Vascular Endothelial Growth Factor_165_ Resolves Glomerular Inflammation and Accelerates Glomerular Capillary Repair in Rat Anti–Glomerular Basement Membrane Glomerulonephritis

AKIRA SHIMIZU, YUKINARI MASUDA, TAKAHIRO MORI, HIROSHI KITAMURA, MASAMICHI ISHIZAKI, YUICHI SUGISAKI, and YUH FUKUDA

Department of Pathology, Nippon Medical School, Tokyo, Japan

Abstract. Vascular endothelial growth factor (VEGF) is essential for maintenance of the glomerular capillary network. The present study investigated the effects of VEGF in rats with progressive crescentic glomerulonephritis (GN). Necrotizing and crescentic GN was induced in rats by injection of anti-rat glomerular basement membrane (GBM) antibody. The alterations of glomerular capillaries and glomerular VEGF expression were assessed. In addition, the effects of continuous VEGF_165_ administration (10 μg/100 g per d) on glomerular capillaries, glomerular inflammation, and the course of crescentic GN were examined. The appropriate timing of VEGF administration in progressive GN also was evaluated. In anti-GBM GN, necrotizing and crescentic glomerular lesions occurred by day 7, and newly formed necrotizing lesions reoccurred by week 3. Expression of VEGF was markedly reduced in necrotizing and crescentic lesions. Capillary repair was impaired after capillary destruction in necrotizing and crescentic glomeruli, which rapidly progressed to sclerotic glomeruli with chronic renal failure. In contrast, in the rats that received VEGF_165_ administration from day 7, the necrotizing and crescentic lesions recovered and renal function significantly improved in week 4. This was evident by proliferating endothelial cells and glomerular capillary repair. In addition, VEGF administration decreased intercellular adhesion molecule-1 and monocyte chemoattractant protein-1 expression in glomeruli (particularly on endothelial cells), reduced glomerular infiltrating CD8-positive and ED-1–positive cells, and inhibited the newly formed necrotizing lesions. VEGF administration was apparently effective against both the inflammatory and necrotizing glomerular lesions. These results suggest that VEGF administration resolves glomerular inflammation and accelerates glomerular recovery in the progressive necrotizing and crescentic GN. The therapeutic application of VEGF may be clinically useful for severe GN accompanied by extensive glomerular inflammation and endothelial injury.

Glomerular capillary and endothelial injury plays an important role in the pathogenesis of renal diseases and is viewed as a crucial factor in disease progression (1–4). Recent studies of experimental glomerulonephritis (GN) have indicated that impairment of the capillary repair process in glomerular damage may be associated with the continuation of mesangial proliferation and the accumulation of mesangial matrix, which results in the development of glomerular sclerosis and renal dysfunction (5–7). However, in recovery models of GN, complete capillary repair in damaged glomeruli can lead to full recovery of the glomerular architecture with resolution of the mesangial proliferation (8–10). These findings demonstrate that capillary repair is a crucial event to allow for glomerular healing as well as recovery from mesangial proliferative GN accompanied by destruction of the glomerular capillary network. One of the most severe types of GN accompanied by destruction of the glomerular capillary network is diffuse necrotizing and crescentic GN. Progression to crescentic GN is determined by the severity of the injury to the glomerular capillary walls (11,12). Our recent study demonstrates that the progression of glomerular sclerosis with renal dysfunction in necrotizing and crescentic GN is associated with destruction of the capillary network in the necrotizing lesions and subsequently the impairment of the glomerular capillary repair process (5). In addition, capillary regression caused by endothelial cell apoptosis contributes to the development of glomerular sclerosis. These results suggest that the development of necrotizing and crescentic GN and renal dysfunction may be prevented by the therapy for stimulation of angiogenesis.

