Lymphatic vessels develop during tubulointerstitial fibrosis

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Recent progress with specific markers of lymphatic vessel endothelium allowed recognition of lymphangiogenic events in various disease states; however, there is little information concerning this process in human chronic renal diseases. To determine this we measured expression of the lymphatic marker D2-40 and vascular endothelial growth factor-C (VEGF-C), a major growth factor in lymphangiogenesis, in 124 human renal biopsy specimens. In the kidneys of control subjects and in uninjured areas of pathologic specimens, lymphatic vessels were detected only around the arcuate and interlobular arteries. An increase in the number of lymphatic vessels was found at the site of tubulointerstitial lesions correlating with the degree of tissue damage and more strongly correlating with areas of fibrosis than inflammation. On serial sections, lymphatic vessel proliferation was found in the tubulointerstitial area at the site of tuft adhesions to Bowman’s capsule. Lymphatic growth was associated with VEGF-C expression in inflammatory mononuclear cells and tubular epithelial cells, mainly of proximal tubules. Lymphangiogenesis and VEGF-C expression was elevated in diabetic nephropathy in comparison to other renal diseases. Our results indicate that lymphangiogenesis is a common feature in the progression of the tubulointerstitial fibrosis.

Most commonly used endothelial markers, such as CD31, CD34, and von Willebrand factor, are non-discriminatory and are expressed by lymphatic endothelial cells. Therefore, the distribution of capillaries derived from the blood system and those of lymphatic origin is not clearly recognized owing to the absence of specific markers. Several markers that are exclusively, or at least predominantly, expressed by lymphatic endothelial cells were recently discovered, including the tyrosine kinase receptor for vascular endothelial growth factor receptor-3 (VEGFR-3), lymphatic endothelial hyaluronan receptor-1, podoplanin, the transcription factor Prox-1, and D2-40.1-6 D2-40 is a novel monoclonal antibody against a 40 000-Da O-linked sialoglycoprotein that reacts with a fixation-resistant epitope on lymphatic vessels.7 An antibody against D2-40 has been shown to detect lymphatic endothelium in formalin-fixed tissues and does not react with the blood vessel endothelium. These new markers of lymphatics enable one to identify neoplastic cells in the Kaposi sarcoma and recognize newly formed lymphatic vessels under diseased conditions.4,8-13

In the majority of organs, the lymphatic network is responsible for the removal of interstitial protein and fluid, thereby maintaining interstitial fluid balance and providing lymphatic clearance of macromolecules. Under physiological conditions, lymphatic vasculature is a rather quiescent system; however, several pathological conditions, including lymphatic vessel injury, inflammation, and cancer, are characterized by lymphatic vessel growth through stimulation by various growth factors. Lymphatic vessels provide key routes for drainage of interstitial fluid from tissues. In lymphangiogenesis, increased attention has been focused on the VEGF-C-VEGF3R system as a potentially important mediator.14-16 Gene transfer of VEGF-C resolved lymphedema through direct augmentation of lymphangiogenesis, whereas overexpression of VEGF-C or -D by gene transfer ameliorated lymph and inflammatory tissue edema in animal models of chronic airway inflammation, diabetic wound healing, and metastatic cancer.9,17-19 In addition to its role in fluid drainage, the lymphatic system also plays an important role in circulating immune surveillance and for metastatic dissemination of tumor cells through preexisting and possibly through newly formed lymphatic vessels.20,21

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Recently, Kerjaschki et al.\textsuperscript{5} showed lymphatic neoangiogenesis in renal transplants. However, the state of lymphatics in chronic renal diseases is unknown. In this study, we investigate the expression of D2-40-positive lymphatic vessels and VEGF-C, the most important mediator of lymphangiogenesis, in human renal diseases, and discuss the role of newly formed lymphatics.

