Blood capillary rarefaction and lymphatic capillary neoangiogenesis are key contributors to renal allograft fibrosis in an ACE inhibition rat model

Hamar P, Kerjaschki D. Blood capillary rarefaction and lymphatic capillary neoangiogenesis are key contributors to renal allograft fibrosis in an ACE inhibition rat model. Am J Physiol Heart Circ Physiol 311: H981–H990, 2016. First published August 5, 2016; doi:10.1152/ajpheart.00320.2016.—Chronic allograft fibrosis is the major cause of graft loss in kidney transplantation. Progression can only be reduced by inhibition of the renin-angiotensin system (RAS). We tested the hypothesis that the protection provided by angiotensin-converting enzyme (ACE) inhibition also decreases capillary rarefaction, lymphangiogenesis, and podocyte injury in allograft fibrosis. Fisher kidneys were transplanted into bilaterally nephrectomized Lewis rats treated with enalapril (60 mg/kg per day) (ACE inhibitor, ACEi) or vehicle. Proteinuria, blood urea nitrogen, and plasma creatinine were regularly assessed, and grafts were harvested for morphological and immunohistological analysis at various times up to 32 wk. In the vehicle group, many new lymphatic capillaries and diffuse mononuclear infiltration of allografts were observed already 1 wk after transplantation. Lymphangiogenesis increased until week 4, by which time inflammatory infiltration became focal. Lymphatic capillaries were often located at sites of inflammation. Progressive interstitial fibrosis, glomerulosclerosis, capillary rarefaction, and proteinuria appeared later, at weeks 4–12. The number of lymphatic capillary cross sections strongly correlated with the interstitial fibrosis score. Podoplanin immunostaining, a marker of healthy podocytes, disappeared from inflamed or sclerotic glomerular areas. ACEi protected from lymphangiogenesis and associated inflammation, preserved glomerular podoplanin protein expression, and reduced glomerulosclerosis, proteinuria, tubulointerstitial fibrosis, and blood capillary rarefaction at 32 wk. In conclusion, ACEi considerably decreased and/or delayed both glomerulosclerosis and tubulointerstitial injury. Prevention of glomerular podoplanin loss and proteinuria could be attributed to the known intraglomerular pressure-lowering effects of ACEi. Reduction of lymphangiogenesis could contribute to amelioration of tubulointerstitial fibrosis and inflammatory infiltration after ACEi.

lymphangiogenesis; capillary rarefaction; chronic allograft fibrosis; glomerular podoplanin; angiotensin-converting enzyme inhibition

NEW & NOTEWORTHY

Beneficial effects of angiotensin-converting enzyme (ACE) inhibition in renal allograft fibrosis included preservation of podocyte podoplanin, reduction of proteinuria, and inhibition of tubulointerstitial fibrosis, lymphangiogenesis, and capillary rarefaction in rats. Reduction of lymphangiogenesis could contribute to amelioration of tubulointerstitial fibrosis and inflammatory infiltration after ACE inhibition.

ALLOGRAFT FIBROSIS IS A LEADING CAUSE of posttransplant graft failure with still largely undiscovered pathomechanisms. Capillary rarefaction (8, 12) has been described in renal fibrosis previously (26). We hypothesized that rarefaction of blood capillaries is accompanied by simultaneous proliferation of lymphatic vessels. Capillary cross sections have been assessed previously with antibodies not differentiating between lymphatic endothelial cells (LECs) or blood capillary endothelial cells (BECs).

The Fischer-to-Lewis (F344-to-LEW) rat kidney allograft is the most frequently used animal model of chronic allograft fibrosis. The two inbred strains differ only in one myosin heavy chain (MHC) I antigen, and a number of non-MHC antigens. If ischemia is short (<25 min) during transplantation, an initial acute rejection episode resolves spontaneously without any immunosuppression but induces a chronic allograft fibrosis. Pathological proteinuria begins around 12 wk after grafting, eventually leading to allograft fibrosis (14, 29).

Our previous study has shown that, during the initial rejection episode, radioactively labeled recipient lymphocytes infiltrate the graft temporarily and leave the graft shortly after infiltration. Labeled lymphocytes were found in the tubulointerstitium, suggesting that lymphocyte migration out of the graft could take place through lymphatic vessels (14). The role of lymphatic capillaries in the organization of inflammatory infiltration (6, 21) and fibrosis (10) has been suggested before. In this model, we did not observe signs of alloimmune-mediated injury with electron microscopy 32, 40, and 52 wk after transplantation, suggesting that the alloimmune process may cease during the late phase of chronic allograft fibrosis (32). The predominance of alloantigen-independent processes in the late phase of allograft fibrosis is also supported by retransplantation experiments (32). Following cessation or reduction of alloimmune processes, an alloantigen-independent mechanism may be primarily responsible for the self-perpetuation and progression of allograft fibrosis (15, 17).