Several studies have indicated that the most important angiogenic factor in glomeruli is vascular endothelial growth factor (VEGF) (13–15). VEGF has been known as a proliferative and survival factor for endothelial cells. It plays a highly dynamic role in the regulation of angiogenesis through specific receptors on endothelial cells (16,17). The systemic administration of VEGF can mediate glomerular endothelial cell proliferation in several experimental models of renal diseases, including thrombotic microangiopathy, renal ablation, and mesangial proliferative GN (6,18–20). In the present study, the diffuse necrotizing and crescentic GN was induced in Wistar-Kyoto (WKY) rats by injection of anti-rat glomerular basement
membrane (GBM) antibody. We determined the serial changes in VEGF production in the glomeruli and the alterations of glomerular capillaries. In addition, we examined the potential beneficial effects of systemic VEGF165 administration in the development of glomerular inflammation and in the progression of crescentic GN.

Materials and Methods

Anti-GBM GN Model in WKY Rats

The Ethics Review Committee for Animal Experimentation of Nippon Medical School approved the animal experiments described in the present study. Inbred male WKY rats (Charles River Japan, Kanagawa, Japan) that weighed 100 g were used for all experiments. Anti-GBM GN was induced by injection of rabbit anti-rat GBM antibody at a dose of 50 μg IgG/100 g body wt on day 0 (5,21). In the present study, we performed three experiments. In experiment 1, for assessing the alterations of VEGF expression and the glomerular capillary network in the development of anti-GBM GN, five rats were killed on day 7 and 2, 3, 4, 6, and 8 wk after the administration of anti-GBM antibody. In experiment 2, for investigating the beneficial effects of exogenous VEGF, rats were treated with recombinant human VEGF165 (Life Technologies BRL, Tokyo, Japan) dissolved in saline (VEGF-treated group) or saline alone (control group) injection. Rats were administered VEGF165 at a dose of 10 μg/100 g body wt per d or saline using an intraperitoneal micro-osmotic pump (Alzet oosmotic pump; Alza, Mountain View, CA) starting at day 7 and ending at day 28 (3 wk). In each group, five rats underwent biopsy or were killed on day 7 (before VEGF administration) and 2, 3, 4, 6, and 8 wk after the disease induction. In experiment 3, for examining the appropriate timing of VEGF administration in anti-GBM GN, rats in the various phases of GN were administered VEGF165 (10 μg/100 g body wt per d) or vehicle using the same method. VEGF165 was initiated on day 2 (glomerular inflammation phase), day 7 (necrotizing glomerular phase), week 2 (proliferative glomerular phase), and week 4 (sclerotic glomerular phase) and continued for 1 wk duration, respectively. In each group, five rats underwent biopsy or were killed on, before, and 1 wk after treatment with VEGF165. For estimating 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).

Histopathologic 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, and periodic acid-methenamine Silver for histopathologic examination.

