Vascular Endothelial Growth Factor Enhances Glomerular Capillary Repair and Accelerates Resolution of Experimentally Induced Glomerulonephritis

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Vascular endothelial growth factor (VEGF) regulates angiogenesis through endothelial cell proliferation and plays an important role in capillary repair in damaged glomeruli. We tested the hypothesis that VEGF might be beneficial in rats with severe glomerular injury in glomerulonephritis (GN) based on its angiogenic and vascular remodeling properties. Acute GN with severe glomerular destruction was induced in rats by injection of anti-Thy-1.1 antibody (day 0) and Habu-snake venom (day 1). Rats were intraperitoneally injected with recombinant human VEGF₁₆₅ (10 µg/100 g body wt/day) or vehicle from day 2 to day 9, and monitored changes in glomerular capillaries, development of glomerular inflammation, and progression to glomerular sclerosis after acute glomerular destruction in both groups. Rats that received anti-Thy-1.1 antibody and Habu-snake venom showed severe mesangiolysis and marked destruction of capillary network on day 2. VEGF was expressed on glomerular epithelial cells, proliferating mesangial cells, and some infiltrating leukocytes, and VEGF₁₆₅ protein levels increased in damaged glomeruli during day 5 to day 7. Normal, damaged, and regenerating glomerular endothelial cells expressed VEGF receptor flk-1. However, endothelial cell proliferation and capillary repair was rare in vehicle-treated rats with severe glomerular damage, which progressed to global sclerosis and chronic renal failure by week 8. In contrast, in the VEGF-treated group, VEGF₁₆₅ significantly enhanced endothelial cell proliferation and capillary repair in glomeruli by day 9 (proliferating endothelial cells: VEGF₁₆₅, 4.3 ± 1.1 ; control, 2.2 ± 0.9 cells on day 7, $P < 0.001$; and glomerular capillaries: VEGF₁₆₅, 24.6 ± 4.8 ; control, 16.9 ± 3.4 capillaries on day 7, $P < 0.01$). Thereafter, damaged glomeruli gradually recovered after development of capillary network by week 8, and significant improvement of renal func-

tion was evident in the VEGF-treated group during week 8 (creatinine: VEGF₁₆₅, 0.3 ± 0.1 ; control, 2.6 ± 0.9 mg/dl, $P < 0.001$; proteinuria: VEGF₁₆₅, 54 ± 15 ; control, 318 ± 60 mg/day, $P < 0.001$). We conclude that the beneficial effect of VEGF₁₆₅ in severe glomerular injury in GN emphasizes the importance of capillary repair in the resolution of GN, and may allow the design of new therapeutic strategies against severe GN. (*Am J Pathol* 2001, 159:599–608)

The glomerulus is a well-developed capillary network and the basic filtration organ in the kidney. Various degrees of injury to glomerular endothelial cells and the glomerular capillary network have been reported in various renal diseases, including several forms of glomerulonephritis (GN).^{1,2} Glomerular endothelial cell injury is also seen in a variety of conditions associated with glomerular hypertension and hyperfiltration.^{1,3}

Recent studies of experimental GN indicate that after glomerular capillary destruction, a complete capillary repair in damaged glomeruli leads to full recovery of the glomerular architecture.^{4–6} However, impairment of capillary repair process in glomerular damage could be associated with the development of glomerular sclerosis and renal dysfunction.⁷ In the recovery model of Thy-1 GN, repair of glomerular capillary network with endothelial cell proliferation is associated with up-regulated expression of vascular endothelial growth factor (VEGF) and its receptor, flk.⁴ In addition, systemic administration of VEGF can mediate glomerular endothelial cell proliferation in an experimental rat model of thrombotic microangiopathy.⁸ On the other hand, in the recovery model of Thy-1 GN, administration of VEGF₁₆₅ antagonist significantly reduced glomerular endothelial cell proliferation and inhibited glomerular capillary repair, leading to glomerular sclerosis.⁹ These findings demonstrate that capillary repair is a crucial event in the recovery from glomerular damage accompanied by destruction of glo-

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merular capillary network, and indicate that VEGF plays an important role in endothelial cell proliferation and capillary repair in damaged glomeruli.