Involvement of the renin-angiotensin system (RAS) is well documented in renal fibrosis, and RAS inhibition is the only approved treatment to slow progression of renal fibrosis (9, 16, 27, 35). RAS inhibition may act by reducing glomerular capillary pressure increase caused by hyperfiltration of remnant
nephrons (5), and RAS inhibition may reduce extracellular matrix accumulation by decreasing matrix production and stimulating matrix degradation (7, 17, 36).

In the present study, we evaluated tubulointerstitial fibrosis, inflammation, and glomerulosclerosis up to 32 wk after Fisher-to-Lewis allograft transplantation. The focus of the study was to follow lymphatic neoangiogenesis and blood capillary rarefaction, and the morphological changes were correlated with changes in blood urea concentration and urinary protein excretion. To reveal the role of RAS in the above changes, one group of rats was treated with enalapril.

**MATERIALS AND METHODS**

*Experimental animals.* Naive male inbred Lewis (LEW, RT1) and Fisher (F-344, RT1+) rats (2) at 8 wk of age were used throughout the experiment. All animals were obtained from Charles River Germany, through Akronym Kft (Budapest, Hungary). The rats were housed under standard conditions and received rat chow and water ad libitum. All experimental procedures were in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996), and the experimental protocol was reviewed and approved by the Institutional Ethical Committee for Animal Care and Use of Semmelweis University (approval number: XIV-1-001/2012-4/2012).

**Renal transplantation, follow-up of graft function, and end points.** Fisher rats served as donors and Lewis rats as recipients. Transplantation was performed as previously described (32). Briefly, the animals were anesthetized with pentobarbital sodium (Nembutal, 50 mg/kg, ip) and atropine sulfate (0.05 mg/kg) (18, 37). The left donor kidney was perfused with 4°C cold ringer lactate, removed, and positioned orthotopically into the recipient, whose renal vessels had been isolated and clamped with the left native kidney removed. End-to-end anastomosis of renal artery, vein, and ureter was performed using 10-0 prolene sutures. Total graft ischemia was set to 25 min. No immunosuppression was applied. Aseptic conditions were maintained throughout all surgical procedures. Postoperative care included morphine hydrochloride (2.5 mg/kg body wt, sc after the operation) and analgesia, and to prevent infectious complications during the perioperative phase, rats received 20 mg/kg per day Cephtriaxone (Rocephin) sc. (Roche Hungary, Budapest, Hungary) during the first 10 postoperative days, at which time the right native kidney was removed.

Animals were placed in metabolic cages (Techniplast, Buguggiate, Italy) for 24 h every 4 wk for the estimation of proteinuria. Body weight was measured, and blood was taken from the tail vein by the end of the urine collection period to measure blood urea nitrogen (BUN) and plasma creatinine. Serum and urine samples were stored at −80°C for later measurements. Urinary total protein concentrations were determined by the biuret method (Bio-Rad Laboratories, Munich, Germany). Absorbance was determined at 595 nm with a Philips PU8700 spectrophotometer. BUN and creatinine concentrations were determined photometrically with a Reflotron IV automat (Boehringer, Mannheim, Germany).

Experiments were terminated at different time points up to 32 wk. Under isoflurane narcosis (30, 42), rats were exsanguinated from the abdominal aorta, and kidney grafts were removed, cut, and fixed in 4% buffered formalin and embedded in paraffin (FFPE). All histopathological procedures were performed on paraffin-embedded sections.

**Experimental design.** Fischer-to-Lewis (F344-to-LEW) rat kidney allografts were investigated in two experimental settings. Nontransplanted naive Lewis rats at 8 wk of age were included as controls (n = 4).

In the first series, grafts were investigated 32 wk after engraftment to study capillary rarefaction and lymphangiogenesis with BEC- and LEC-specific antibodies (JG12 aminopeptidase and LF3 podoplanin, respectively). Transplanted animals received either usual drinking water (no therapy group, TxNoTh; n = 4) or an angiotensin-converting enzyme (ACE) inhibitor (ACEi) enalapril (Merck Sharp and Dohme, Whitehouse Station, NJ) (60 mg/l ACEi group, TxACEi; n = 4) throughout the study period.

In the second series, untreated allografts were harvested 1, 2, 4, or 16 wk after engraftment (n = 4/time) to investigate the time course and progression of allograft fibrosis, the podoplanin expression in glomeruli, the lymphangiogenesis, and lymphocytic infiltration.