RESULTS

Lymphatic vessels in control kidney tissues

Peritubular capillaries and glomerular endothelial cells were positive for CD31 in the normal structure of the renal cortex (Figure 1a and c). However, D2-40-positive lymphatic vessels were infrequently encountered in the normal cortical tubulointerstitial area (Figure 1b and d), and were restricted to the periarterial adventitia of large and intermediate-sized arteries. As shown in Figure 1d, D2-40-positive lymphatic vessels were detected adjacent to larger intrarenal branches of the renal artery to the level of the arcuate artery. Lymphatic endothelial cells positive for D2-40 were sometimes positive for CD31 (Figure 1e and f), which is consistent with previous reports that CD31 is not a specific marker for vascular endothelial cells.\textsuperscript{8}

Lymphatic vessels proliferated tubulointerstitial fibrosis

Lymphatic vessels in renal diseases. Increases in D2-40-positive lymphatics in tubulointerstitial injury are generally associated with glomerulonephritis and diabetic nephropathy (Figures 1g–k, 2 and 4). The uninjured areas of renal cortical interstitial tissues were universally indistinguishable from the interstitial patterns of sparse lymphatics, as described above in the control kidney. In the case of minimal change nephrotic syndrome, immunoglobulin A nephropathy (IgAN), and focal glomerulosclerosis without interstitial fibrosis, D2-40-positive lymphatic endothelial cells were rare in the tubulointerstitial area (grade 0, Figure 4b). In contrast, increased lymphatics were identified within injured tubulointerstitial areas in IgAN, FGS, type 2 diabetic nephropathy, tubulointerstitial nephritis (TIN), lupus nephritis, and anti-neutrophil cytoplasmic antibody-related glomerulonephritis (grade II and III, Figure 4a and b). Lymphatic endothelial cells were detected in tubulointerstitial fibrotic lesions (Figure 1g–j) and also in the interstitial area with numerous infiltrating cells (Figure 1k). Small lymphatic vessels are frequently filled with inflammatory cells in various renal diseases. Interestingly, there is much less proliferation of lymphatics in acute TIN than in chronic TIN (Figure 2b). In periglomerular fibrotic lesions, lymphatic vessels were often detected in diabetic nephropathy but not in non-diabetic glomerular diseases (Figure 1e and f). A significant correlation between the extent of tubulointerstitial fibrosis and the number of lymphatics per surface area was observed (Figure 2). Although there was a good correlation between the increase in lymphatic growth and \( \alpha \)-smooth muscle actin (\( \alpha \)-SMA)-positive fibroblasts and CD68-positive macrophages in the group of glomerular diseases other than

![Figure 1](https://example.com/figure1.png) Distribution of lymphatic vessels in control and diseased kidneys. D2-40-positive lymphatic vessels were rarely detected in the normal architecture of the cortical tubulointerstitial area (a, b). Lymphatics were observed around the arcuate artery in control kidneys (c, d). D2-40-positive lymphatic endothelial cells were sometimes positive for CD31 (e, f). Arrows indicate the same lymphatic vessels. Lymphatics proliferated in tubulointerstitial fibrosis and areas with interstitial infiltration (h, j, k). Clusters of inflammatory cells were observed in D2-40-positive vasculature (inset, k). Immunohistochemistry for CD31 (a, c, e), D2-40 (b, d, f, h, j, k), and Masson-Trichrome stain (g, i) on serial sections. Control kidney (a–d), diabetic nephropathy (e, f, i, j, k), IgA nephropathy (g, h). Bars = 100\( \mu \)m.
diabetic nephropathy, no significant association was seen in DMN. The number of lymphatic vessels also showed a stronger correlation with α-SMA-positive fibroblasts than with macrophages in glomerular diseases other than diabetic nephropathy and TIN (Figure 3).

**Lymphatic vessel proliferation was prominent in diabetic nephropathy.** The number of lymphatics was significantly higher in diabetic nephropathy, particularly with mild-to-moderate levels of tubulointerstitial fibrosis (grade I (P<0.0001) and II (P<0.05)), as compared with the same extent of tubulointerstitial injury in non-diabetic renal diseases (Figure 4a). When we compared the number and extension of lymphatic vessels in DMN against those in other renal diseases, lymphatics were significantly increased in DMN specimens, particularly when compared with IgAN (grade I (P<0.005) and II (P<0.05)), and TIN (grade III (P<0.05) (Figure 4b)).