However, the changes of VEGF production and alteration of glomerular capillaries in the development of glomerular sclerosis in GN have not been fully investigated. In the present study, we determine the serial changes in VEGF production in damaged glomeruli, alteration of glomerular capillaries, development of glomerular inflammation, and progression to glomerular sclerosis after severe glomerular destruction in experimental GN. In addition, we examined the potential beneficiary effects of VEGF₁₆₅ in glomerular repair and resolution of GN. Acute GN with severe glomerular destruction was induced in rats by injection of both anti-Thy-1.1 antibody and Habu-snake venom, and is termed here as Thy-1/Habu-snake venom GN. Injection of anti-Thy-1.1 antibody induces diffuse mesangial cell lysis,^{10,11} and administration of Habu-snake venom, which has a strong proteolytic enzyme activity, mainly induces mesangial matrix lysis.² Because mesangial cells and matrix provide a strong support for glomerular capillaries,¹² injection of both anti-Thy-1.1 antibody and Habu-snake venom induces marked mesangiolysis with extremely severe capillary destruction of glomeruli, leading to the development of GN with irreversible glomerular damage and chronic renal failure.

Materials and Methods

Thy-1/Habu-Snake Venom GN Model in Rats

Animal experiments described in the present study were approved by the Ethics Review Committee for Animal Experimentation of Nippon Medical School. Male Wistar rats (Sankyo Laboratory, Tokyo, Japan) weighing 180 g were used for all experiments. Thy-1/Habu-snake venom GN was induced by injection of both monoclonal anti-Thy 1.1 antibody (OX7; Cedarlane Laboratories, Ontario, Canada) at a dose of 60 μ g IgG/100 g body wt on day 0 and Habu-snake venom (*Trimeresurus flavoviridis*; Wako Pure Chemical Industries, Osaka, Japan) at a dose of 75 μ g/100 g body weight on day 1. Rats were then treated with VEGF₁₆₅ (Gibco BRL, Tokyo, Japan) dissolved in saline (VEGF-treated group) or saline alone (control group) injections. We have demonstrated that, in the recovery models of Thy-1 or Habu-snake venom GN, glomerular capillary regeneration in the early phase after injury, before occurrence of massive mesangial cell proliferation, is necessary to complete glomerular repair.^{5,6} Rats, therefore, were administered by VEGF₁₆₅ or vehicle using intraperitoneal micro-osmotic pumps (Alzet osmotic pump, model 2ML1; Alza, Mountain View, CA) starting at day 2 and ending at day 9 (1 week). The pumps were filled with VEGF₁₆₅ (140 μ g/2 ml saline) and had a delivery time of 7 days, therefore, VEGF₁₆₅ delivery was calculated as $\sim$ 10 μ g/100 g body weight/day. In each group, five rats were biopsied or sacrificed on days 0, 2, 5, 7, and 9, and 2, 3, 4, 6, and 8 weeks after the disease induction. To estimate renal function, urine and blood samples were collected for measurement of urinary

protein, plasma creatinine, and blood urea nitrogen using an autoanalyzer (SRL, Tokyo, Japan).

Histopathological and Immunohistochemical Examination

After removal of the kidney, renal tissues were fixed in 20% buffered formalin and embedded in paraffin for light microscopic examination. Tissues were stained with hematoxylin and eosin, periodic acid-Schiff (PAS), and periodic acid-methenamine silver for histopathological examination. The naphthol AS-D chloroacetate esterase staining was performed to detect infiltrating neutrophils.