**Histology.** For histology, FFPE kidney sections were stained with hematoxylin/eosin (HE), periodic acid-Schiff (PAS), or acid fuchsin orange-G (SFOG) to quantify fibrosis as protein deposits (1). We quantified the extent of glomerular sclerosis (GSI), interstitial fibrosis (IF), tubular atrophy (TA), and tubulointerstitial inflammation (TII) according to the BANFF scoring system (33, 34). Glomerulosclerosis was defined as an increase in glomerular mesangial matrix, focal adhesions to the Bowman’s capsule, or collapse of the glomerular capillaries (12). Slides were scored in a blinded fashion, semi-quantitatively from 0 to 4 [0: no histopathologic change; 1: mild, <25%; 2: moderate, 25–50%; 3: severe, >50%; 4: very severe change, >75% of the analyzed structures (glomeruli, tubules, interstitial fields) show pathologic changes (GSI, IF, TA, TII)] (35). Additionally, the percentage of normal glomeruli is given. A minimum of 50 glomeruli or

**Table 1. Body weight (g)**

<table>
<thead>
<tr>
<th>Week</th>
<th>Healthy</th>
<th>16 wk</th>
<th>TXACEi</th>
<th>TXNoTh</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>207 ± 13</td>
<td>183 ± 45</td>
<td>206 ± 22</td>
<td>210 ± 27</td>
</tr>
<tr>
<td>1</td>
<td>279 ± 27</td>
<td>264 ± 15</td>
<td>295 ± 21</td>
<td>290 ± 24</td>
</tr>
<tr>
<td>4</td>
<td>318 ± 18</td>
<td>303 ± 35</td>
<td>309 ± 27</td>
<td>304 ± 25</td>
</tr>
<tr>
<td>8</td>
<td>358 ± 39</td>
<td>330 ± 71</td>
<td>348 ± 22</td>
<td>336 ± 22</td>
</tr>
<tr>
<td>16</td>
<td>407 ± 48</td>
<td>350 ± 82</td>
<td>401 ± 32</td>
<td>334 ± 28*</td>
</tr>
<tr>
<td>24</td>
<td>507 ± 68</td>
<td>465 ± 31*</td>
<td>435 ± 32*</td>
<td>346 ± 26†</td>
</tr>
<tr>
<td>32</td>
<td>546 ± 72</td>
<td>350 ± 57†</td>
<td>465 ± 31*</td>
<td>346 ± 26†</td>
</tr>
</tbody>
</table>

Values are means ± SD. In the 16 wk experiment, only animals that survived ≥16 wk are included. *P < 0.05, †P < 0.001 vs. healthy (ANOVA). TxACEi, 32-wk-old allografts with angiotensin-converting enzyme inhibitor treatment. TXNoTh, 32-wk-old allografts with no treatment.

**Table 2. Proteinuria (mg/24 h)**

<table>
<thead>
<tr>
<th>Week</th>
<th>Healthy</th>
<th>16 wk</th>
<th>TXACEi</th>
<th>TXNoTh</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>27 ± 15</td>
<td>37 ± 13</td>
<td>16 ± 3</td>
<td>21 ± 6</td>
</tr>
<tr>
<td>8</td>
<td>28 ± 11</td>
<td>20 ± 3</td>
<td>31 ± 10</td>
<td>47 ± 6</td>
</tr>
<tr>
<td>12</td>
<td>33 ± 8</td>
<td>59 ± 13*</td>
<td>34 ± 14</td>
<td>62 ± 15*</td>
</tr>
<tr>
<td>16</td>
<td>40 ± 10</td>
<td>86 ± 15†</td>
<td>49 ± 9</td>
<td>114 ± 16†</td>
</tr>
<tr>
<td>20</td>
<td>45 ± 13</td>
<td>177 ± 24†</td>
<td>43 ± 9</td>
<td>80 ± 7†</td>
</tr>
<tr>
<td>24</td>
<td>30 ± 8</td>
<td>54 ± 12*</td>
<td>54 ± 12*</td>
<td>170 ± 28†</td>
</tr>
<tr>
<td>28</td>
<td>43 ± 11</td>
<td>78 ± 19†</td>
<td>78 ± 19†</td>
<td>257 ± 35†</td>
</tr>
<tr>
<td>32</td>
<td>40 ± 9</td>
<td>110 ± 12†</td>
<td>110 ± 12†</td>
<td>246 ± 43†</td>
</tr>
</tbody>
</table>

Values are means ± SD. In the 16-wk experiment, only animals that survived ≥16 wk are included. *P < 0.05, †P < 0.001 vs. healthy (ANOVA).
Antibodies and immunohistochemistry reagents. Monoclonal antibodies against macrophages (ED-1) were purchased from Serotec Camon Labor-Service (Wiesbaden, Germany). Lymphocytes were detected with CD45R (B cells) and CD43 (T cells). Endothelial cells were labeled with LF3 podoplanin found on LECs and podocytes or JG12, an aminopeptidase, found on vascular (blood) endothelial cells (25). Color reactions were performed with commercial kits for 3-aminon-9-ethilcarbasol (brick red) (ID Laboratories, London, Ontario, Canada), diaminobenzidine (brown) (Perbio 9b; Pierce, Rockford, IL), vector-blue (blue) (Vector Laboratories, Burlingame, CA), and vector-red (red) (Vector Laboratories).