**Lymphatic vessels grew in inflammatory interstitium and interstitial fibrosis induced by misdirected filtration.** To explore the possible roles of proliferation of the lymphatic vessel system in the tubulointerstitial area, we immunohistochemically examined the presence of D2-40 on sequential sections. Lymphatic growth was observed to transmigrate into the interstitial area accompanied by infiltration of mononuclear cells (Figure 5). The lumens of lymphatic vessels were frequently occupied by mononuclear cells in a high-power field on light microscopy (Figures 1k and 5). Lymphatic vessels with peripheral extension were confirmed to contact the trunk stream of the lymphatics adjacent to the interlobular arteries on serial sections (Figure 5).

In 10 out of 38 cases of IgAN and in 4 out of 20 case of DMN, we found tubular degeneration and interstitial fibrosis induced by misdirected filtration in glomerular injury with tuft adherence. Urinary fluid can be filtrated into the interstitium at tuft adherences to Bowman’s capsule (Figure 6, arrow heads in panels c and d, arrow in panel e). An analysis of serial sections in these cases confirmed numerous lymphatics in these areas. These networks might function to drain the misdirected filtrated fluid into the tubulointerstitial area (Figure 6). In these biopsy specimens, the distribution of lymphatic vessels was restricted around the affected nephron (Figure 6a and b, arrows).

In DMN, lymphatic vessels were often detected around the glomerulus in association with fibrotic changes (Figures 1e, f and 7). In these glomeruli, the glomerular tuft connects to the periglomerular interstitium through a gap in Bowman’s capsule (arrow in Figure 7, serial section numbers 7–11). Misdirected urinary filtration into the periglomerular space appears to enhance periglomerular fibrosis and lymphangiogenesis. These lymphatics were connected to the trunks (Figure 7). In some glomeruli in diabetic kidneys, lymphatic vessels were detected in the surroundings of the glomerulus without periglomerular fibrosis (Figure 1e and f). In contrast, these findings were not seen in non-diabetic renal diseases, including IgAN.

**Expression of VEGF-C in tubulointerstitial area**

**VEGF-C expression is accompanied by lymphatic proliferation in tubulointerstitial fibrosis.** In the normal control kidney, VEGF-C was weakly detected in tubular epithelial cells. In the tubulointerstitial area with tissue injuries, VEGF-C was expressed by interstitial mononuclear cells and was upregulated in tubular epithelial cells in association with the proliferation of lymphatic vessels. More than 80% of the VEGF-C expressing mononuclear cells were CD68 positive. VEGF-C was detected in the proximal tubules and in some parts of the collecting tubules in the injured tubulointerstitial area (Figure 8i–k). The VEGF-C expression score was higher in DMN (tubulointerstitial injury score, 2.17 ± 0.18) than in IgAN (tubulointerstitial injury score, 2.14 ± 0.15), with the same extent of tubulointerstitial damage (VEGF-C expression score, 2.59 ± 0.12 vs 1.52 ± 0.19, Figure 9).
Expression of VEGF-C in proximal tubular epithelial cells

To clarify the expression of VEGF-C by proximal tubular epithelial cells, we explored the expression of VEGF-C in rat proximal tubular epithelial cells harvested by Laser capture microdissection and in two human proximal tubular epithelial cell lines (Figure 10). VEGF-C mRNA and 18S rRNA were seen in normal rat proximal tubular epithelial cells. In cultured proximal tubular epithelial cells and PC-3 cells (prostate adenocarcinomas as a positive control), PCR products of VEGF-C and glyceraldehyde-3-phosphate dehydrogenase were detected, but no PCR product was yielded in the absence of cDNA (data not shown). enzyme-linked immunosorbent assay showed that VEGF-C protein was secreted into the supernatants by both HK-2 and RPTEC under serum-free conditions.