For immunohistochemistry, 20% buffered formalin-fixed paraffin-embedded tissue sections were used, and the specimens were stained by the standard avidin-biotin-peroxidase complex technique. The following antibodies were used for immunohistochemistry. 1) Polyclonal rabbit anti-rat thrombomodulin (TM) antibody¹³ (kindly provided by Dr. David Stern, Columbia University, New York, NY), which reacts with the surface of endothelial cells. This antibody has been used as a marker for endothelial cells.^{5-7,13} Unfortunately, this antibody sometimes may detect free form of TM in glomerular sclerosis or glomerular proliferative areas, we, therefore, determined glomerular endothelial cells carefully, using not only expression of TM but also its location and morphology. In addition, endothelial TM expression alters in renal disorders. Confirmation of the glomerular endothelial cells displayed by TM was performed by staining tissue with the antibody against a surface antigen expressed on all rat endothelial cells (RECA-1)⁴ or *Griffonia (Banerare) simplicifolia* lectin.¹⁴ The results demonstrated similar endothelial cell patterns in glomeruli; neither was comparable in quality or specificity to TM (data not shown). This indicates that loss of TM activity in this model reflects a loss of glomerular endothelial cells, confirming data of a previous study.⁵⁻⁷ 2) Monoclonal mouse anti-human α -smooth muscle actin (α -SMA) antibody (1A4; DAKO, Glostrup, Denmark), which is a marker for activated mesangial cells. 3) Monoclonal mouse anti-proliferating cell nuclear antigen (PCNA) antibody (PC10, DAKO), which is a marker for cellular proliferation. 4) Polyclonal goat anti-type IV collagen antibody (Southern Biotechnology Associates, Birmingham, AL), which is used for evaluation of mesangial matrix accumulation and glomerular sclerosis. 5) Polyclonal rabbit anti-VEGF antibody,¹⁵ which can detect VEGF-producing cells. 6) Monoclonal mouse anti-flk-1 antibody (A-3; Santa Cruz Biotechnology, Santa Cruz, CA), which can detect cells that express VEGF receptor. 7) Polyclonal rabbit anti-human CD3 antibody (DAKO), which can detect infiltrating rat T cells. 8) Monoclonal mouse anti-rat ED1 antibody (BMA, Augst, Switzerland), which can detect infiltrating macrophages. For TM, type IV collagen, VEGF, flk-1, CD3, and ED-1, tissue sections were incubated with 0.1% pepsin for 60 minutes, 0.1% pepsin for 30 minutes, and 0.1% proteinase for 5 minutes, 0.4% pepsin for 20 minutes, 0.1% proteinase for 5 minutes, 0.1% pepsin for 45 minutes, and 0.1% pepsin for 45 minutes, respectively, before incubation with the

Figure 1. Marked mesangiolysis and severe destruction of glomerular capillary network in rats with Thy-1/Habu-snake venom GN on day 2. **A:** Damaged glomeruli are characterized by extensive mesangiolysis with multiple large capillary aneurysmal balloonings (periodic acid-methenamine silver stain; original magnification, $\times 200$). **B:** Glomerular capillary network is destroyed with loss of endothelial cells in ballooning lesions (**asterisk**). Endothelial cell proliferation is only rarely noted in damaged glomeruli [double stain with PCNA (blue) and TM (brown); original magnification, $\times 600$]. **C:** Disappearance of endothelial cells in capillary ballooning is evident with prominent platelets and fibrin deposition (original magnification, $\times 3000$).

primary antibody. To optimize the detection of PCNA, sections were microwaved for 10 minutes in 0.01 mol/L sodium citrate (pH 6.0) after dewaxing. Proliferating endothelial cells were identified after double-immunohistochemistry staining with TM and PCNA, using the color modification method of 3,3'-diaminobenzidine (DAB) precipitation by nickel chloride, which changes DAB color from brown to black.^{6,7} Sections were incubated with anti-rat TM antibody, a peroxidase conjugated goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, West Grove, PA) followed by DAB containing hydrogen peroxide (H_2O_2). Sections were then incubated with PCNA followed by a peroxidase-conjugated goat anti-mouse IgG (BML, Nagoya, Japan), and H_2O_2 , nickel chloride containing DAB (DAB substrate kit for peroxidase; Vector Laboratories, Burlingame, CA). Double immunostaining with PCNA and α -SMA was also performed to detect proliferating and activated mesangial cells, using the same technique. Sections were first stained with anti- α -SMA antibody, a peroxidase-conjugated goat anti-mouse IgG (BML) followed by H_2O_2 containing DAB. Sections were then incubated with PCNA followed by a peroxidase-conjugated goat anti-mouse IgG (BML), and H_2O_2 , nickel chloride containing DAB. To detect VEGF-producing mesangial cells, double immunostaining with VEGF and Thy-1 (rat mesangial cell marker) was also performed. Four- μ m frozen sections were stained with polyclonal rabbit anti-VEGF antibody and followed by fluorescein isothiocyanate-labeled goat anti-rabbit IgG antibody (Zymed, San Francisco, CA). Sections were then incubated with anti-Thy-1 antibody (OX7, Cedarlane Laboratories) and followed by Texas Red-conjugated goat anti-mouse IgG antibody (Leinco Technologies, St. Louis, MO), and the nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories). Specimens were examined under a confocal laser scanning microscope (CLSM, TCS-SP; Leica Lasertechnik, Heidelberg, Germany) based on an upright microscope (DMRB, Leica Lasertechnik) equipped with a krypton/argon laser. For all biopsies, negative controls were used in which the primary antibody was substituted with equiv-

alent concentrations of an irrelevant antibody or normal rabbit IgG (DAKO). All control sections were negative.

