Increased Expression of Vascular Endothelial Growth Factor in Kidney Leads to Progressive Impairment of Glomerular Functions

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ABSTRACT
Vascular endothelial growth factor (VEGF) is an important mediator in maintaining normal kidney functions. In addition, several lines of evidence suggest that upregulation of VEGF in glomeruli may be associated with or cause renal dysfunction such as diabetic nephropathy. For elucidation of the pathologic consequences of high levels of VEGF in glomeruli, transgenic (Tg) rabbits that express human VEGF165 isoform in both kidney and liver under the control of the human α1-antitrypsin promoter were generated and characterized. With the use of heterozygous Tg rabbits and their littermates aged 8 to 55 wk, renal functions and structures were investigated. Compared with control rabbits, Tg rabbits exhibited progressive proteinuria with increased GFR at the early stage and decreased GFR at the later stage. Histologic examinations revealed that Tg rabbit kidneys were characterized by considerable glomerular hypertrophy as a result of increased proliferation of both glomerular capillaries and mesangial cells accompanied by prominent podocyte hypertrophy. With increasing age starting from 20 wk, Tg rabbit kidneys showed prominent formation of microaneurysms and capillary proliferation at the vascular pole area. At a later stage (55 wk), many glomeruli showed sclerosis and tuft collapse with the formation of glomerular cysts on a background of tubular atrophy and interstitial fibrosis. This study provides the first evidence that increased expression of VEGF in glomeruli directly causes the glomerular hypertrophy that is associated with proteinuria, suggesting that VEGF exerts multiple effects on the glomerular pathophysiologic processes.


Vascular endothelial growth factor (VEGF) is an important mediator in maintaining normal kidney functions. In addition, several lines of evidence suggest that upregulation of VEGF in glomeruli may be associated with or cause renal dysfunction such as diabetic nephropathy. For elucidation of the pathologic consequences of high levels of VEGF in glomeruli, transgenic (Tg) rabbits that express human VEGF165 isoform in both kidney and liver under the control of the human α1-antitrypsin promoter were generated and characterized. With the use of heterozygous Tg rabbits and their littermates aged 8 to 55 wk, renal functions and structures were investigated. Compared with control rabbits, Tg rabbits exhibited progressive proteinuria with increased GFR at the early stage and decreased GFR at the later stage. Histologic examinations revealed that Tg rabbit kidneys were characterized by considerable glomerular hypertrophy as a result of increased proliferation of both glomerular capillaries and mesangial cells accompanied by prominent podocyte hypertrophy. With increasing age starting from 20 wk, Tg rabbit kidneys showed prominent formation of microaneurysms and capillary proliferation at the vascular pole area. At a later stage (55 wk), many glomeruli showed sclerosis and tuft collapse with the formation of glomerular cysts on a background of tubular atrophy and interstitial fibrosis. This study provides the first evidence that increased expression of VEGF in glomeruli directly causes the glomerular hypertrophy that is associated with proteinuria, suggesting that VEGF exerts multiple effects on the glomerular pathophysiologic processes.


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VEGF exerts a number of physiologic and pathophysiologic effects on the kidneys. It is widely known that VEGF is essential for normal nephrogenesis, particularly glomerulogenesis, and homozygous deletion of VEGF genes in glomeruli resulted in perinatal lethality of knockout mice. Ample data suggest that increased VEGF may be involved in the pathogenesis of glomerulopathies such as diabetic nephropathy and FSGS. VEGF and its receptors are upregulated in the diabetic states of experimental animals. Eremina et al. showed that podocyte-specific overexpression of the VEGF164 isoform in mice resulted in renal failure as a result of a collapsing glomerulopathy. Several reports showed that there may be a close relationship between plasma levels of VEGF and proteinuria. The data collected thus far suggest that constitutive expression of VEGF in the glomerular epithelial cells essentially maintains normal glomerular functions and provides a filtration barrier, whereas aberrant increased expression of VEGF may potentially be detrimental in terms of renal functions, although this hypothesis has not been verified.

Although previous studies so far have established a link between increased VEGF and glomerular injury and proteinuria, it remains unknown whether increased VEGF is causative of or simply a consequence of these pathologic processes. In fact, there is still controversy regarding the beneficial and deleterious effects of VEGF on the kidney because some studies even suggested that VEGF may protect against renal injury. However, the precise elucidation of the direct pathophysiologic effects of VEGF in the previous studies may have been complicated by the presence of other factors, such as advanced glycation end products, plasminogen, and high levels of plasma glucose. It is also not clear whether glomerular VEGF can directly affect the filtration of the glomerulus or plays a central role in the pathogenesis of glomerulopathies and proteinuria.