Immunohistochemistry. Immunohistochemistry was performed on FFPE slides. Following deparaffination, antigens were retrieved in an auto-clave (20 min, 1.2 Bar, citrate buffer). The avidin-biotin method was used. Signals were retrieved with streptavidin and horseradish or alkaline phosphatase methods. Samples were counterstained with Giemsa. Lymphatic cross sections or cells staining positive were counted and expressed as lymphatic capillaries or cells per fv. At least 20 fv sections per specimen were counted at ×400 magnification.

Statistics. Results are presented as means ± SD. The normality of data was checked by Shapiro-Wilk W-test, and homogeneity of variances was checked by Bartlett’s test. Discrete variables were compared using one-way ANOVA, followed by Dunnett’s multiple-comparison post hoc test vs. healthy rats. Continuous variables were compared using Kruskal-Wallis test followed by Dunn’s multiple-comparison post hoc test vs. healthy rats. Discrete variables were compared using Student’s unpaired t-test or Mann-Whitney U-test. Linear correlation was assessed by Pearson product-moment correlation coefficient. The null hypothesis was rejected if the P value reached statistical significance (P < 0.05).

RESULTS

Functional studies. Body weight increased during the observation period. Weight gain was slower in the TxNoTh group vs. the TxACEi group from week 16 (Table 1).

In all recipients, proteinuria progressed over time (Table 2). Pathological proteinuria started to develop at week 12 in untreated animals. Proteinuria initiated later and progressed slower in the TxACEi group.

Table 3. BUN retention (mg/dl)

<table>
<thead>
<tr>
<th>Exp. Group</th>
<th>1</th>
<th>4</th>
<th>8</th>
<th>16</th>
<th>20</th>
<th>24</th>
<th>28</th>
<th>32</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>13 ± 5</td>
<td>20 ± 6</td>
<td>22 ± 8</td>
<td>19 ± 11</td>
<td>24 ± 6</td>
<td>17 ± 3</td>
<td>21 ± 9</td>
<td>20 ± 7</td>
</tr>
<tr>
<td>1 wk</td>
<td>20 ± 5</td>
<td>17 ± 2</td>
<td>30 ± 6</td>
<td>29 ± 7</td>
<td>38 ± 13*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16 wk</td>
<td>15 ± 3</td>
<td>21 ± 2</td>
<td>32 ± 7</td>
<td>28 ± 4</td>
<td>25 ± 6</td>
<td>41 ± 9†</td>
<td>38 ± 14*</td>
<td>53 ± 22†</td>
</tr>
<tr>
<td>TxACEi</td>
<td>22 ± 4</td>
<td>18 ± 2</td>
<td>33 ± 9</td>
<td>61 ± 12†</td>
<td>102 ± 7†</td>
<td>88 ± 16†</td>
<td>134 ± 9†</td>
<td>152 ± 9†</td>
</tr>
</tbody>
</table>

Values are means ± SD. *P < 0.01, †P < 0.001 vs. healthy (ANOVA). BUN, blood urea nitrogen.

The deterioration of renal function further manifested in elevated BUN. BUN retention reached pathological range only 16–20 wk after transplantation, and the most severe retention was in the TxNoTh group (Table 3).

Plasma creatinine was below the detection limit of the Reflotron method (<44 μmol/l) during the first 16 wk of the study. Plasma creatinine started to rise at week 16 in the TxNoTh group but reached detectable levels in the TxACEi group only on week 32 (Table 4).

Light microscopy and immunohistology: Inflammation and fibrosis of the allografts. In the time-course experiment on SFOG- and PAS-stained slides (Table 5, Fig. 1) at week 1, severe, diffuse mononuclear infiltration was the predominant finding. Mononuclear infiltration was occasionally accompanied by interstitial edema. Interstitial edema was detected as unstained, clear areas between tubuli with filamentous structures in inflamed areas. Signs of fibrosis were absent at week 1. At week 2, infiltration was still diffuse in some grafts but became focal in others. Tubuli themselves, however, were not visibly damaged. Sclerosis of glomeruli (FSGS) was focal if present. FSGS and TA progressed by week 4. Fibrotic matrix appeared (IF) in the interstitium; however, inflammatory infiltration did not change significantly.

At week 8, IF, TA, and FSGS became severe and progressed further by week 16, whereas inflammatory infiltration remained focal.

PAS and SFOG staining of 32-wk-old grafts (Table 5, Fig. 1) revealed focal sclerosis (grade I–II) of glomeruli in the TxACEi group compared with a more global (grade III-IV) sclerosis in the TxNoTh group. Most glomeruli showed some sclerosis in the TxNoTh group, whereas many glomeruli were still normal appearing in TxACEi grafts. IF was mild or absent in TxACEi grafts in contrast to significant IF in TxNoTh. Many tubuli were severely atrophic with flat epithelial cells, and hyaline-filled tubular cross sections were common in the TxNoTh but not in the TxACEi group. Inflammatory foci were seldom in the TxACEi group, but mononuclear infiltration was common and diffuse in TxNoTh rats.

