2 ± 1.9</td>
<td>3.5 ± 0.3</td>
<td>3.1 ± 0.6</td>
<td>16.2 ± 5.6</td>
<td>2.1 ± 0.4</td>
<td>29.7 ± 5.7</td>
<td>2.8 ± 0.8</td>
</tr>
<tr>
<td>Control</td>
<td>20</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0.1 ± 0.1</td>
<td>36.4 ± 2.4</td>
<td>NA</td>
<td>NA</td>
<td>59.7 ± 5.3</td>
</tr>
</tbody>
</table>

Abbreviations are: CAN, chronic allograft nephropathy; CR, chronic rejection; CHR, chronic humoral rejection; Control, 0 hour pretransplant control kidneys; Cr, creatinine; NA, not available; PTCs, peritubular capillaries. Data are mean ± SD.

glomerular capillaries undergoing the process of CAN, as judged by the TUNEL method (Fig. 4E and F), although they were almost undetectable in the 0-hour control biopsies. In electron microscopic studies, injured PTCs appeared small with narrow-lumen capillaries, together with activated endothelial cells, the detachment of endothelial cells from the basement membrane (BM), and increased lamination of the BM (Fig. 5A to D). In areas with severe interstitial injury, PTC lumina were barely detectable due to the complete loss of their original shape, and the interstitium was widened and filled with fibrotic material (Fig. 5E). In the glomeruli, injured capillaries were characterized by narrow lumen and activated endothelial cells with double contour, mesangial interposition, lamination, and thickening of the GBM (Fig. 5F), similar to injured PTCs. αSMA-positive myofibroblasts and activated mesangial cells accumulated in areas with interstitial and glomerular injury, characterized by the dilated interstitium and mesangial areas with loss or abnormal shape of capillaries (Fig. 4G and H).

PTCs and interstitial fibrosis in CAN

We examined whether PTC injury contributed to tubulointerstitial scarring, graft dysfunction, and proteinuria
Fig. 2. Correlation between CAN severity as defined by Banff’s classification and the impairment of graft function (A), the development of proteinuria (B), or the duration after kidney transplantation (C). Also, the relationship between serum creatinine (D) and proteinuria (E) levels to the duration after transplantation. (○) and (△) indicate CR and CHR cases, respectively. The severity of CAN based on Banff’s classification strongly correlates with graft dysfunction ($r = 0.82$, $P < 0.001$), and weakly correlates with daily proteinuria ($r = 0.44$, $P < 0.05$). However, no correlation is evident between the duration after transplantation and the severity of CAN ($r = 0.13$, $P > 0.05$), serum Cr levels ($r = 0.14$, $P > 0.05$), or proteinuria levels ($r = 0.05$, $P > 0.05$). CR and CHR cases seem to progress into the moderate to severe CAN within a short period after transplantation, accompanied by the development of proteinuria and graft dysfunction.

Fig. 3. Relationship between the score of vascular occlusion (vascular fibrous intimal thickening) and the severity of CAN (A), graft dysfunction (B), or the duration after kidney transplantation (C). (□) indicates the results of pretransplant control kidneys. The score of vascular occlusion does not correlate significantly with the severity of CAN ($r = 0.34$, $P > 0.05$), the graft dysfunction ($r = 0.29$, $P > 0.05$), or the duration after kidney transplantation ($r = 0.19$, $P > 0.05$).
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Fig. 4. Renal microvasculature injury (PTCs and glomerular capillaries) in CAN. Infiltration by CD3-positive T cells are seen in PTCs (A) and glomerular capillaries (B) (A and B: CD3 stain, ×400). Some CD3-positive infiltrating T cells are in contact with endothelial cells in PTCs (arrow). Infiltration by cytotoxic cells is also evident in PTCs (TIA-1 stain, ×400) (C). Immunofluorescence for C4d shows diffuse and intense staining along PTCs (C4d stain, ×200) (D). TUNEL-positive apoptotic dead cells (arrows) are detected in PTCs (E) and glomerular capillaries (F) (TUNEL method, ×500). α-SMA-positive interstitial myofibroblasts (G) and α-SMA-positive activated mesangial cells (H) accumulate in the dilated interstitium and mesangial areas with the loss or abnormal shapes of capillaries (α-SMA stain, ×400).