One of the valid and powerful methods to answer this “cause-and-effect” question may rely on the application of transgenic (Tg) animal models in which VEGF is overexpressed in glomeruli while excluding other interfering factors such as high levels of blood glucose. To achieve this goal, our laboratory generated Tg rabbits to study the relationship between VEGF and glomerulopathies. The rabbit was selected for this undertaking because of its usefulness in the study of vascular biology and because the large size of rabbits compared with rodents may also facilitate many biochemical analyses of the plasma and surgical intervention. In this study, we characterized VEGF Tg rabbits and found that overexpression of VEGF in the glomerular cells has profound effects on the kidney pathologies.

**RESULTS**

Characterization of VEGF Expression in Tg Rabbits

Analysis using real-time reverse transcriptase–PCR revealed that the renal expression of human VEGF (hVEGF) was 4.5% (arbitrary units) of the hepatic expression (Figure 1A) and the total VEGF mRNA expression (sum of human transgene and rabbit endogenous gene) in the whole kidneys of Tg rabbits was slightly increased compared with that of control rabbits (19% increase over the control kidneys on average, arbitrary units). To determine whether hVEGF was expressed in glomeruli, we isolated glomeruli from tubules by a sieving method and found that the expression of hVEGF indeed occurred in the glomeruli of Tg rabbits (Figure 1B). Tg rabbit glomeruli were hypertrophic compared with control glomeruli (Figures 3 and 4). Immunohistochemical staining showed that the hypertrophic podocytes in the hypertrophic glomeruli of Tg rabbits exhibited discernible VEGF staining, whereas the tubules of both Tg and control rabbits expressed VEGF (Figure 1C).

To determine whether a certain amount of hVEGF may be present in the circulation of Tg rabbits, we measured the plasma VEGF using two different ELISA with human VEGF as a positive control. As shown in Table 1, Biotrak ELISA kits revealed that the plasma levels of VEGF were 1.2-fold higher in Tg rabbits than that in control rabbits, although not statistically significant ($P = 0.272$). CyELISA (hVEGF specific) kits

<table>
<thead>
<tr>
<th>Group</th>
<th>Biotrak ELISA</th>
<th>CyELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tg ($n = 12$)</td>
<td>85.6 ± 12.2 pg/ml</td>
<td>ND</td>
</tr>
<tr>
<td>Control ($n = 7$)</td>
<td>68.4 ± 3.8 pg/ml</td>
<td>ND</td>
</tr>
</tbody>
</table>

*The data for transgenic (Tg) rabbits were combined from rabbits aged 10 ($n = 4$), 20 ($n = 4$), and 50 wk ($n = 4$). Antibody used in the Biotrack ELISA kits can react with both rabbit and human vascular endothelial growth factor (hVEGF), whereas antibody used in the CyELISA is hVEGF specific. Human recombinant VEGF was used as a positive control. ND, not detected.
along with Western blotting analysis (data not shown), however, did not detect the presence of human VEGF in the plasma of Tg rabbits. These data suggest that the transgenic hVEGF proteins were indeed expressed locally or associated with extracellular matrix (ECM), and little, if any, were released into the circulation. Compared with control rabbits, Tg rabbits had slightly lower body weight with relatively enlarged kidneys (Table 2).

Renal Functional Changes in Tg Rabbits

To evaluate the effects of increased expression of VEGF on renal functions, we first examined whether Tg rabbits exhibited proteinuria. Proteinuria was not detected in Tg rabbits until 12 wk and gradually became prominent thereafter with increasing age and reached a plateau at 28 wk (Figure 2A). To quantify the contents of urinary proteins, we analyzed the 24-h urinary proteins that were collected from rabbits at various ages when proteinuria was markedly increased. As shown in Table 3, the levels of urinary proteins in Tg rabbits were 12.7-, 34-, and 33-fold higher than those in control rabbits at 23, 28, and 42 wk. SDS-PAGE analysis demonstrated that the major urinary protein was albumin on the basis of its molecular size of approximately 68 kD (Figure 2A, insert). In addition, GFR of Tg rabbits at 23 wk was increased by 10% but progressively declined by 45 and 35% at 30 and 42 wk, respectively, compared with that in control rabbits (Figure 2B), suggesting that upregulation of VEGF impairs the glomerular barrier functions of Tg rabbits. Biochemical analysis of the plasma showed that Tg rabbits had hyperlipidemia and lower level of albumin at 30 wk, whereas their glucose, blood urea nitrogen, creatinine, and total proteins were not significantly different from control rabbits (Table 4). To exclude the possibility that hepatic dysfunctions of Tg rabbits may be responsible for lower albumin levels in plasma, we measured the plasma glutamic-oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) and found that there was no significant difference between control and Tg rabbits at 30 wk (GPT 32.2 ± 9.10 IU/L in control versus 44.3 ± 15.9 IU/L in Tg; GOT 40.5 ± 9.1 IU/L in control versus 46 ± 13.3 IU/L in Tg).