Type of interstitial capillaries. Double immunostaining of 32-wk-old allografts for podoplanin (LF3) on LECs and podocytes and aminopeptidase (JG12) on BECs (Table 6, Fig. 2)
demonstrated a significantly higher number of lymphatic and lower number of blood capillary cross sections in the TxNoTh group compared with healthy native kidneys or TxACEi grafts. Blood capillaries were reduced in number, and the regular appearance of peritubular capillaries was lost as diameter inhomogeneity and tortuosity of JG12-positive capillaries were observed in fibrotic areas of allografts in the TxNoTh group.

### Table 5. Allograft fibrosis and inflammation

<table>
<thead>
<tr>
<th>Group</th>
<th>Time</th>
<th>Normal Glomeruli, %</th>
<th>GSI Score</th>
<th>IF Score</th>
<th>TA Score</th>
<th>TII Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td></td>
<td>98.0 ± 1.0</td>
<td>0.1 ± 0.1</td>
<td>0.0 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>16 wk</td>
<td></td>
<td>82.0 ± 17.0</td>
<td>0.4 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>3.4 ± 0.4</td>
</tr>
<tr>
<td>2 wk</td>
<td></td>
<td>47.0 ± 11.0</td>
<td>1.4 ± 0.2</td>
<td>0.3 ± 0.2</td>
<td>0.8 ± 0.4</td>
<td>2.2 ± 0.6</td>
</tr>
<tr>
<td>4 wk</td>
<td></td>
<td>12.0 ± 2.0</td>
<td>2.1 ± 0.4</td>
<td>1.8 ± 0.3</td>
<td>1.3 ± 0.5</td>
<td>1.6 ± 0.6</td>
</tr>
<tr>
<td>8 wk</td>
<td></td>
<td>4.0 ± 4.0</td>
<td>3.4 ± 0.3</td>
<td>2.0 ± 0.8*</td>
<td>2.2 ± 0.6*</td>
<td>1.3 ± 0.7*</td>
</tr>
<tr>
<td>16 wk</td>
<td></td>
<td>6.0 ± 3.0</td>
<td>3.1 ± 0.5</td>
<td>2.4 ± 0.8†</td>
<td>2.5 ± 0.5†</td>
<td>1.1 ± 0.3†</td>
</tr>
<tr>
<td>TxACEi</td>
<td>32 wk</td>
<td>18.0 ± 3.0</td>
<td>1.8 ± 0.2</td>
<td>1.0 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>TxNoTh</td>
<td></td>
<td>0.0</td>
<td>3.6 ± 0.4</td>
<td>2.5 ± 0.4‡</td>
<td>2.7 ± 0.2‡</td>
<td>1.9 ± 0.3‡</td>
</tr>
</tbody>
</table>

Values are means ± SD. Scores are based on 50 glomeruli or 20 fields of view at ×400. *P < 0.05, †P < 0.01 vs. healthy, ‡P < 0.05 vs. TxACEi. GSI, glomerular sclerosis; IF, interstitial fibrosis; TA, tubular atrophy; TII, tubulointerstitial inflammation.

Fig. 1. Evolution of tubulointerstitial fibrosis and inflammation up to 32 wk after transplantation. Fischer-to-Lewis (F344-to-Lew) allografts 1, 2, 4, 8, 16, and 32 wk after engraftment are shown with acid fuchsin orange-G staining; magnification ×400, scale bar = 100 μm. A: healthy rat kidney. B: week 1. Severe interstitial inflammatory infiltration is seen, with no signs of fibrosis. C: week 2. Varying severity or focal interstitial infiltration and interstitial edema (pale areas between tubular cross sections) are seen. Extracellular matrix deposition is in parts of glomeruli. D: week 4. Focal interstitial inflammation and matrix deposition (interstitial fibrosis, IF) are shown with low-grade (grade I–II) sclerosis of glomeruli (FSGS) in some glomeruli. E: week 8. Severe IF and tubular atrophy (TA) with extensive cast formation are seen, with sclerotic or collapsed glomeruli (grade III–IV). F: week 16. Extensive IF and TA are seen. Casts are in tubuli. G: week 32 (transplanted animal group with angiotensin-converting enzyme inhibitor, TxACEi). FSGS grade: I, mild tubulointerstitial fibrosis. H and I: week 32, transplanted animal group with no therapy (TxNoTh). These groups had almost completely obliterated glomeruli (FSGS grade IV), severe IF, and severe tubulointerstitial inflammatory infiltration (TII). I: TA and hyaline-filled tubular cross sections are seen.
Table 6. Immunostaining: Lymphatic and blood capillary cross sections and podoplanin-negative glomeruli