Figure 3 | Correlation between lymphangiogenesis and expression of α-smooth muscle actin (α-SMA)-positive fibroblasts and CD68-positive macrophages. (a, b) Glomerular diseases other than diabetic nephropathy. (c, d) Diabetic nephropathy. (e, f) Tubulointerstitial nephritis. (a, c, e) α-SMA. (b, d, f) CD68.
to transfer extravasated plasma protein and cells back into circulation. Recently, increases in lymphatic vessels have been reported in several conditions, including tumor metastasis, respiratory inflammatory diseases, wound healing, and granulation tissues in myocardial infarction. In the renal transplant rejection, lymphatic neoangiogenesis was seen as early as 72 h after transplantation. In the transplanted kidney, lymphatic neoangiogenesis was closely associated with inflammatory infiltrates and was correlated to allograft function at 1 year after transplantation. These reports suggested that lymphatic vessel proliferation in the cortex of a renal allograft may alter immune response to the graft, possibly owing to the attenuation of pathogenicity of cellular infiltrates. Under these physiological and the pathological conditions, inflammation and the accompanying fluid overload are considered to stimulate lymphangiogenic response.

Here, we investigated the expression of lymphatics in human renal diseases. In the normal kidney, lymphatic vessels are found only around interlobular and arcuate arteries, whereas in cortical areas without tubulo-interstitial injury, lymph vessels are rarely observed. However, in tubulo-interstitial fibrosis and inflammatory interstitial areas, lymphatic vessels proliferated and were often found to be filled with mononuclear cells in the lymphatic lumen. We showed that the extent of lymphatic growth was significantly correlated with tubulo-interstitial injury. Fibrosis is the final common pathway for almost all forms of renal disease that progress to end-stage renal failure, including immunologically mediated glomerulonephritis and TIN, hemodynamic and metabolic disorders, and hereditary diseases. Fibrosis is often accompanied by infiltration of inflammatory cells and tubular damage, which plays a role in accelerating the progression of interstitial fibrosis by production and secretion of cytokines and growth factors. Lymphatic vessels have been shown to expand into the tubulo-interstitial area with accumulation of mononuclear cells and/or with tubular atrophy and degeneration in chronic renal diseases. Although lymphatic vessels were grown in chronic TIN, lymphangiogenesis was not prominent in acute TIN, as recently reported by Heller et al. This suggests that chronic inflammation may trigger lymphangiogenesis. Kriz et al. used several animal models and human studies to establish that misdirected filtration of the tubulo-interstitium in glomerular injury with tuft adherence is an important pathway that leads to tubular degeneration and interstitial fibrosis. In the context of focal and segmental glomerulosclerosis (FSGS), misdirected filtration and peritubular filtrate spread may extend injury to the corresponding tubulo-interstitium, thus leading to severely injured nephrons. However, the final process of overflow by misdirected filtration is uncertain. We observed that lymphatic vessel density increased markedly in the tubulo-interstitial area, where urine is filtered in the wrong direction and inflammatory cells infiltrate. We also found that the lymphatic network developed around the glomerulus, with the paraglomerular and paratubular space filled with extracellular matrix. On the basis of recent reports.
of the possible role of lymphangiogenesis in diseased conditions, our findings suggest that filtered fluid and inflammatory cells may stimulate local lymphangiogenesis, and might be eliminated through proliferated lymphatics in chronic renal diseases. Lymphangiogenesis was correlated more strongly with α-SMA-positive expression than with infiltration of CD68-positive cells. In addition, growth of the lymphatic vessels was not prominent in acute TIN. Our observations suggest that lymphangiogenesis is affected by the duration of inflammation and progression of fibrosis, rather than by acute inflammation. It is noted that the extent of lymphangiogenesis with VEGF-C expression was higher in DMN than in other renal diseases. Furthermore, no correlation was seen between lymphatic growth, fibrosis, and infiltration in DMN. These results suggest that diabetic conditions, such as high glucose and AGE, may be related to the higher growth of lymphatics in DMN.