Histopathologic Examinations

Because Tg rabbits exhibited apparent renal dysfunction as described above, we next examined the pathologic changes of the kidneys of Tg rabbits at various ages. For the convenience of descriptions of glomerular changes, we arbitrarily divided the renal lesions of Tg rabbits into (1) early stage (preproteinuria stage, before 8 wk), (2) intermediate stage (12, 20, and 35 wk), and (3) later stage (55 wk) according to the appearance and extent of proteinuria and severity of renal pathology. At first glance, the renal histologic morphology of Tg rabbits starting from 8 to 20 wk was characterized by progressive glomerular hypertrophy with increased cell numbers per glomerulus (Figure 3). The glomerular size in Tg rabbits was increased by a factor of approximately 1.5-2 times compared with that in control rabbits. The glomeruli appeared uniformly enlarged throughout the cortical area of the kidney. The capillary loops of glomeruli were dilated with an increased number of cells per glomerulus. Marked mesangial matrix deposition was evident in the glomeruli. The tubules showed hydronephrosis with increased tubular cell numbers. In addition, the interstitium contained increased inflammatory cells and fibrosis. At 35 wk, the glomeruli showed further enlargement with increased cell number and mesangial matrix deposition. The tubules showed marked interstitial fibrosis and mild tubular atrophy. The interstitium contained increased inflammatory cells and fibrosis. At 55 wk, the glomeruli showed further enlargement with increased cell number and mesangial matrix deposition. The tubules showed marked interstitial fibrosis and mild tubular atrophy. The interstitium contained increased inflammatory cells and fibrosis.

Table 2. VEGF Tg rabbit body and kidney weight

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n = 11)</th>
<th>Tg (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>3511 ± 99</td>
<td>3014 ± 151b</td>
</tr>
<tr>
<td>Kidney (g)</td>
<td>18.2 ± 0.4</td>
<td>21.4 ± 2.6</td>
</tr>
<tr>
<td>Ratio of kidney to body weight (%)</td>
<td>0.52 ± 0.02</td>
<td>0.72 ± 0.08b</td>
</tr>
</tbody>
</table>

aData are means ± SEM. Male rabbits were 32 ± 3 wk of age at the time of analysis. Kidney weight was total weight of both right and left kidneys. bP < 0.05, P < 0.01 versus control.

Table 3. The 24-h urine proteins of VEGF Tg and control rabbits

<table>
<thead>
<tr>
<th>Age (wk)</th>
<th>Control (mg/d)</th>
<th>Tg (mg/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>23</td>
<td>41.1 ± 8.1</td>
<td>523.9 ± 212.4b</td>
</tr>
<tr>
<td>28</td>
<td>34.2 ± 4.3</td>
<td>1168.3 ± 399.5c</td>
</tr>
<tr>
<td>42</td>
<td>38.4 ± 3.8</td>
<td>1277.9 ± 381.6c</td>
</tr>
</tbody>
</table>

aData are means ± SEM. All rabbits were male, and n = 4 to 6 for each group. bP < 0.05, cP < 0.01 versus control.
creased by 1.17-, 1.76-, and 2.4-fold at 8, 12, and 20 wk compared with that in control rabbits (Figure 4A). The glomerular hypertrophy in Tg rabbits was caused by an increase of the number of cells, including both endothelial cells and mesangial cells on the basis of the morphologic features and immunohistochemical staining with CD31 mAb (Figures 4, B through D, and 5).

It should be noted that in the early stage, even though proteinuria was still not detected, the number of endothelial cells in Tg glomeruli was significantly increased. In the intermediate stage, proteinuria started to become obvious starting from 12 wk; however, mesangial cells along with endothelial cells were concomitantly increased in Tg rabbits compared with those of control rabbits (Figure 4, C and D). Furthermore, the mesangial matrix contents were significantly increased at 20 wk in Tg rabbits compared with the controls (Figures 3 and 4E).