The following primary antibodies were used for immunohistochemistry. (1) Polyclonal rabbit anti-rat thrombomodulin (TM) antibody (provided by Dr. David Stern, Columbia University, New York, NY), which has been used as a marker for endothelial cells (5,6,9,10). Biotinylated anti-rat TM antibody was prepared using a biotin labeling kit (Boehringer Mannheim, Mannheim, Germany). (2) Monoclonal mouse anti-rat endothelial cell antigen-1 (RECA-1) antibody (Serotec, Oxford, UK), which has also been used as a marker for endothelial cells. (3) Monoclonal mouse anti-proliferating cell nuclear antigen (PCNA) antibody (PC10; DAKO, Glostrup, Denmark), which is a marker for cellular proliferation. (4) Polyclonal rabbit anti-VEGF antibody (6), which can detect VEGF-producing cells. Biotinylated anti-VEGF antibody was prepared using a biotin labeling kit (Boehringer Mannheim). (5) Monoclonal mouse anti-flk-1 antibody (A-3; Santa Cruz Biotechnology, Santa Cruz, CA), which can detect cells that express VEGF receptor-2 (VEGFR-2). (6) Monoclonal mouse anti-rat CD8 antibody (Nichirei, Tokyo, Japan). (7) Monoclonal mouse anti-rat ED-1 antibody (BMA, Nagoya, Japan), which can detect infiltrating macrophages. (8) Monoclonal mouse anti-intercellular adhesion molecule-1 (anti-ICAM-1) antibody (G-5; Santa Cruz Biotechnology). (9) Polyclonal goat anti-monozygotic chemoattractant protein-1 (anti-MCP-1) antibody (R-17; Santa Cruz Biotechnology). (9) Polyclonal goat anti-type IV collagen antibody (Southern Biotechnology Associates, Birmingham, AL), which is used for evaluation of mesangial matrix accumulation and glomerular sclerosis. For immunohistochemistry for TM, PCNA, CD8, ED-1, and type IV collagen, 20%-buffered, formalin-fixed, paraffin-embedded tissue sections were used and the specimens were stained by the standard avidin-biotin-peroxidase complex technique. For TM, VEGF, flk-1, ED-1, and type IV collagen, tissue sections were incubated with 0.1% pepsin for 60 min, 0.4% pepsin for 20 min, 0.1% proteinase for 5 min, 0.1% pepsin for 45 min, 0.1% pepsin for 30 min, and 0.1% proteinase for 5 min, respectively, before incubation with the primary antibody. For optimizing the detection of PCNA and CD8, sections were microwaved for 10 min in 0.01 M sodium citrate (pH 6.0) and in 4% urea, respectively, after dewaxing, respectively, after dewaxing. Proliferating endothelial cells were identified after double immunohistochemistry staining with PCNA and TM using the color modification method of 3,3’-diaminobenzidine (DAB) precipitation by nickel chloride, which changes DAB color from brown to black (5,6,10). Sections were incubated with PCNA followed by a peroxidase-conjugated goat anti-mouse IgG and H2O2, nickel chloride–containing DAB. Sections were then incubated with biotinylated anti-rat TM antibody and an avidin-biotin peroxidase complex followed by H2O2 containing DAB. For detecting ICAM-1 and MCP-1 expression in glomeruli, 4-μm frozen sections were stained by the standard indirect technique and were observed with a fluorescence microscope. For detecting ICAM-1 and MCP-1 expression on endothelial cells, double immunohistochemistry staining with RECA-1 and ICAM-1 or MCP-1 was performed. Four-micrometer frozen sections were stained with anti–RECA-1 antibody (mouse IgG1) and followed by FITC-labeled goat anti-mouse IgG1 antibody (ZYMED, San Francisco, CA). Sections were then incubated with anti–ICAM-1 (mouse IgG2a) or anti–MCP-1 (goat IgG) and followed by Texas-red conjugated goat anti-mouse IgG2a or rabbit anti-goat IgG antibodies (Biomedia, Foster City, CA). 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 equivalent 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 Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate and then examined with an electron microscope (model H7100, Hitachi Corp., Tokyo, Japan).

Isolation of Glomeruli and Western Blot Analysis for VEGF165, ICAM-1, and MCP-1

For examining the production of VEGF, ICAM-1, and MCP-1 in glomeruli before and after disease induction, Western blotting was performed using polyclonal rabbit anti-VEGF antibody (147; Santa Cruz Biotechnology), monoclonal mouse anti–ICAM-1 antibody
(G-5; Santa Cruz Biotechnology), or monoclonal mouse anti–MCP-1 antibody (MB10; IBL, Gunma, Japan), respectively. For this purpose, glomeruli were isolated using a standard three-stage sieving method (6). Isolated glomeruli were homogenized in lysis buffer. After centrifugation at 15,000 × g for 30 min at 4°C, the supernatant was collected and used for analysis. Samples that contained 10 μg of protein per lane were separated on 10% acrylamide gel by SDS-PAGE. After electrophoresis, the separated protein was transferred to a Hybond-P nitrocellulose membrane (Amersham Life Science, Buckinghamshire, UK) and incubated with anti-VEGF antibody (1:2000), anti–ICAM-1 antibody (1:1000), or anti–MCP-1 antibody (1:1500). Bound antibody was detected with peroxidase-conjugated anti-rabbit IgG antibody (1:1000) or peroxidase-conjugated anti-mouse IgG antibody (1:5000), respectively, with the enhanced chemiluminescence detection system (ECL Western blotting detection regents; Amersham). Membranes were washed and then exposed to film. For confirming equal loading of each protein, the membrane was stripped and rebotted with anti–β-actin antibody (Sigma, St. Louis, MO). Densitometric analysis of the bands was performed with NIH Image software.