For electron microscopic examination, the kidney tissue was fixed in 2.5% glutaraldehyde solution in phosphate buffer (pH 7.4) and postfixed with 1% osmium tetroxide, dehydrated, and embedded in Epok 812. Ultrathin sections were stained with uranyl acetate and lead citrate, and then examined with an electron microscope (model H7100; Hitachi Corp., Ibaragi, Japan).

Figure 2. VEGF expression (**A–C**) and VEGF receptor flk-1 expression (**D–F**) in glomeruli before (**A** and **D**), 2 days (**E**), and 7 days (**B**, **C**, and **F**) after induction of GN. **A** and **B:** VEGF stain; original magnifications, $\times 800$. **C:** double stain with VEGF (green) and Thy-1 (red); original magnification, $\times 700$. **D–F:** flk-1 stain, original magnifications, $\times 600$. **A:** In normal glomeruli, VEGF expression is confined to podocytes. **B:** In the proliferative lesions, visceral epithelial cells, proliferating mesangial cells, and infiltrating leukocytes ($\downarrow$) express VEGF. **C:** In double stain with VEGF (green) and Thy-1 (red), the expression of VEGF in proliferative mesangial cells is indicated by yellow color. **D–F:** VEGF receptor flk-1 is expressed on endothelial cells in normal, damaged, and regenerating glomerular capillaries.

Figure 3. Up-regulation of VEGF in damaged glomeruli after induction of experimental GN. Western blot analysis shows that bands of molecular weight corresponding to the VEGF₁₆₅ are detected in lane of recombinant human VEGF₁₆₅ (lane rh), and lane of protein lysate extracted from isolated glomeruli in control (lane C), 2 days (lane 2d), 5 days (lane 5d), 7 days (lane 7d), 14 days (lane 14d), and 28 days (lane 28d) after disease induction. VEGF₁₆₅ is up-regulated in damaged glomeruli between days 5 and 7.

Isolation of Glomeruli and Western Blot Analysis for VEGF₁₆₅

To examine the production of VEGF in glomeruli before and after disease induction, Western blotting was performed using polyclonal rabbit anti-VEGF antibody (147; Santa Cruz Biotechnology). For this purpose, glomeruli were isolated as described previously,¹¹ using a standard three-stage sieving method. Isolated glomeruli were homogenized in lysis buffer (150 mmol/L NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mmol/L NaF, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1 mmol/L Na₃VO₄, 1 mmol/L phenylmethylsulfonyl fluoride, and 20 mmol/L Tris-HCl, pH 7.4). After centrifugation at 15,000 $\times g$ for 30 minutes at 4°C, the supernatant was collected and used for analysis. Samples containing 10 μ g of protein per lane were separated on 10% acrylamide gel by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After electrophoresis, the separated protein was transferred to a Hybond-P nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK) and incubated with anti-VEGF antibody (1:2000). Bound antibody was detected with peroxidase-conjugated anti-rabbit IgG antibody (1:1000; Jackson ImmunoResearch) with the enhanced chemiluminescence detection system (ECL Western blotting detection reagents, Amersham). Membranes were washed and then exposed to film. Densitometric analysis of the bands was performed with NIH Image software.

Quantification of Histopathological Findings

In each kidney sample, more than 20 cross-sections of glomeruli were examined sequentially for the following parameters: 1) total number endothelial cells, the mean number of nuclei of TM+ cells per glomerular cross-section; 2) proliferating endothelial cells; the mean number of both PCNA+ and TM+ cells per glomerular cross-section; 3) capillary repair; the mean number of glomerular capillary lumina surround by TM+ cells, per glomerular cross-section; 4) proliferating and activated mesangial cells; the mean number of both PCNA+ and α -SMA+ cells per glomerular cross-section; 5) infiltrating neutrophils; the mean number of naphthol AS-D chloroacetate esterase+ cells per glomerular cross-section; 6) infiltrating T cells; the mean number of CD3+ cells per glomerular cross-section; and 7) infiltrating macrophages; the mean number of ED-1+ cells per glomerular cross-section. In addition, for evaluation of 8) activated

mesangial cell area and 9) sclerotic area in glomeruli, more than 20 cross-sections of glomeruli were graded semiquantitatively on specimens stained for α -SMA and type IV collagen, respectively, using a graded system^{9,16} (0 = diffuse, very weak, or absent glomerular staining and no localized increases of staining; 1+ = up to 25% of the glomerular tuft showed focal increase of staining; 2+ = 25 to 50% of the glomerular tuft showed focal increase of staining; 3+ = 50 to 75% of the glomerular tuft showed focal increase of staining; 4+ = >75% of the glomerular tuft showed focal increase of staining). Glomerular cross-sections containing only a small portion of the glomerular tuft were excluded from the analysis. All histopathological evaluations were performed by investigators blinded to the treatment modality (saline versus VEGF). These results were expressed as the mean $\pm$ SD, and statistical analysis was performed using the Student's *t*-test.