Another important histologic feature of the glomeruli of Tg rabbits was the appearance of pronounced hypertrophy of

Table 4. Comparison of plasma parameters of VEGF Tg rabbits at various ages

<table>
<thead>
<tr>
<th>Parameter</th>
<th>12 Wk Control</th>
<th>12 Wk Tg</th>
<th>20 Wk Control</th>
<th>20 Wk Tg</th>
<th>30 Wk Control</th>
<th>30 Wk Tg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg/dl)</td>
<td>125.9 ± 4.7</td>
<td>124.9 ± 3.4</td>
<td>111.5 ± 3.7</td>
<td>122.1 ± 7.9</td>
<td>113.5 ± 2.7</td>
<td>122.0 ± 3.4</td>
</tr>
<tr>
<td>TC (mg/dl)</td>
<td>49.0 ± 4.6</td>
<td>105.1 ± 5.9b</td>
<td>35.5 ± 5.3</td>
<td>91.3 ± 19.6c</td>
<td>41.8 ± 5.1</td>
<td>76.9 ± 15.3c</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>60.6 ± 4.2</td>
<td>209.5 ± 59.2c</td>
<td>33.4 ± 1.4</td>
<td>193.1 ± 68.9</td>
<td>45.1 ± 4.6</td>
<td>169.3 ± 74.8</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>15.0 ± 1.8</td>
<td>16.3 ± 1.7</td>
<td>16.2 ± 1.6</td>
<td>19.3 ± 1.6</td>
<td>18.5 ± 2.6</td>
<td>21.8 ± 0.2</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.6 ± 0.2</td>
<td>0.6 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>2.1 ± 0.1</td>
<td>2.2 ± 0.1</td>
<td>1.9 ± 0.1</td>
<td>2.0 ± 0.1</td>
<td>2.0 ± 0.1</td>
<td>1.7 ± 0.1b</td>
</tr>
<tr>
<td>TP (g/dl)</td>
<td>7.4 ± 0.4</td>
<td>7.8 ± 0.2</td>
<td>7.3 ± 0.4</td>
<td>7.3 ± 0.3</td>
<td>7.6 ± 0.2</td>
<td>7.8 ± 0.3</td>
</tr>
</tbody>
</table>

aData are means ± SEM, and n = 4 to 6 for each group. BUN, blood urea nitrogen; TC, total cholesterol; TG, triglycerides; TP, total protein.

bP < 0.01, cP < 0.05 versus control.

Figure 3. Micrographs of glomeruli of control and Tg rabbits at 12 wk. Paraffin sections from rabbits were stained with either periodic acid-Schiff (PAS; top) or periodic acid-methenamine silver (PAM; bottom). Compared with the controls, the glomeruli of Tg rabbits showed increased proliferation of endothelial cells and mesangial cells and increased mesangial matrix contents.

Figure 4. Morphometric analysis of glomerular area and cell number. PAM-stained sections were examined for glomerular area (×10²/µm²) and total cell number per glomerulus (A and B). The number of endothelial cells and mesangial cells were further calculated on the basis of the morphology on PAS-stained sections (C and D). PAM-stained area was measured and expressed as mesangial matrix content (%; E). Data are means ± SEM; n = approximately 5 to 6 rabbits for each group. *P < 0.05, **P < 0.01 versus control.
podocytes (Figure 6A), whereas the number of podocytes (Wilms' tumor-1 [WT-1]-positive cells) per glomerulus was slightly increased (average podocyte number per glomerulus 7.7 ± 1.8 in control versus 9.6 ± 3.0 in Tg at 20 wk; \( P < 0.001 \); Figure 6B). Hypertrophic podocytes were larger in size than those of the controls (three-fold increase on average; \( P < 0.001 \)) and were characterized by a prominent large single nucleus or occasionally two nuclei (Figure 6A; see also transmission electron microscopy [TEM] results).

TEM examinations (Figure 7A) confirmed that podocyte hypertrophy was the major feature of Tg glomeruli, as shown by light microscopy. In addition, compared with normal rabbit podocytes, Tg rabbit podocytes showed loss of slit pores, and their foot processes appeared fused together and effaced (Figure 7B). Hypertrophic podocytes were larger in size than those of the controls (three-fold increase on average; \( P < 0.001 \)) and were characterized by a prominent large single nucleus or occasionally two nuclei (Figure 6A; see also transmission electron microscopy [TEM] results).