<table>
<thead>
<tr>
<th>Group</th>
<th>Time</th>
<th>Lymphatic Cross Sections/fv</th>
<th>Blood Capillary Cross Sections/fv</th>
<th>LF3-Negative Glomeruli, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>1 wk</td>
<td>1.2 ± 0.3</td>
<td>25.0 ± 3.0</td>
<td>3.0 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>16 wk</td>
<td>7.0 ± 3.0</td>
<td>21.0 ± 5.0</td>
<td>2.0 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>2 wk</td>
<td>10.0 ± 6.0*</td>
<td>18.0 ± 4.0</td>
<td>11.0 ± 4.0</td>
</tr>
<tr>
<td></td>
<td>4 wk</td>
<td>8.0 ± 6.0</td>
<td>6.0 ± 5.0</td>
<td>25.0 ± 8.0</td>
</tr>
<tr>
<td></td>
<td>8 wk</td>
<td>11.0 ± 5.0*</td>
<td>7.0 ± 6.0</td>
<td>29.0 ± 9.0</td>
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<tr>
<td></td>
<td>16 wk</td>
<td>13.0 ± 7.0†</td>
<td>5.0 ± 4.0</td>
<td>44.0 ± 6.0†</td>
</tr>
<tr>
<td>TxACEi</td>
<td>32 wk</td>
<td>5.0 ± 3.0</td>
<td>11.0 ± 9.0</td>
<td>57.0 ± 9.0†</td>
</tr>
<tr>
<td>TxNoTh</td>
<td></td>
<td>11.0 ± 4.0‡</td>
<td>4.0 ± 4.0</td>
<td>83.0 ± 5.0*‡</td>
</tr>
</tbody>
</table>

Values are means ± SD. Lymphatic endothelial cell (LF3 podoplanin) and blood capillary endothelial cell (JG12 aminopeptidase) staining was conducted. *P < 0.05, †P < 0.01 vs. healthy kidneys. ‡P < 0.05 vs. TxACEi. fv: field of view at ×400.

Fig. 2. Changes in the density of peritubular and lymphatic capillaries up to 32 wk after transplantation. F344-to-Lew allografts 2, 4, 16, and 32 wk after engraftment are shown with double immunostaining. An overview of interstitial capillary types (A–F, magnification ×200) is provided. Glomeruli and a lymphatic capillary with inflammatory cells (G–I, magnification ×400, scale bar = 100 μm) are seen. LF3:podoplanin (1:1,000) on podocytes and lymphatic capillaries (3-amino-9-ethylcarbasol, AEC: brick red) + JG12:aminopeptidase (vector blue) are shown. A: healthy rat kidney. Two lymphatic (*) and 41 blood (x) peritubular capillary cross sections are shown. B–D: weeks 2, 4, and 16. In several lymphatic capillary cross sections and peritubular capillary rarefaction, peritubular capillaries become irregular in shape and distribution and less in number. E: week 32, TxNoTh. Many (25) lymphatic (*) capillaries, severely dilated peritubular capillaries, and peritubular capillaries are irregular in size and distribution. Almost completely obliterated glomeruli (+, FSGS grade IV) with missing podoplanin staining are shown. F: week 32, TxACEi. Lymphatic capillaries are rare (5), and peritubular capillaries appear in regular fashion like in a healthy kidney. Glomerular podoplanin staining is present. G–I: glomeruli became podoplanin negative already at weeks 2 (G) and 4 (H), but glomerular podoplanin was preserved to some extent in TxACEi (I). G: mononuclear infiltration and 2 lymphatic capillary cross sections in the interstitium. Lymphatic capillaries are shown with mononuclear cells (arrow).
Investigating the grafts at earlier times with (LF3) immunostaining (Table 6, Fig. 3) or double staining (Fig. 2) demonstrated that already 1 wk after transplantation numerous LF3+/H11001 lymphatic capillaries appeared in the tubulointerstitium. Lymphatic capillaries were often associated with mononuclear infiltration (lymphangiogenesis with nodular infiltrates) (Fig. 4). Thereafter, lymphatic cross sections with nodular infiltrates were present throughout the 16-wk observation period. The number of lymphatic capillary cross sections correlated with the severity of tubulointerstitial fibrosis (IF score) (Fig. 6). Aminopeptidase (JG12)-positive blood capillaries were present in high numbers still 2 wk after transplantation but were significantly reduced and irregular thereafter (Fig. 2). On the other hand, ACE inhibition preserved the morphology and number of peritubular capillaries (Fig. 2).

Type of inflammatory infiltration. Investigating the time course of triple immunostaining for T and B lymphocytes and podoplanin revealed that mononuclear infiltrating cells were mostly T (CD43+/H11001) and less B (CD45R+/H11001) lymphocytes and only a few macrophages (ED1+) (Fig. 5) in nonimmunosuppressed F344-to-LEW renal allografts.