Results

Thy-1/Habu-Snake Venom GN

Thy-1/Habu-snake venom GN is produced by injecting on day 0 an antibody to the Thy-1 antigen, which is present on mesangial cells. This results in an acute, complement-dependent mesangial cell-lysis.^{10,11} Subsequently, Habu-snake venom is administered on day 1 to induce mesangial matrix decomposition, because of proteolytic enzyme activity.² Although anti-thymocyte serum or Habu-snake venom classically results in a focal lesion,^{5,10} the rats developed diffuse and marked mesangiolysis on day 2 after injection of both anti-Thy-1.1 antibody and Habu-snake venom. In accordance with the failure of the capillary support function of mesangial cells and matrix, the capillary network was destroyed, and mesangiolytic lesions progressed to multiple and large microaneurysmal ballooning. Multiple and large microaneurysmal ballooning lesions were observed in >60% of glomeruli on day 2, when the most severe glomerular damage was noted in this model (Figure 1A). Glomerular endothelial cells were lost in the ballooning lesions with destruction of glomerular capillary network as evident in both immunostaining for endothelial surface protein, TM (Figure 1B), and ultrastructural studies (Figure 1C).

Before disease induction, glomerular podocytes in normal tissues expressed VEGF (Figure 2A). In addition to VEGF staining in podocytes in kidney tissue samples of Thy-1/Habu-snake venom GN, proliferating mesangial cells and infiltrating leukocytes also expressed VEGF on day 5 to week 2 (Figure 2, B and C). In parallel with the increase in the relative area of VEGF expression in glomeruli, the protein level of VEGF₁₆₅ increased in isolated glomeruli between days 5 and 7 (Figure 3). However, VEGF₁₆₅ protein level subsequently decreased at a rapid rate until week 2. VEGF receptor flk-1 was expressed on glomerular endothelial cells in normal, damaged, and regenerating capillaries (Figure 2; E, F, and G). Little or no expression of flk-1 was detected in proliferating mesangial cells.

Figure 4. Damaged glomeruli in a representative vehicle-infused rat on day 7. **A:** PCNA+/TM+-proliferating endothelial cells (↓) are rarely seen in damaged glomeruli [double stain with PCNA (blue) and TM (brown); original magnification, ×800]. **B:** Many PCNA+/α-SMA+-activated and -proliferating mesangial cells (↓) are seen in damaged glomeruli [double stain with PCNA (blue) and α-SMA (brown); original magnification, ×800]. **C:** Endothelial cells and capillary formation are rarely detected in proliferative lesions (original magnification, ×1500).

Although VEGF production was up-regulated after disease induction and flk-1 was expressed on the capillary endothelium in damaged glomeruli, only a few proliferating endothelial cells (PCNA+/TM+) were present in severely damaged aneurysmal lesions on days 5 to 9 (Figure 4A). In contrast, many proliferating and activated mesangial cells (PCNA+/α-SMA+) were found in these lesions (Figure 4B). In proliferative lesions, glomerular

endothelial cells and capillary structures were rarely detected on days 5 to 9 (Figure 4C). Mesangial hypercellularity and mononuclear cell infiltration were persistently seen without capillary regeneration in damaged glomeruli on weeks 2 to 4 (Figure 5). Thereafter, glomerular inflammation progressed without capillary repair, and accumulation of mesangial matrix including type IV collagen advanced with the development of glomerular sclerosis. In

Figure 5. Damaged glomeruli progress to global sclerosis without capillary regeneration in a representative vehicle-infused rat on day 7 (**A** and **E**), week 2 (**B** and **F**), week 4 (**C** and **G**), and week 8 (**D** and **H**). **A–D:** Marked proliferative lesions are noted on day 7 and gradually progress to global sclerosis by week 8. **E–H:** During the progression of proliferative lesions to global sclerosis, TM+ capillary regeneration is rare in damaged glomeruli.

Figure 6. Renal function in rats treated with VEGF or vehicle only. Serum creatinine and blood urea nitrogen levels significantly improve in VEGF-treated rats compared with those in vehicle-infused rats on week 8. Urinary protein levels also diminish significantly in VEGF-treated rats on weeks 4 to 8. Note that the level of proteinuria does not increase during VEGF administration between days 2 and 9. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

parallel with the histopathological and immunohistochemical evidence of a progressive glomerular damage, changes in blood and urine indicated a gradual deterioration of renal function evident by proteinuria and worsening of plasma creatinine and blood urea nitrogen (Figure 6).