through the progression of CAN severity. Since visualization of PTCs is difficult by routine light microscopy, PTC structures were identified using immunohistochemical staining for CD34, which is known as an endothelial cell marker [14, 15]. In the pretransplant control kidneys (0 hour biopsied specimens) (Fig. 6A and E), a well-developed PTC network was evident in contact with the tubules and very few focal type I collagen areas in the interstitial space. In grafts with mild CAN (Fig. 6B and F), the CD34-positive PTC lumina appeared compressed with abnormal shape due to mild expansion of the interstitium caused by fibrosis. Focal and mild tubular atrophy was accompanied by PTC changes, and there was an increasingly mild accumulation of type I collagen in the interstitium. In grafts with moderate CAN (Fig. 6C and G), the number of CD34-positive PTC lumina that retained their original shapes were markedly decreased, and a large part of the interstitium was expanded due to accumulation of fibrotic materials including type I collagen. Tubular atrophy was prominent in the corresponding interstitium. In grafts with severe CAN (Fig. 6D and H), only a few CD34-positive PTC lumina were noted; they were displaced by fibrotic material and the lumina of the majority of remaining PTCs were compressed, disintegrated, or dilated. Marked atrophy of tubules and loss of tubular structure were prominent in the affected area of the interstitium. Thus, more severe CAN was associated with a greater reduction of CD34-positive PTCs (Table 1, Fig. 6). The decrease in the number of CD34-positive PTCs in the interstitium correlated significantly with the grading of the severity of CAN (r = −0.74, P < 0.001), the score of interstitial fibrosis (r = −0.75, P < 0.001), and an increase in serum creatinine levels (r = −0.69, P < 0.001) (Fig. 7A to C). In addition, there was a weak correlation between the number of CD34-positive PTCs and the development of proteinuria (r = −0.45, P < 0.05) (Fig. 7D). However, no correlation was evident between the number of CD34-positive PTCs and the duration after transplantation (r = −0.21, P > 0.05) (Fig. 7E). The degree of interstitial fibrosis also did not correlate with the duration after transplantation (r = 0.26, P > 0.05) (Fig. 7F).

Glomerular capillaries and glomerular sclerosis in CAN

In the pretransplant 0-hour biopsies (Fig. 8A to C), glomerular capillaries were wide open, and type IV collagen was distributed in the GBM and mesangial matrix of the glomeruli. In CR and CHR cases, chronic allograft glomerulopathy was evident with segmental sclerosis and diffuse thickening of the GBM (Fig. 8D). In CAN cases
Fig. 5. Morphologic changes in the injured PTCs and glomerular capillaries. With mononuclear cell infiltration (asterisk) under the endothelial cell in the PTC, note the detachment of endothelial cells from their BM (arrows) (\( \times 2000 \)) (A). Detachment of endothelial cells from the PTC BM (arrows) is also detected in PTCs without cell infiltration (\( \times 2000 \)) (B). Injured capillaries are small in size with a narrow lumen and lamination of the BM (\( \times 2500 \)) (C). An endothelial cell (asterisk) is activated and swollen, and its fenestration cannot be recognized. The endothelial cell also protrudes into the lumen of the capillary. In the fibrotic area, which is markedly atrophic, collapsed and occlusive capillaries (arrow) are still seen with marked lamination of the BM (\( \times 2000 \)) (D). Note the expansion of the interstitium, which is filled with fibrotic material and the loss of PTCs, evident around tubules with interstitial fibrosis (\( \times 1000 \)) (E). The glomerular capillary shows narrowing of the lumen with mesangial interposition and double contour, lamination, and thickening of the GBM (\( \times 7100 \)) (F). Also, an endothelial cell is activated with partial loss of fenestration.