Vascular endothelial growth factor-C and -D, members of the VEGF family of polypeptide growth factors, drive lymphangiogenesis through VEGF receptor-3 (VEGFR-3, known as Flt4) signaling and partly through VEGFR-2 (Flk-1). VEGF-D, as well as VEGF-C, appears to induce angiogenesis through VEGFR-2. Other mediators, such as VEGF-A and PDGF, may induce lymphatic growth under certain conditions. Of these, the VEGF-C-VEGF3R system has been considered to be the main signaling pathway to induce lymphangiogenesis. In our analysis, lymphangiogenesis was found to be associated with VEGF-C expression in human renal biopsy specimens. VEGF-C was expressed by inflammatory CD68 positive mononuclear cells as reported. In addition, we found a strong expression of VEGF-C in the tubular epithelial cells, and that proximal tubules are one of the important sources of VEGF-C in renal diseases. Expression of VEGF-C by tubular epithelial cells may contribute to lymphangiogenesis, which shows a stronger correlation with fibrosis than inflammation. Stimulation of lymphatic growth was recently considered as a possible new target in some disease states. The relative importance of lymphangiogenesis will have to be established by manipulation of VEGF-C or -D levels in chronic renal injury models.

There have been some reports showing increased angiogenesis in DMN. Nevertheless, the role of VEGF-C in DMN has not yet been studied in detail. VEGFR-2, which is
one of the ligands for VEGF-C and stimulates vascular endothelial growth and angiogenesis, was shown to be elevated in streptozotocin-induced diabetic kidney.\textsuperscript{14,16,38} Upregulation of VEGF-C may be linked with both lymphangiogenesis and dysregulated blood vessel endothelial cell proliferation in DMN. Furthermore, the extent of lymphangiogenesis and VEGF-C expression was higher in DMN. Future studies are necessary to investigate the regulation and interaction between VEGF-C, and lymph and vascular neangiogenesis under diabetic conditions in animal and culture experiments.

In summary, we showed that lymphatic vessels proliferate and show good correlation with tubulointerstitial injury in chronic renal diseases. Inflammatory mononuclear cells and interstitial fluid, particularly owing to misdirected filtration, may trigger regional lymphatic angiogenesis, which leads to their transport. The clinical impact of local increases of lymphatic vessels in the tubulointerstitial area remains uncertain. Further enhancement of VEGF-C expression and lymphangiogenesis, and their role in chronic renal diseases and in DMN, will have to be established in the future.

### MATERIALS AND METHODS

#### Patient profiles and histological stratification

A total of 121 specimens from control (\(n = 9\)) and human diseased kidneys (\(n = 115\)) were studied (Table 1). Kidney specimens were obtained from patients undergoing diagnostic evaluation at Nagoya University Hospital (Nagoya, Japan). Biopsy kidney specimens taken 1 h after renal transplantation were used as controls. Histological diagnoses are listed in Table 1. These include minimal change nephrotic syndrome, IgAN, DMN, focal glomerulosclerosis, lupus nephritis, anti-neutrophil cytoplasmic antibody-related glomerulonephritis, TIN, and other renal diseases. All cases of acute TIN were drug-induced TIN, and chronic TIN was mainly because of Schögen’s syndrome. The mean duration from initiation of symptoms to renal biopsy of acute and chronic TIN was \(0.9 \pm 0.2\) and \(31.4 \pm 18.7\) months, respectively. Histological diagnosis was performed by light microscopy, immunofluorescence microscopy, and electron microscopy. Six representative serial sections for DMN (\(n = 3\)) and IgAN (\(n = 3\)) were subjected to immunohistochemistry, and were carefully examined to explore the role of lymphatic vessel development in the tubulointerstitial area.