VEGF₁₆₅ Modified the Course of Thy-1/Habu-Snake Venom GN

We also examined the effects of VEGF₁₆₅-induced angiogenesis on the course of Thy-1/Habu-snake venom GN. In the treatment group, rats were infused intraperitoneally with recombinant human VEGF₁₆₅ (10 μ g/100g body weight/day) from days 2 to 9 when minimal endothelial cell proliferation and impaired capillary repair occurred after disease induction in the control group.

Administration of VEGF₁₆₅ significantly enhanced endothelial cell proliferation and glomerular capillary repair by day 9. Numerous proliferating endothelial (PCNA+/TM+) cells were found in the mesangiolytic and ballooning lesions (Figure 7). Furthermore, the number of proliferating endothelial cells was increased significantly on days 5 to 7 (Figure 8), which was followed by a rapid recovery in the number of total TM+ glomerular endothelial cells by week 2 (Figure 8). Glomerular capillary repair occurred through capillary regeneration from the remaining endothelial cells as well as new capillary growth from the glomerular vascular pole (Figure 7; A, B, and C). The regenerating capillaries with activated endothelial cells

Figure 7. Glomerular capillary repair with proliferating endothelial cells in damaged glomeruli during treatment with VEGF. **A–C:** Numerous PCNA+/TM+ proliferating endothelial cells ($\downarrow$) are seen in damaged glomeruli on day 5 (**A** and **B**) and day 7 (**C**) [double stain with PCNA (blue) and TM (brown); original magnifications, $\times 600$ (**A**), $\times 900$ (**B**), $\times 800$ (**C**)]. Glomerular capillary repair occurs through the process of capillary regeneration from remaining endothelial cells as well as new capillary growth from the glomerular vascular pole ($\downarrow$). **D:** PCNA+/ α -SMA+ activated and -proliferating mesangial cells ($\downarrow$) are seen on day 7 [double stain with PCNA (blue) and α -SMA (brown); original magnification, $\times 800$]. However, the number of these cells is significantly lower in VEGF-treated group compared with those in the control group. **E:** The developing capillary ($\downarrow$) with activated endothelial cells is seen in the proliferative lesions on day 5 (original magnification, $\times 1200$). **F:** Many endothelial cells (asterisks) in capillaries are detected in the proliferative lesions on day 7 (original magnification, $\times 2000$).

Figure 8. Correlation between number of PCNA+/TM+-proliferating endothelial cells (A), TM+ total endothelial cells (B), and TM+ glomerular capillary lumina (C) per glomerular cross-section in VEGF-treated (filled circle) or control (open circle) groups. Filled square represents values at day 0 and day 2 (before VEGF or vehicle treatment) in both groups. Values are expressed as mean $\pm$ SD. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

were seen in the proliferative lesions (Figure 7, E and F). Thereafter, new glomerular capillary network developed by week 8 (Figure 9). In parallel with capillary regeneration, the number of glomerular capillary lumina per glomerular cross-section also increased (Figure 8). On the other hand, numerous PCNA+/ α -SMA+-proliferating and -activated mesangial cells were found in proliferative lesions from the early phase of GN (Figure 7D). Subsequently, mesangial hypercellularity was noted on day 7 with marked mesangial proliferative GN (Figure 9). However, this process was transient, and correlated with the development of glomerular capillary network; mesangial cell hypercellularity and matrix expansion gradually sub-

sided in damaged glomeruli (Figure 9). By week 8, most of the damaged glomeruli healed and only minimal segmental sclerotic lesions were noted with α -SMA+ or type IV collagen+ areas (Figure 10). During recovery, VEGF-treated rats showed a rapid fall in mesangial cell proliferation and/or activation, and leukocyte infiltration, together with a significant decrease in the number of these cells (Figure 11).

Finally, VEGF-treated rats showed significant improvement in renal function on week 8 compared to the vehicle-treated control rats. Although the level of proteinuria was similar in rats treated with VEGF₁₆₅ and vehicle between days 2 and 9, proteinuria gradually improved in VEGF-treated group (Figure 6), in parallel with the recovery from damaged glomeruli.