Mononuclear infiltration was most severe and diffuse at day 7, at which time T cells were predominant. Infiltration became focal in most allografts at day 14 when B cells also appeared in large numbers. One month after transplantation mononuclear infiltration became focal and contained numerous B cells besides T lymphocytes.

In 32-wk-old allografts, focal mononuclear infiltrations were accompanied by LF3+/H11001 lymphatic capillary cross sections (Fig. 6). Some lymphatic capillaries contained various numbers of

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Fig. 3. Changes in the immunohistochemical staining of podoplanin in glomeruli and lymphatic capillaries up to 32 wk after transplantation. F344-to-Lew allografts 1, 2, 4, 8, 16, and 32 wk after engraftment are shown with magnification ×400; scale bar = 100 μm. LF3 (podoplanin on podocytes and lymphatic capillaries is shown; diaminobenzidine, DAB, is brown). A: normal kidney. Podoplanin-positive glomeruli and no lymphatic capillaries are seen. B: week 1. Severe, diffuse inflammatory infiltration with lymphatic capillary cross sections in the interstitium is seen. C: week 2. Focal infiltration with edema and lymphatic capillaries is seen. Partially sclerotic glomerulus with podoplanin positivity is in the healthy part. D: week 4. Reduced glomerular podoplanin staining in 2 glomeruli and moderate interstitial inflammation with 3 lymphatic cross sections are seen. E: week 8. Eight lymphatic cross sections, interstitial inflammation, and tubular hyaline are shown. F: week 16. Podoplanin-negative glomerulus and lymphatic capillaries in the interstitium with mononuclear infiltration are shown. G: lymphatic capillary with infiltrating mononuclear cells (arrow) (2 wk after engraftment). H: week 32, TxACEi. Podoplanin-positive glomerulus and normal interstitium are shown. I: week 32, TxNoTh. Podoplanin-negative glomerulus, focal inflammation, and several (14) lymphatic capillaries are shown.
mononuclear cells in their lumen. The inflammatory infiltrates were still mostly CD43+ T lymphocytes both at 8 and 32 wk after engraftment, and most of the infiltrating cells were ED-1 negative both in the TxNoTh and TxACEi groups.

Glomerular podoplanin staining. In healthy rat kidneys (Fig. 2A), glomerular capillaries have a double-positive contour for both the blood (aminopeptidase JG12) and the lymphatic (podoplanin LF3) endothelial markers. At sites of inflammatory infiltration, glomeruli were LF3 (podoplanin) negative already 1 wk after transplantation (Figs. 2 and 3). Besides areas of interstitial infiltration, sclerotic areas of glomeruli were also podoplanin negative (Figs. 2 and 3). In 32-wk-old allografts, most of the glomeruli were podoplanin negative in the TxNoTh group, whereas more glomeruli expressed some podoplanin in the TxACEi group (Table 6). The ratio of unstained glomeruli correlated closely with proteinuria (Fig. 7).

DISCUSSION
The main finding of the study was that the F344-to-LEW chronic allograft fibrosis model can be characterized by lymphangiogenesis accompanied by nodular tubulointerstitial lymphocyte infiltration and fibrosis as well as a gradual loss of podoplanin from glomeruli. Besides areas of nodular interstitial inflammation, lymphatic capillaries were observed around sclerotic glomeruli; thus lymphatic capillaries may be involved in inflammatory and fibrotic processes. The observed rarefaction of peritubular capillaries may contribute to the progression
of fibrosis by inducing hypoxia, as reviewed recently (12). As angiotensin II induces vascular dysfunction (22), antifibrotic benefits of ACE inhibition may be attributed to prevention of blood capillary rarefaction (3).

Lymphangiogenesis as a contributor to renal fibrosis is well established (19). Similar lymphangiogenesis with nodular infiltrates has been described in kidney fibrosis also in the remnant kidney model (25) and in human kidney allografts (10). The novelty of our study is the demonstration of lymphangiogenesis with nodular infiltrates in the F344-to-LEW allograft model of chronic allograft fibrosis. Furthermore, the correlation of lymphatic neoangiogenesis with nodular infiltrates and the severity of allograft fibrosis is described here for the first time. Similar progressive rarefaction of blood capillaries was associated with renal fibrosis, and the antifibrotic effects of angiotensin antagonism were attributed to preservation of renal blood capillaries recently (31). However, the importance of blood capillaries in allograft fibrosis has not been demonstrated before.