### Table 1 | Renal biopsy cases evaluated for lymphatic vessels

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Number</th>
<th>Gender M/F</th>
<th>Age (years)</th>
<th>Hypertension</th>
<th>Proteinuria (g/day)</th>
<th>eGFR (ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9</td>
<td>6/3</td>
<td>37 ± 5.0</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Minimal change nephrotic syndrome</td>
<td>10</td>
<td>9/1</td>
<td>44.5 ± 6.0</td>
<td>4/10</td>
<td>4.9 ± 1.5</td>
<td>60.0 ± 6.9</td>
</tr>
<tr>
<td>IgA nephropathy</td>
<td>38</td>
<td>27/11</td>
<td>45.2 ± 3.0</td>
<td>20/38</td>
<td>2.5 ± 0.5</td>
<td>48.3 ± 3.6</td>
</tr>
<tr>
<td>DMN</td>
<td>20</td>
<td>16/4</td>
<td>60.7 ± 3.0</td>
<td>14/20</td>
<td>5.8 ± 1.1</td>
<td>41.4 ± 5.8</td>
</tr>
<tr>
<td>Nephrosclerosis</td>
<td>8</td>
<td>8/0</td>
<td>44.5 ± 6.3</td>
<td>8/8</td>
<td>1.6 ± 0.4</td>
<td>28.8 ± 2.7</td>
</tr>
<tr>
<td>TIN</td>
<td>16</td>
<td>10/6</td>
<td>52.7 ± 5.5</td>
<td>7/16</td>
<td>0.49 ± 0.11</td>
<td>23.2 ± 3.7</td>
</tr>
<tr>
<td>Acute TIN</td>
<td>7</td>
<td>5/2</td>
<td>40.6 ± 9.2</td>
<td>3/7</td>
<td>0.43 ± 0.13</td>
<td>27.5 ± 7.2</td>
</tr>
<tr>
<td>Chronic TIN</td>
<td>9</td>
<td>5/4</td>
<td>62.1 ± 5.1</td>
<td>4/9</td>
<td>0.50 ± 0.16</td>
<td>18.2 ± 2.9</td>
</tr>
<tr>
<td>Focal and segmental glomerulosclerosis</td>
<td>4</td>
<td>3/1</td>
<td>61.5 ± 6.7</td>
<td>3/4</td>
<td>4.3 ± 2.9</td>
<td>42.1 ± 14.2</td>
</tr>
<tr>
<td>Lupus nephritis</td>
<td>3</td>
<td>1/2</td>
<td>34.7 ± 4.2</td>
<td>0/3</td>
<td>6.0 ± 3.9</td>
<td>57.2 ± 26.8</td>
</tr>
<tr>
<td>ANCA-related glomerulonephritis</td>
<td>3</td>
<td>1/2</td>
<td>70.3 ± 9.4</td>
<td>3/3</td>
<td>1.7 ± 1.2</td>
<td>9.5 ± 4.1</td>
</tr>
<tr>
<td>Others</td>
<td>13</td>
<td>9/4</td>
<td>44.8 ± 5.6</td>
<td>8/13</td>
<td>3.2 ± 1.0</td>
<td>27.9 ± 6.7</td>
</tr>
</tbody>
</table>

Total: 124

Means ± s.e.

ANCA, anti-neutrophil cytoplasmic antibody; DMN, type 2 diabetic nephropathy; eGFR, endothelial growth factor receptor; IgA, immunoglobin A; TIN, tubulointerstitial nephritis.
Histology and immunohistochemistry

Formalin-fixed paraffin sections were deparaffinized with xylene, rehydrated, and washed with phosphate-buffered saline. After blocking endogenous peroxidase activity with 0.3% hydrogen peroxide and free protein-binding sites with normal goat serum (Dako, Glostrup, Denmark), tissues were incubated with primary murine monoclonal antibodies against D2-40 (Dako), α-SMA (1A4; Dako), CD68 (PG-M1; Dako), and CD31 (Dako) for 2 h at room temperature. After washing with phosphate-buffered saline, the sections were treated with a conjugate of polyclonal goat anti-rabbit IgG antibodies or anti-mouse IgG antibodies, and with horseradish peroxidase-labeled polymer (Histofine Simple Stain, Nichirei, Tokyo, Japan) as secondary reagents. Enzyme activity was finally developed with 3,3′-diaminobenzidine tetrahydrochloride and counterstained with hematoxylin.