Discussion

We have demonstrated in the present study that angiogenic capillary repair plays an important role in recovery from severe glomerular damage in rats with experimentally induced GN. Systemic administration of VEGF₁₆₅ after acute glomerular injury successfully induced glomerular repair and resolution of GN, associated with stimulation of angiogenesis, and vascular remodeling. The use of VEGF₁₆₅ as an angiogenic factor, therefore, could be potentially useful clinically for the treatment of GN

Figure 9. Recovery of damaged glomeruli with capillary repair in VEGF-treated group on day 7 (A and E), week 2 (B and F), week 4 (C and G), and week 8 (D and H). A-D: periodic acid-methenamine silver stain; original magnification, $\times 600$. E-H: TM stain; original magnifications, $\times 700$ (E and F), $\times 600$ (G and H). A-D: Although mesangial proliferation occurs on day 7, the mesangial hypercellularity gradually subsides by weeks 2 to 4, and the glomerular lesions recover by week 8. E-H: During the recovery, capillary regeneration occurs and the capillary network develops by week 8.

Figure 10. Activated mesangial cells (**A** and **C**) and mesangial matrix areas (**B** and **D**) in vehicle (**A** and **B**) or VEGF (**C** and **D**)-treated rats on week 8 [α -SMA stain; original magnifications, $\times 600$ (**A** and **C**); type IV collagen stain; original magnification, $\times 600$ (**B** and **D**)]. In vehicle-treated rats, damaged glomeruli are characterized by global sclerosis with presence of numerous α -SMA+ activated mesangial cells and type IV collagen deposition. In contrast, in VEGF-treated rats, damaged glomeruli recover by week 8 and show minimal activated mesangial cells and minimal type IV collagen+ mesangial matrix accumulation.

accompanied by severe endothelial injury and capillary destruction.

VEGF is as an endothelial cell-specific mitogen and could play a highly dynamic role in the regulation of both physiological and pathological angiogenesis.^{17–19} Through alternative splicing of RNA, VEGF could exist in one of four different isoforms: two isoforms are diffusible (VEGF₁₂₁, VEGF₁₆₅), and two (VEGF₁₈₉, VEGF₂₀₆) are mostly bound to the extracellular matrix. Its binding sites, shown recently to include the tyrosine kinase receptors flt-1 (VEGFR-1), flk-1/KDR (VEGFR-2), and flt-4 (VEGFR-3), are present in endothelial cells,^{17–21} in agreement with the hypothesis that VEGF is an endothelial cell-specific factor. Consistent with certain features of VEGF, systemic administration of VEGF₁₆₅ in the present study significantly enhanced glomerular capillary repair with endothelial cell proliferation in Thy-1/Habu-snake venom GN. Importantly, even in rats treated with VEGF₁₆₅, endothelial cell proliferation is precisely regulated and VEGF-mediated endothelial cell proliferation can be inhibited before occurrence of endothelial cell overproliferation. Indeed, in the present study, proliferating endothelial cells were rapidly reduced in VEGF-infused rats on day 9. Moreover, in this model, administered VEGF₁₆₅ at a dose of 10 μ g/100 g body weight/day did not mediate endothelial cell proliferation in normal organs, including liver, lungs, heart, and digestive tract (data not shown).

Recent studies have indicated that VEGF receptors are also expressed on mesangial cells,^{22,23} and VEGF₁₆₅ induces mesangial cell proliferation,²³ suggesting an autocrine mechanism of VEGF/VEGF receptors in mesangial cell proliferation in GN. In the present study, however, proliferation and/or activation of mesangial cells even in rats treated with VEGF₁₆₅ significantly decreased compared with the control group, indicating that treatment with VEGF₁₆₅ at a dose of 10 μ g/100 g body weight/day does not enhance the proliferation and activation of mesangial cells. Because impaired capillary regeneration in severely damaged regions relates to the continuation of mesangial cell proliferation and activation, capillary regeneration can either directly or indirectly influence the suppression of mesangial cell proliferation

and activation, may associate with a reciprocal interaction between endothelial cells and mesangial cells. VEGF increases the permeability of capillaries and is sometimes referred to as vascular permeability factor,^{17–19,24} and is also thought to be involved in the development of proteinuria.^{25,26} However, recent studies have shown that VEGF does not affect the development of proteinuria in renal diseases.^{27–29} Our results in the present study also demonstrated that administration of VEGF₁₆₅ did not worsen proteinuria in GN. VEGF is also known as a chemoattractant for leukocytes.^{18,19} In our study, however, we could not detect a significant effect of VEGF₁₆₅ on infiltration of naphthol AS-D chloroacetate esterase+ neutrophils, CD3+ T lymphocytes, and ED-1+ macrophages in the glomeruli during treatment. Our results, therefore, indicate that VEGF₁₆₅ specifically mediates