Quantification of Histopathologic Findings

In each kidney sample, >30 cross-sections of glomeruli were examined sequentially for the following parameters: (1) Glomerular endothelial cells: The mean number of nuclei of TM-positive cells per glomerular cross-section; (2) proliferating endothelial cells: The mean number of both PCNA- and TM-positive cells per glomerular cross-section; (3) glomerular capillaries: The mean number of glomerular capillary lumina surround by TM-positive cells per glomerular cross-section; (4) infiltrating CD8+ cells: The mean number of CD8-positive cells per glomerular cross-section; (5) infiltrating macrophages: The mean number of ED-1–positive cells per glomerular cross-section; (6) necrotizing glomeruli: The mean percentage of glomeruli with necrotic lesions in periodic acid-methenamine Silver–stained sections; (7) glomerular sclerosis: The mean semiquantitative staining score of type IV collagen per glomerular cross-section (6) (scores 0 to 4; score 0, no localized increase of staining; score 1, up to 25% of the glomerular tuft showing focally increased staining; score 2, 25 to 50% of the glomerular tuft showing focally a strong staining; score 3, 50 to 75% of the glomerular tuft stained strongly in a focal manner; score 4, >75% of the glomerular tuft stained strongly). Glomerular cross-sections that contained only a small portion of the glomerular tuft were excluded from the analysis. All histopathologic evaluations were performed by investigators who were blinded to the treatment modality (saline versus VEGF). These results were expressed as the mean ± SD, and statistical analysis was performed using the t test.

Results

Glomerular VEGF and Impaired Capillary Repair in Anti-GBM GN

A severe necrotizing and crescentic GN was produced in WKY rats by a single injection of anti-rat GBM antibody on day 0. Many leukocytes infiltrated the glomeruli, and, subsequently, severe necrotizing lesions occurred with cellular crescents by day 7 (Figure 1A). Newly formed necrotizing glomerular lesions occurred repeatedly by week 3 (Figure 1B), and these injured areas progressed to global sclerosis of the glomeruli between week 4 and week 8 (Figure 1, C and D). In necrotizing lesions, TM-positive endothelial cells disappeared concordant with destruction of the glomerular capillary network (Figure 1E).

The podocytes expressed VEGF in normal glomeruli before disease induction (data not shown). However, the expression of VEGF in podocytes was markedly decreased or lost around necrotizing lesions or within cellular crescents (Figure 2A). In addition, the expression of VEGF was not upregulated in segmental necrotizing and crescentic lesions, although VEGF expression was detected in podocytes or cells in mesangial areas (probably activated mesangial cells and infiltrating macrophages) in remaining histologic well-preserved glomerular segments (Figure 2A). In accordance with the expansion of the injured glomerular areas by week 8, remaining podocytes and cells in mesangial areas with VEGF expression gradually reduced (Figure 2B). In parallel with the decrease in the relative area of VEGF expression in glomeruli, the protein level of VEGF165 gradually decreased in isolated glomeruli by week 8 (Figure 3).

VEGFR-2 (flk-1) was expressed on glomerular endothelial cells in the remaining capillaries in damaged glomeruli (Figure 2C). However, in accordance with the decreased expression of glomerular VEGF, rare proliferating endothelial cells were present along with impaired capillary regeneration in necrotizing lesions (Figure 2D). In damaged glomeruli, capillary repair was rarely seen between week 2 and week 4, and the TM-positive capillary lumina gradually reduced during the development of glomerular sclerosis by week 8 (Figure 1, E through H).
VEGF165 Accelerates Glomerular Capillary Repair in Anti-GBM GN