In the later stage (55 wk), most glomeruli in Tg rabbits showed more severe and more complicated lesions, including evidence of the healing process, after glomerular injury. In addition to the lesions (proliferation of capillary and mesangial cells and microaneurysms) described, the majority of glomeruli showed either global or segmental sclerosis or tuft collapse (partially or completely) or glomerular cysts (Figure 10, A and B). In sclerotic glomeruli, some cells around the Bowman’s capsule were positively stained with mAb against \( \alpha \)-smooth muscle actin (Figure 10, C through E). Apart from the lesions seen in glomeruli, tubular atrophy accompanied by interstitial fibrosis was frequently found throughout in Tg rabbit kidneys (data not shown). There was no macrophage infiltration in the glomeruli of either Tg or control rabbits, as confirmed by immunohistochemical staining (data not shown).

DISCUSSION

In this study, we characterized Tg rabbits that expressed the hVEGF\(_{165}\) isoform in the kidney in an attempt to evaluate the effect of increased VEGF on kidney pathophysiology. Human VEGF transgene expression was under the control of \( \alpha \)-1-antitrypsin promoter, so the transgene was mainly expressed in the liver and, to a lesser extent, in the kidney.\(^{13} \) Although this promoter is not kidney specific, we found that Tg rabbits showed a mild increase of VEGF expression in the kidney (approximately 19% increase of mRNA expression over that in control rabbit kidneys). hVEGF proteins were not detected in the plasma of Tg rabbits by ELISA and Western blotting. This result was initially surprising but may suggest that either hVEGF was associated with plasma inhibitors or the levels of plasma hVEGF were too low to be detected or hVEGF may be bound to the ECM. Despite this, our studies suggest that increased VEGF expression in kidney...
and liver has dramatic and multiple biologic effects on both glomerular cells and ECM production.

**VEGF and Glomerular Hypertrophy**

Compared with control rabbit glomeruli, the glomeruli of Tg rabbits were markedly hypertrophic, and the hypertrophy was associated with increased capillaries and mesangial cell proliferation. It is unlikely that these lesions were mediated by immune dysfunctions, because IgG staining was negative (data not shown). It seems that increased VEGF expression initially induces proliferation of endothelial cells and later induces that of mesangial cells, even though both types of cells express VEGF receptors.\(^4\,14\) In this respect, Ostendorf *et al.*\(^\text{15}\) showed that administration of VEGF\(_{165}\) antagonist led to a reduction of glomerular endothelial cell proliferation and an increase in endothelial cell death in nephritis, whereas proteinuria was not improved. Of note, podocytes were markedly hypertrophic (up to three-fold increase in size over the control) in Tg rabbits, accompanied by the loss of podocyte processes as observed by TEM. The mechanism by which VEGF induces podocyte hypertrophy...
Figure 10. Representative micrographs of glomeruli from Tg rabbits at 55 wk. Paraffin sections were stained with PAS (A), PAM (B and C), Azan (D), and α-smooth muscle actin (E). (A) Glomeruli showed either global or partial tuft collapse accompanied by cystic formation. (B) At higher magnification, glomeruli showed obvious tuft collapse with sclerosis and Bowman’s capsule fibrosis. A glomerulus with severe global sclerosis is shown (C through E), and increased fibrosis (D) and α-smooth muscle actin–positive cells were present (E).

is unknown but may possibly be mediated in an autocrine manner because podocytes not only produce VEGF but also express VEGF receptors.16,17 Although this hypothesis remains to be verified, podocytes may have the potential to bind to the VEGF that they secrete themselves. Consistent with the previous finding that VEGF can also stimulate podocytes to proliferate and increase their survival in vitro,18 we found that hypertrophic podocytes in Tg rabbits were increased in number and had two or more nuclei, suggesting that VEGF may enhance podocyte growth in vivo.