without CR and CHR, glomeruli with nonspecific segmental sclerosis were noted without diffuse thickening of the GBM (Fig. 1C). In both chronic allograft glomerulopathy and nonspecific segmental glomerular sclerosis, the number of CD34-positive glomerular capillaries was reduced within the sclerotic lesions (Fig. 8E and F). However, in cases free of CR and CHR, glomerular sclerosis tended to be minimal even in those with severe CAN and severe deterioration of graft function (Fig. 8G to I). Also, the severity of the glomerular changes did not parallel the severity of CAN (\( r = -0.24 \), \( P > 0.05 \)) (Fig. 9A). The loss of identifiable glomerular capillaries was more prominent in CR and CHR cases than in other CAN cases (Table 1, Fig. 9A to D). There was a significant correlation between the number of CD34-positive glomerular capillaries and the score of glomerular sclerosis (\( r = -0.66 \), \( P < 0.001 \)) or the development of proteinuria (\( r = -0.65 \), \( P < 0.001 \)) (Fig. 9B and D). However, there was no correlation between the number of glomerular capillaries and serum Cr levels (\( r = -0.35 \), \( P > 0.05 \), or with the duration after kidney transplantation (\( r = 0.03 \), \( P > 0.05 \)) (Fig. 9C and E). The degree of glomerular sclerosis also did not correlate with the duration after transplantation (\( r = -0.04 \), \( P > 0.05 \)) (Fig. 9F).

DISCUSSION

In the present study, we demonstrated that, irrespective of whether CR, CHR, or other factors lead to the
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Fig. 6. PTCs and interstitial fibrosis through the progression of CAN severity (A to H: CD34 stain; A to D: ×400; E to H: ×600; I to L: type I collagen stain, ×400; inset in I to L: PAM stain, ×400). In the 0-hour control kidney, PTCs are well preserved and there is little type I collagen in the interstitium (A, E, I). In the case with mild CAN, note the low number of CD34-positive PTCs, narrow lumina, and the increase in the interstitial area positive for type I collagen (B, F, J). In the case with moderate CAN, note the narrow lumina and the loss of PTC, together with the low number of PTCs and the progression of interstitial fibrosis (C, G, K). In the case with severe CAN, there is severe interstitial fibrosis with accumulation of type I collagen (D, H, L). Collapsed and a decreased number of CD34-positive PTCs are evident in the fibrotic interstitium.

development of CAN, the processes involved in its development appear similar, and are characterized by irreversible injury and loss of renal microvasculature, with the development of renal scarring. In particular, irreversible injury and progressive loss of PTCs seem to be crucial events in the progression of CAN severity, as well as chronic deterioration of graft function.

PTCs, representing a network of interstitial vessels, are essential for maintenance of proper renal hemodynamics and renal function, and for supplying oxygen to the entire kidney [7–10]. Persistent PTC injury may directly cause loss of PTCs [16, 24], and is also associated with an increase in PTC pressure and an increase in postglomerular resistance, resulting in further glomerular injury and augmentation of PTC injury [7, 25, 26]. In addition, PTC injury triggers the release or activation (angiotensin II, adenosine, renal sympathetic nerves) or inhibition (nitric oxide, prostaglandin, dopamine) of vasoactive mediators and vascular constriction, as well as reduction of PTC plasma flow that further enhances local ischemia of the tubules and interstitium [25, 26]. Following PTC injury, the PTC endothelium is activated, and such a process may correlate with the enhancement of inflammation and activation of the coagulation system that favors further interstitial and capillary injury [27–31]. Hence, PTC injury can potentially cause localized hypoxia, chronic ischemia, and interstitial inflammation, leading to tubular atrophy with phenotypic changes in tubular epithelial cells, development of interstitial injury with fibroblast activation, and stimulation of the interstitial scarring process [9, 10, 24, 26]. Indeed, in the present study, marked interstitial fibrosis, tubular atrophy, and consequent tubulointerstitial scarring were colocalized within areas of severe injury and loss of PTCs.