Figure 7 | Immunohistochemistry for D2-40 on serial sections from patient with diabetic nephropathy. D2-40-positive lymphatic vessels are present around the glomerulus with periglomerular fibrosis. Glomerulus (g) with tuft adhesion to Bowman’s capsule (arrow) is associated with thick paraglomerular and paratubular space filled with matrix (indicated by open arrowhead, serial section numbers 7–26). Urinary orifice is narrowed by these spaces. Scheme indicates that the lymphatic vessel system connects to the lymphatic trunk. A, a: interlobular artery, g: glomerulus, *: urinary pole. Bars = 100 μm.

Figure 8 | Expression of vascular endothelial growth factor C (VEGF-C) and D2-40-positive lymphatic vessels in tubulointerstitial lesions on serial sections. VEGF-C is expressed in the proximal tubules (arrowhead) and in some parts of the collecting tubules (*) in the injured tubulointerstitial area. VEGF-C is negative in some parts of the proximal tubules (arrow). (a, c, e, g) D2-40 staining. (b, d, f, h, k) VEGF-C staining. (i) Aquaporin 1 staining. (j) Aquaporin 2 staining. (a, b) Control kidney. (c, d, g, h, i, j, k) Diabetic nephropathy. (e, f) IgA nephropathy. gl: glomerulus, GL: another glomerulus.
detected using a 3,3′-diaminobenzidine tetrahydrochloride liquid system (Dako).\textsuperscript{39,40} Immunohistochemistry for VEGF-C was performed on frozen sections in 53 cases, including 9 controls, 17 cases of DMN, and 27 cases of IgAN. The sections (4 μm) were cut with a cryostat, air-dried, and fixed in acetone at room temperature for 10 min. Endogenous peroxidase activity was inhibited with 0.1% NaN\textsubscript{3} and 0.3% hydrogen peroxide in phosphate-buffered saline, and nonspecific protein-binding sites were blocked with normal goat serum. The sections were incubated with rabbit anti-VEGF-C antibody (Zymed Laboratories, South San Francisco, CA, USA) or anti-D2-40 antibody, followed by a conjugate of anti-rabbit IgG antibodies, and with horseradish peroxidase-labeled polymer (Histofine Simple Stain, Nichirei) as secondary reagent. Enzyme activity was detected by 3-amino-9-ethyl-carbazole (Dako).\textsuperscript{39,40} To identify the tubular segments expressing VEGF-C in the diseased kidney, serial sections were stained with rabbit anti-Aquaporin 1 (marker of proximal tubules, Chemicon, Temecula, CA, USA) and rabbit anti-Aquaporin 2 (marker of collecting duct, Calbiochem, Darmstadt, Germany), as described previously.\textsuperscript{41}

**Correlation between number of lymphatic vessels and tubulointerstitial injury**

To assess the correlation between the number of interstitial lymphatic vessels and tubulointerstitial injury, D2-40-positive vessels, CD68-positive macrophages, and VEGF-C-positive cells were identified and counted in the whole cortex of biopsy specimens using Zeiss Z1 image microscopy and Axiovision Windows software, version 4.4 (Carl Zeiss, Oberkochen, Germany). Lymphatic vessel density in the whole cortical area was calculated and was expressed as lymphatic vessel density per square millimeter (/mm\textsuperscript{2}). Lymphatic vessels in the vicinity of interlobular arteries were not taken into account, as they were considered to be physiologically present in the normal kidney cortex.\textsuperscript{42} Tubulointerstitial injury was assessed as a percentage of blue stained area on Masson-trichrome staining, and was measured using MetaMorph 6.3 image analysis software (Universal Imaging Co., West Chester, PA, USA), followed by classification into four groups according to the extent of cortical interstitial fibrosis, and tubular atrophy and degeneration: (0) normal; (I) mild interstitial fibrosis and tubular atrophy (< 25% of cortical area); (II) moderate interstitial fibrosis and tubular atrophy (26–50% of cortical area); (III) severe interstitial fibrosis and tubular atrophy/loss (> 50% of cortical area). This is comparable with the chronic changes score in the Banff classification of kidney transplant pathology.\textsuperscript{43} VEGF-C expression in the tubulointerstitial area was analyzed and semiquantitatively classified into four groups by positive staining area: (0) no staining; (1) mild; (2) moderate; (3) pronounced staining. The α-SMA positive area was also assessed using MetaMorph 6.3 image analysis software.