The pathologic changes that were observed in Tg rabbits at later stages were also interesting. For example, glomerular sclerosis as defined by the periodic acid-methenamine silver (PAM)-stained area was markedly increased with age and accompanied by severe proteinuria. Such features of Tg rabbit glomeruli may mimic many pathologic characteristics of renal diseases, such as FSGS in humans,19 suggesting that the up-regulation of VEGF may be a potentially causative factor for the progression of FSGS. Two mechanisms may be operative in terms of VEGF-induced glomerulosclerosis. First, it has been reported that glomerular hypertrophy can directly cause segmental glomerulosclerosis via podocyte damage.20,21 Second, VEGF derived from podocytes may stimulate mesangial cells to proliferate and synthesize ECM.22 In support of this speculation, both mesangial cells and ECM were markedly increased in the glomeruli of Tg rabbits after 20 wk. Of note, massive formation of glomerular microaneurysms was another feature of the Tg rabbits after 20 wk. Glomerular microaneurysms can be induced in experimental mesangioproliferative anti–Thy-1 nephritis.12 Although the pathologic significance of such lesions is unknown, mesangial cell damage (mesangiolysis) is proposed to be responsible for the formation of microaneurysms. If this is really operative, it remains to be determined whether VEGF either directly or indirectly induces mesangiolysis in future.

VEGF and Proteinuria

Podocytes play an important role in maintaining the glomerular filtration barrier because podocytes contribute structurally by the provision of the slit diaphragm and glomerular basement membrane.23 Glomerular VEGF derived from podocytes is also involved in the maintenance of the glomerular endothelium (including maintaining fenestration) and/or selective permeability to macromolecules.15,23 Therefore, it is reasonable to speculate that hypertrophic podocytes that are observed in Tg rabbits may lose their normal physiologic function of maintaining the macromolecular selectivity of the glomerular filtration barrier as one of the mechanisms that are responsible for the prominent proteinuria that is observed in Tg rabbits. In Tg rabbits, the proteinuria started to become obvious from 12 wk, suggesting that the deleterious effect of VEGF was progressive and required a certain time rather than starting from embryogenesis. Previous reports have shown that increased plasma levels of VEGF may be associated with proteinuria in patients with diabetes,9 and the administration of VEGF antibodies ameliorates proteinuria in diabetic nephropathy.24,25 Our results are in support of the notion that VEGF is an important mediator of glomerular functions and that the chronic upregulation of VEGF in podocytes leads to prominent and progressive proteinuria. Increased VEGF in the glomeruli may result in proteinuria through two possible different yet closely overlapping or related mechanisms. In the early stage, in addition to the abnormalities of hypertrophic podocytes discussed, high levels of VEGF derived from podocytes can strongly bind to capillary endothelial cells through specific VEGF receptors, which may result in increased permeability or glomerular hyperfiltration by altering capillary fenestration or basement membrane components26 or indirectly through the induction of nitric oxide and prostacyclin.27 In our study, we found that many endothelial cells in glomeruli showed subendothelial edema (Figure 7C). It has been reported that VEGF stimulates increased synthesis of collagenase by endothelial cells, which would result in the proteolytic disruption of the basement membrane.22 In the later stage, however, there were severe secondary glomerular injuries, including microaneurysms, tuft collapse, and glomerular sclerosis along with tubular injuries, all of which may participate in the enhancement of proteinuria. Therefore, the mechanisms that are responsible for proteinuria in Tg rabbits may be different in the early stage (mainly primary effect directly mediated by VEGF) from those in the later stage (both VEGF-induced effects and/or secondary effects on glomerular injury). Consistent
with this notion, our study showed that in Tg rabbits, there was a increase of GFR at 23 wk, but later on, GFR was progressively decreased, possibly reflecting the severe glomerulosclerosis.

Although Eremina et al. reported that podocyte-specific overexpression of the hVEGF164 led to a striking collapsing glomerulopathy in mice, our Tg rabbits apparently differed from Tg mice. First, unlike Tg mice, which showed kidney abnormalities and albuminuria as early as 5 d of age, Tg rabbits showed normal kidney development until 8 wk and showed proteinuria after 12 wk. This discrepancy between Tg mice and rabbits is probably due to the species differences and gene expression (levels and specificity) or developmental expression (from capillary loop stage of mice) versus later expression (in the rabbit model). Tg rabbits showed a mild increase of VEGF expression (1.2-fold increase over control) compared with Tg mice (30-fold increase over control), and transgene expression in Tg rabbits may not be podocyte-specific because some VEGF may be derived from tubuli or liver. In addition, Tg rabbits exhibited dynamic and diverse lesions in the kidneys, including glomerular hypertrophy (at the early stage), prominent glomerulosclerosis, microaneurysms, and vascular pole sclerosis (from the intermediate to later stages), whereas collapsing glomerulopathy was observed only at the age of 55 wk. Importantly, VEGF-induced glomerulopathies have several implications regarding the pathologic roles of VEGF in human kidney diseases such as diabetic nephropathies and FSGS, in which VEGF is upregulated and have been proposed to play a critical role in the pathogenesis of proteinuria. High concentrations of ambient glucose induce the expression of VEGF in both podocytes and mesangial cells.