Injury and loss of PTCs are also recognized in progressive renal diseases, and PTC loss may be due to both destruction and regression of capillaries [8, 9, 32]. In renal transplantation, microvascular destruction has been
In the present study, however, active destruction of PTCs, characterized by capillary disruption and fragmentation of the PTC BM and desquamation of endothelial cells, were not evident in our CAN cases. On the other hand, injured PTCs exhibited a decrease in their size with narrowing of the lumina, appearance of a few TUNEL-positive apoptotic cells, activated endothelial cells, detachment of endothelial cells from their BM, protrusion of endothelial cells into the lumen, and multilayering of the BM. Since this structure resembles that of capillary regression [34], we termed it “angioregression type” of injured capillaries. Thus, our findings suggest that the loss of PTCs in CAN may be ascribed to injury and subsequent angioregression of PTCs. Importantly, our results indicate that the loss of PTCs correlated significantly with the severity of CAN, the degree of interstitial fibrosis, and the impairment of graft function. These findings illustrate that capillary regression and the loss of PTCs are essentially involved in progressive renal dysfunction and the development of CAN. In determining the prognosis of CAN, a recent study demonstrated that the quantitative measurement of fibrosis by computerized image analysis appears to be a useful prognostic indicator for estimating long-term graft function in CAN [35]. Particularly, the cortical fractional interstitial fibrosis volume in protocol biopsies at 6 months’ post-transplant may provide an early surrogate for time to graft failure in renal allograft recipients [36]. Presently in our laboratory, a study is in progress to examine the predictive value of the number of PTCs in the estimation of long-term allograft outcome. In addition, further research is necessary to understand the mechanisms, the signaling pathway(s), and the process of vascular regression in injured PTCs in CAN.

The phenomenon of capillary regression with apoptosis of endothelial cells is well described in the development of glomerular sclerosis in progressive renal diseases [37, 38]. In this present study, the angioregression type of microvascular injury was also seen in glomerular capillaries, similar to that in PTCs. The loss of glomerular capillaries correlated significantly with the degree of glomerular sclerosis in glomeruli, with nonspecific segmental sclerosis, and chronic allograft glomerulopathy. In arteries, occlusive changes were noted mainly in CR and
CHR cases. The presence of glomerular sclerosis, chronic transplant glomerulopathy, or arterial occlusive lesions in CAN suggests that these lesions probably contribute to the development of PTC injury and the progression of CAN [26]. However, in contrast with tubulointerstitial fibrosis, CAN cases without CR and CHR showed relatively minor glomerular sclerosis and arterial occlusive lesions. Furthermore, the severity of glomerular and arterial changes did not parallel the severity of CAN. The loss of glomerular capillaries and the severity of vascular occlusion did not correlate significantly with graft dysfunction. These findings suggest that glomerular capillary and artery injuries may not be essential events in the development of CAN, but rather, the loss of PTCs can lead to CAN, independent of glomerular and large blood vessel injury.

The pathogenesis of CAN involves a complex network of immunologic, metabolic, and hemodynamic changes in renal allografts [2–6]. In the present study, although we could not determine the pathogenesis of CAN in each case, it is likely to be multifactorial, including immunologic and nonimmunologic events. Indeed, our cases included CHR cases with C4d deposition and cases with or without morphologic features of CR. However, the present study demonstrated that, irrespective of CR, CHR, or other CAN, the process of progression of CAN severity is characterized by injury and loss of PTCs with the development of interstitial scarring. Several studies indicated recently that the factors involved in CAN, such as chronic cellular or chronic humoral rejection [16], ischemia-reperfusion injury [39], cyclosporine A–induced nephrotoxicity [40], salt-sensitive hypertension [41], aging-associated renal injury [22, 42], and reduced functioning renal mass [43] may facilitate renal microvascular injury, especially PTC injury, ultimately resulting in end-stage renal failure. These findings and those of the present study suggest that the chronic immunologic and nonimmunologic events in CAN may induce similar graft injuries, in particular, microvascular injury. Alternatively, since many factors involved in CAN induce similar graft
microvascular injury, resulting in a similar pattern of graft pathology, it is often difficult pathologically to ascertain the relative contribution of each factor to the development of CAN in the biopsies. With regard to the treatment of CAN, it is critically important to elucidate the pathogenic process by examining biopsy samples to help in the decision making regarding the early selection and use of specific therapy before the pathologic process progresses to end-stage renal scarring [44]. However, this is usually a very difficult task. Our results in the present study provide evidence that CAN is characterized by a progressive injury and loss of the renal microvasculature, especially PTCs, together with impairment of graft function. We propose, therefore, that maintenance of the PTC network is beneficial for the prevention of the development of CAN, and may represent a novel therapeutic strategy to combat progressive CAN.

CONCLUSION

Microvascular injury, subsequent capillary regression, and progressive loss of PTCs play important roles in the development of CAN and contribute to graft dysfunction.

A careful analysis of PTC injury in biopsies is important in the diagnosis of CAN and determination of the severity of graft damage. Furthermore, the PTC is a potential target for therapeutic intervention in CAN.

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