Proteinuria, BUN, and creatinine were similar to previously reported data in control animals (11, 27). Better-preserved renal function and less proteinuria manifested in better preserved body weight gain in ACEi-treated rats. Proteinuria has been suggested to induce tubulointerstitial lymphangiogenesis (40) before the onset of interstitial inflammation and fibrosis (39). Proteinuria-induced lymphangiogenesis could explain the observed increase in interstitial lymphatic neoangiogenesis in our proteinuric rats. Lymphatic capillaries were predominantly

![Image](https://example.com/image1)

**Fig. 5.** Scarce macrophage but intense T-cell infiltration at 8 and 32 wk after transplantation. F344-to-Lew allografts 32 and 8 wk after engraftment are shown. Immunostaining, with magnification ×400 was performed; scale bar = 100 μm. ED1 (macrophage marker, vector red), CD43 (T cells, DAB, brown) was used. A and B: week 32. Foci of mononuclear infiltration were mostly ED1 negative in both the No Therapy (A) and ACEi (B) groups. C and D: CD43 staining (DAB) of 32-wk-old (C) and 8-wk-old (D) allografts. Most infiltrating cells are CD43 positive T cells.

![Image](https://example.com/image2)

**Fig. 6.** Correlation between the number of lymphatic capillary cross sections (LF3) and IF score. LF3-positive lymphatic capillary cross sections were counted per field of view under ×400 magnification and correlated with the semiquantitative IF score. Correlation statistics: y = 0.1198x + 0.337. R² = 0.3475.

![Image](https://example.com/image3)

**Fig. 7.** Correlation between the percentage of podoplanin-negative glomeruli and daily urinary protein excretion. Correlation statistics: y = 0.2668x + 19.43. R² = 0.9119.
The other hand, proteinuria and edema of the fibrotic tissue associated with proteinuria, similarly to the effect of anti-podoplanin described previously (4). Loss of podoplanin could be associated with glomerular sclerosis, podoplanin loss, and proliferation of lymphatic capillaries. Glomerular podoplanin loss correlated with proteinuria, which has been suggested to induce proliferation of lymphatic capillaries and tubulointerstitial fibrosis. Tubulointerstitial fibrosis can cause blood capillary rarefaction, which in turn can worsen fibrosis attributable to tissue hypoxia. ACE inhibition has been described to reduce intraglomerular pressure and inhibit fibrosis.

observed in inflamed areas. Thus lymphatic neangiogenesis may be triggered by proteinuria, but the strong association of lymphatic neangiogenesis with inflammatory infiltration of the tubulointerstitium suggests a possible active role of lymphatic capillaries in inflammation. Indeed, lymphangiogenesis influences the progression of fibrosis by promoting inflammatory infiltration (13, 23). Also, lymphangiogenesis can develop as a consequence of fibrosis (19). Following subtotal nephrectomy (25) or renal transplant rejection (20) as well as left ventricular remodeling (38, 41), VEGF-C produced by inflammatory macrophages induces massive lymphangiogenesis, and angiotensin II promotes monocyte and macrophage recruitment (28). Taken together, a vicious circle of fibrosis-induced lymphangiogenesis and inflammation promoted by lymphatic capillaries propagates fibrosis.

Glomerular podoplanin loss was related to inflammatory infiltration in the surrounding tubulointerstitium or was related to sclerosis of the glomeruli. Glomerular podoplanin loss may be an early marker of glomerular damage, even before the appearance of fibrotic matrix deposition in glomeruli. Loss of podoplanin from podocytes in glomerular disease has been described previously (4). Loss of podoplanin could be associated with proteinuria, similarly to the effect of anti-podoplanin IgG injection reported earlier (24). Our study underlines the important role of podocytes in the hyperfiltration-induced glomerular sclerosis.

Conclusions. The known beneficial effects of ACEi therapy in reducing allograft fibrosis may be explained in part by reducing glomerular capillary pressure and consequent podocyte damage. Podoplanin loss in glomeruli is a marker of podocyte damage, which ultimately causes proteinuria. Proteinuria is thought to initiate and sustain tubulointerstitial fibrosis accompanied with blood capillary rarefaction. Rarefaction correlated with fibrosis. The amelioration of rarefaction by ACEi therapy suggests an ethological role of rarefaction dependent on hypoxia in the progression of fibrosis (Fig. 8). On the other hand, proteinuria and edema of the fibrotic tissue stimulate lymphangiogenesis. The observation of lymphatic capillaries with T and B lymphocytes at sites of fibrosis and the fact that the extent of lymphangiogenesis correlated with proteinuria and fibrosis suggest an etiological role of lymphatic capillary neangiogenesis in inflammation and fibrosis. Neither focal infiltration nor lymphatic capillary numbers progressed significantly during the observation period, suggesting a self-limiting process in this inflammation. We hypothesize that lymphatic capillaries may have a regulatory role contributing to the self-limiting nature of the inflammation.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

P.H. and D.K. conception and design of research; P.H. and D.K. performed experiments; P.H. and D.K. analyzed data; P.H. and D.K. interpreted results of experiments; P.H. prepared figures; P.H. drafted manuscript; P.H. and D.K. edited and revised manuscript; P.H. and D.K. approved final version of manuscript.

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