**CONCLUSION**

Our study demonstrated that local overexpression of the hVEGF165 gene in the podocytes of Tg rabbits resulted in glomerular hypertrophy and increased proliferation of both endothelial cells and mesangial cells and glomerular sclerosis associated with progressive proteinuria. Podocytes were markedly hypertrophic and showed effacement of their foot processes. These data support the hypothesis that VEGF is involved in the maintenance of the glomerular barrier and in the pathogenesis of proteinuria. It is interesting that Tg rabbits exhibited many pathologic features similar to those found in human FSGS. These results suggest that local upregulation of VEGF may play diverse roles in glomerulopathies and proteinuria. Inhibition of VEGF in the glomeruli may become a potential target for the treatment of kidney diseases such as diabetic nephropathies and FSGS.

**CONCISE METHODS**

**Tg Rabbits Expressing hVEGF**

Tg rabbits that express hVEGF165 cDNA under the control of the human α-1-antitrypsin promoter were generated in our laboratory. A previous study showed that the α-1-antitrypsin promoter contained an element that was able to direct the expression of the transgene in the kidney in addition to the liver. Three founders were obtained, but one founder died before breeding. Autopsy examination revealed that this founder had hepatic and kidney lesions. The blood levels of hVEGF of all three founders were below the detection.

For this study, heterozygous male Tg rabbits and control littermates from V58 line were analyzed at the ages of 8 to 55 wk. All rabbits were kept individually in a room where the temperature and humidity were maintained at 24 ± 2°C and 55 ± 15%, respectively. The rabbits were submitted to a regular 12-h light/dark cycle and given water and a standard chow diet (CBR-1; CLEA, Tokyo, Japan). All experimental protocols were approved by the Saga University Animal Experimentation Committee and performed under the Saga University Guidelines for Animal Experimentation. For monitoring the health status of Tg rabbits, blood was collected from rabbits after overnight fasting (16 h), and the plasma albumin, blood urea nitrogen, creatinine, glucose, total protein, GOT, and GPT were determined using a compact VetScan unit (Abaxia, Union City, CA) in a Point-of-care blood analyzer (Abaxia, Sunnyvale, CA).

**Reverse Transcriptase–PCR Analysis**

Total RNA from different tissues was rapidly isolated using Trizol reagent (Invitrogen, Tokyo, Japan) and reverse-transcribed into cDNA using Qiagen reverse transcription reagents (Tokyo, Japan), and real-time reverse transcriptase–PCR (DNA Engine Opticon; MJ Research, Tokyo, Japan) was performed using DyNAmo SYBR Green Point-of-care blood analyzer (Abaxia, Sunnyvale, CA).

**Table 5.** Primers for real-time reverse transcriptase–PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Product (bp)</th>
<th>T_a (°C)</th>
<th>GeneBank Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>hVEGF</td>
<td>AGGGAGGAGGGCAGAATCATCAT</td>
<td>151</td>
<td>57.0</td>
<td>BC058855</td>
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<tr>
<td>Rabbit VEGF</td>
<td>CATCAAGGGGACACACAGGAT</td>
<td>152</td>
<td>56.0</td>
<td>BC058855</td>
</tr>
<tr>
<td>Human and rabbit VEGF</td>
<td>ACCAGAGGCACCGAGGA</td>
<td>149</td>
<td>56.0</td>
<td>BC058855/AY196796</td>
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<tr>
<td>Rabbit GAPDH</td>
<td>ATCACTGCAATCAAGAACAGAC</td>
<td>146</td>
<td>58.3</td>
<td>L23961</td>
</tr>
</tbody>
</table>

^GAPDH, glyceraldehyde-3-phosphate dehydrogenase; T_a, annealing temperature.
qPCR kits (Finnzymes, Espoo, Finland) according to the manufacturer’s instructions. We measured the human-specific VEGF and rabbit endogenous VEGF using specific primers as shown in Table 5. In addition, we separated glomeruli from tubules using a sieving method to examine the hVEGF mRNA expression in glomeruli.