Figure 11. The number of naphthol AS-D chloroacetate esterase+ neutrophils (**A**), CD3+ T lymphocytes (**B**), ED-1+ macrophages (**C**), PCNA+/ α -SMA+ proliferating and activated mesangial cells (**D**), and the score of α -SMA+ areas (**E**) and IV collagen+ areas (**F**) per glomerular cross-section in VEGF-treated (filled circle) or control (open circle) groups. Filled square represents values at day 0 and day 2 (before VEGF or vehicle treatment) in both groups. Values are expressed as mean $\pm$ SD. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

capillary repair through endothelial cell proliferation in GN.

Glomerular VEGF can be released by both resident glomerular cells and infiltrating leukocytes in GN.^{4,21,30–33} Of glomerular cells, epithelial,^{4,30} endothelial,³¹ and mesangial^{4,21,32,33} cells can produce VEGF *in vitro*. Several workers have reported that podocytes express VEGF in normal glomeruli.^{21,33} In addition, activated mesangial cells also produce VEGF in proliferative GN.^{4,32} Our results showed that glomerular epithelial cells, proliferating mesangial cells, and some infiltrating leukocytes expressed VEGF, and the protein levels of VEGF₁₆₅ increased in damaged glomeruli in the early phase of experimentally induced GN. VEGF receptor flk-1 was expressed on normal, damaged, and regenerating glomerular endothelial cells. A variety of cytokines and growth factors are involved in the pathogenesis of GN,³⁴ and several of these, such as transforming growth factor- β , platelet-derived growth factor, interleukin-1 β , epidermal growth factor, and insulin-like growth factor stimulate VEGF production.^{17–19} In addition, VEGF is stimulated by hemodynamic changes, including hypoxia stress, mechanical stretch, and hypertension.^{17–19,35} Similarly, known inducers of VEGF receptors include platelet-derived growth factor and hypoxic stress.^{19,20} Thus, a series of stimuli involved in GN can induce VEGF/VEGF receptor.

In Thy-1/Habu-snake venom GN, however, proliferation of endothelial cells and capillary repair was rarely seen in severely damaged glomeruli. Rather, mesangial cell proliferation and/or activation continued, and the sequence of events showed progression from damaged glomeruli to global sclerosis associated with chronic renal failure. These results suggest that impaired angiogenic capillary repair is associated with progression of GN and irreversible glomerular damage. Angiogenesis, vascular maturation, and vascular remodeling are controlled by local actions of chemical mediators, extracellular matrix, metabolic gradients, as well as physical angiogenic, angiogenesis-inhibitory, and vascular remodeling-induced factors.^{36–39} It is probable that these parameters are not precisely regulated in this model. In addition, severe capillary destruction with severe injury and marked loss of endothelial cells may be associated with impaired angiogenesis. Because glomerular epithelial cells express desmin, a marker of glomerular epithelial injury in rats,⁴⁰ from the early phase of Thy-1/Habu-snake venom GN (Dr. Y Masuda, personal communication), glomerular epithelial cell injury may also influence impaired angiogenesis. Although the mechanisms of impaired glomerular capillary repair in this model are not fully understood at present, acceleration of angiogenic capillary repair is necessary in the treatment of GN accompanied by severe endothelial injury and capillary destruction.

Recently, the therapeutic effects of angiogenic growth factors have been investigated clinically and in animal models, and administration of VEGF, basic fibroblast growth factor (FGF-2), or vectors including their encoding DNA results in improvement of hemodynamics and increased capillary density in ischemic tissues.^{19,41,42} In

renal disease, two angiogenic growth factors, FGF-2 and VEGF, are known to mediate endothelial cell proliferation in damaged glomeruli.^{4,8} However, intravenous injection of FGF-2 results in glomerular podocyte injury and promotes glomerular sclerosis.^{43–45} FGF-2 is also known as a strong mitogen for kidney fibroblasts and may promote interstitial fibrosis.⁴⁶ Based on this background, we examined the beneficial effects of angiogenesis in GN, using VEGF₁₆₅ and demonstrated that stimulation of angiogenic capillary repair by VEGF₁₆₅ enhanced glomerular repair and accelerated resolution of GN. Our results in the present study suggest that systemic administration of VEGF₁₆₅ could be therapeutically effective in GN accompanied by extensive endothelial damage.

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