**Laser capture microdissection using normal rat kidney**

Seven-week-old male Sprague Dawley rats (Japan SLC, Hamamatsu, Japan) were used. Frozen tissues were sectioned at 8 μm and mounted on glass slides using the RNase-free technique. The tissues were stained with an Ambion LCM (laser capture microdissection) Staining kit (Ambion, Austin, TX, USA) and stored until microdissection. Regions of proximal tubular epithelial cells were
dissected using the MB III System (PALM, Bernried, Germany), and were captured with PALM AdhesiveCaps 500 clear (PALM).

**Cell culture study**

Two human proximal tubular cell lines, HK-2 and RPTEC, were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and Takara Bio (Oshtsu, Shiga, Japan), respectively, and were maintained according to the manufacturers’ guidelines. Briefly, HK-2 cells and RPTEC were grown in complete medium containing Dulbecco’s Modified Eagle’s Medium and Ham’s F-12 (Sigma, Tokyo, Japan) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA) or 2% fetal bovine serum, and hormones in humidified air with 5% CO2 at 37°C, as described previously.4 Under subconfluent conditions, both cells were washed twice with phosphate-buffered saline, and the culture medium was replaced with a serum-free medium for 16 h.

**Secretion of VEGF-C into the cell culture supernatant** was determined using the Human VEGF-C Assay kit (IBL, Takasaki, Japan), according to the manufacturer’s instructions.45

**Polymerase chain reaction**

For proximal tubular cell lines, total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Total RNA concentrations were estimated using a spectrophotometer (Ultrospec 3300 pro, Amersham Biosciences, Tokyo, Japan). For laser-captured cells, RNA was isolated with RNeasy Micro Kits (Qiagen), and RNA integrity was assessed using the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA).46

**RNA preparation**

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**Polymerase chain reaction**

For cultured cells, first-strand cDNA was synthesized using the Omniscript Reverse Transcription Kit (Qiagen), according to the manufacturer’s instructions. PCR was performed using the HotStarTaq PCR kit (Qiagen), as described previously.45 PCR products for VEGF-C (865 bp) and glyceraldehydes-3-phosphate dehydrogenase (188 bp) were electrophoresed on 2% agarose gels in Tris-acetate EDTA buffer, followed by staining with ethidium bromide. Synthetic oligonucleotides sense (5’-AGTTTTTGCCAAATCCA CTTCTCGT-3’) and antisense (5’-GTCAATGTCAGAAAACCG TCTT-3’), and sense (5’-ATCATCCTGGCCTCTACTGG-3’) and antisense (5’-CCCTCGAGCGCTGCTCAC-3’) were used as specific primers for VEGF-C and glyceraldehydes-3-phosphate dehydrogenase, respectively.45

For laser capture microdissected samples, cDNA was synthesized with a Senscript RT Kit (Qiagen), preamplified by the TaqMan PreAmp Master Mix Kit using the GeneAmp PCR System 9700, and the TaqMan Gene Expression Assays for VEGF-C (assay identification number Rn00586458_m1) and 18S ribosomal RNA (4319413E), according to the manufacturer’s specifications (Applied Biosystems Inc., Foster City, CA, USA). PCR analysis was then performed with an Applied Biosystems Prism 7500HT Sequence Detection System with the same primers. PCR products for VEGF-C (115 bp) and 18S rRNA (187 bp) were then electrophoresed.

**Statistical analysis**

Values are expressed as means ± s.e. Comparisons among groups were performed by one-way analysis of variance, followed by Dunnett’s or Tukey’s HSD multiple comparison test. For statistical analysis between DMN and non-diabetic diseases for lymphatic vessel expression, the two-tailed Student’s t-test was performed. A comparison between groups for VEGF-C expression score was performed by the Mann-Whitney test. Differences were considered to be statistically significant at P < 0.05. All analyses were performed using SPSS (Chicago, IL, USA).

**DISCLOSURE**

All the authors declared no competing interests.

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**REFERENCES**