Urinary Protein Examination Urine was collected in the morning and examined for urobilinogen, blood, bilirubin, ketones, glucose, protein, pH, leukocytes, specific gravity, and ascorbic acid using Protest 10I strips (Wako Pure Chemical Industries, Osaka, Japan). The total amounts of urinary proteins excreted during 24 h were measured from rabbits at 23, 28, and 42 wk using protein assay kits (Bio-Rad Laboratories, Hercules, CA). In addition, urinary proteins were fractionated by 10% SDS-PAGE.

Evaluation of Renal Functions The GFR was indirectly evaluated by measurement of the inulin clearance rate in the blood. Briefly, inulin (Wako) was dissolved in saline and infused into rabbits (which had fasted for 16 h) at a dosage of 100 mg/kg body wt at a constant rate over 30 s through the auricular vein. Blood samples were collected through the intermediate auricular artery at 3, 10, 20, 40, 80, 120, 180, and 240 min. The concentration of serum inulin was determined by the indole-3-acetic acid (Sigma, St. Louis, MO) colorimetric assay as described previously. GFR was estimated by measurement of serum clearance of inulin according to the formula derived from Brown: 

\[
GFR (\text{ml/min per kg body wt}) = \frac{D}{AUC/\text{body wt}}
\]

where D is the amount of injected inulin, and AUC (mg × min/ml) is the total area under the serum inulin disappearance curve. The AUC was calculated according to the trapezium rule described previously.

Histologic Examinations and Immunohistochemistry For the morphologic analysis of kidneys, Tg and littermate control rabbits were killed at various ages and the kidneys were removed and fixed in 10% neutral buffered formalin solution. The middle part of the left and right kidneys was embedded in paraffin, and serial sections were stained with hematoxylin and eosin, periodic acid-Schiff, Azan, and PAM, which were used for morphometric analysis (see next section). In addition, paraffin sections were immunohistochemically stained using mAb against rabbit macrophages (RAM11; 1:200; Dako, Carpenteria, CA); α-smooth muscle actin (1:400; Dako), and hVEGF (ab3; 1:100; Neomarkers, Fremont, CA), which showed cross-reaction with rabbit VEGF. In addition, we performed immunohistochemical staining using two cellular markers for human kidney, including endothelial cell markers CD31 and podocyte markers WT-1. We found that two CD31 and WT-1 could cross-react with rabbit kidneys. Immunohistochemistry was performed with the Dako En-vision system following the manufacturer’s instructions.

Morphometric Analysis For the quantitative analysis of glomerular changes including glomerular size, cellular number, matrix expansion, or fibrosis, PAM-stained sections were examined under a light microscope and 40 constitutive images for each section were recorded using an Olympus DP70 digital camera (Tokyo, Japan). Glomerular size and mesangial matrix area were manually traced and measured using the Lumina Vision image analysis system (Mitani, Tokyo, Japan). Mesangial matrix area was defined as the PAM-stained area (%) within the tuft area as described by Inada et al. We calculated the cellular numbers using periodic acid-Schiff–stained sections on the basis of the cellular morphology and structure under light microscopy. All analyses were performed by two independent researchers blindly. For each assay, at least 40 consecutive intact glomeruli from each section were evaluated.

TEM A small piece of kidney from rabbits at the age of 25 wk was fixed in 2.5% glutaraldehyde and postfixed in 1% osmium tetroxide using a standard protocol. Ultrathin sections were doubly stained with uranyl acetate and lead citrate and were observed under an JEM-100CX electron microscope (JEOL Ltd, Tokyo, Japan) as described previously.

Analysis of Plasma VEGF To determine whether Tg rabbits had high levels of VEGF in plasma, we used two ELISA kits that had different specificity. CytELISA kits (CytImmune Sciences, Rockville, MD) can detect both human and rabbit endogenous VEGF, whereas Biotrak ELISA kits (Amersham Pharmacia Biotech, Buckinghamshire, UK) were specific for hVEGF (without cross-reaction with rabbit VEGF). To ensure the precise determination of hVEGF in Tg rabbit plasma, we used human recombinant VEGF (R&D Systems, Minneapolis, MN) as a positive control in all ELISA assays. Because VEGF may bind to plasma proteins, which might interfere with ELISA results, we also collected the plasma from a normal rabbit that was administered an intravenous injection of 400 ng/kg human recombinant VEGF as a positive control. The same samples were also fractionated by 10% SDS-PAGE and analyzed by Western blotting.

Statistical Analyses Results are expressed as means ± SEM. Statistical analyses were performed using either the t test when the F value was equal or Welch t test when the F value was not equal. P < 0.05 was considered statistically significant.
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DISCLOSURES

None.

REFERENCES