We next examined the effects of VEGF165-induced angiogenesis on the course of anti-GBM GN. Systemic administration of VEGF165 significantly enhanced endothelial cell proliferation and glomerular capillary repair by week 4. Numerous proliferating endothelial cells (both PCNA- and TM-positive cells) were found within necrotizing lesions (Figure 4, A and B), and regenerating capillary networks developed near the crescentic or the adhesional lesions (Figure 4, C and D). The regenerating capillaries had morphologically activated endothelial cells (Figure 4, E and F). The number of proliferating endothelial cells was increased significantly by week 2 (Figure 5A), which was followed by a rapid recovery in the number of total TM-positive glomerular endothelial cells by week 4 (Figure 5B). In parallel with capillary regeneration, the number of glomerular capillary lumina per glomerular cross-section also increased (Figure 5C). Even in rats that were treated with VEGF165 continuously by week 4, VEGF-mediated endothelial cell proliferation could be inhibited before occurrence of endothelial cell overproliferation (Figure 5). VEGF165, which was administered at a dose of 10 μg/100 g body wt per d, did not mediate endothelial cell proliferation in normal organs, including liver, lungs, heart, and the digestive tract (data not shown).

It is interesting that the cellular crescents, which developed around necrotizing lesions by day 7 (Figure 6A), were transient in nature. Recovery of necrotizing lesions correlated with the gradual subsidence of extraglomerular cellular crescents between week 2 and week 4 (Figure 6, B through D). Although small adhesive lesions were evident in damaged glomeruli,
even in the VEGF-treated group, the capillary structure developed around and/or within the adhesive lesions (Figures 4D and 6D).

VEGF$_{165}$ Resolves Glomerular Inflammation in Anti-GBM GN

We examined the effect of systemic administration of VEGF$_{165}$ on glomerular inflammation leading to necrotizing crescentic glomerular injury, because newly formed fibrinoid necrotizing lesions were markedly reduced after VEGF administration (Figure 6, B through D). This model is characterized by the accumulation and activation of CD8-positive lymphocytes and ED-1–positive macrophages in glomeruli, through MCP-1 and ICAM-1 pathways, which are crucial for the initiation and subsequent progression of anti-GBM GN (22–25). ICAM-1 and MCP-1 were expressed at detectable levels on day 7 and significantly in week 2 in damaged glomeruli (Figure 7, A and C), especially on glomerular endothelial cells, glomerular epithelial cells, and cells in crescent in the vehicle-infused control group (Figure 7, E and G). In contrast, VEGF$_{165}$ administration strongly suppressed the upregulation of ICAM-1 and MCP-1 in damaged glomeruli (Figure 7, B and D), particularly on glomerular endothelial cells (Figure 7, F and H). Western blotting analysis also showed that protein levels of ICAM-1 and MCP-1 in isolated glomeruli increased noticeably in week 1 and markedly in week 2 after disease induction in the control group (Figure 8). VEGF administration strongly suppressed the upregulation of ICAM-1 and MCP-1 protein levels in week 2. Many CD8-positive cells and ED-1–positive macrophages infiltrated the glomeruli on day 7. In the vehicle-infused control group, infiltration of these cells continued together with the occurrence of newly formed necrotiz-
ing lesions by week 3 (Figures 9, A and C, and 10). In contrast, the glomerular CD8-positive cells and ED-1–positive cells were rapidly reduced after administration of VEGF (Figure 10, A and B), and a few of these cells were seen in damaged glomeruli in the VEGF-treated group (Figure 9, B and D). After the resolution of the cell infiltration, newly formed necrotizing lesions in glomeruli were markedly diminished during VEGF treatment (Figure 10C). In parallel with the resolution of the glomerular inflammation and the recovery from necrotizing and crescentic GN, VEGF-treated rats showed significant improvement in the degree of glomerular sclerosis, renal function, and proteinuria in week 4 compared with the vehicle-treated control rats (Figure 11).

**After Stopping of VEGF165 Treatment in Anti-GBM GN**

After stopping treatment with VEGF, marked cellular infiltration recurred with the formation of severe necrotizing lesions and crescent by week 6 (Figure 12, A through D). Necrotizing and crescentic glomeruli progressed to sclerotic glomeruli with proteinuria and renal dysfunction by week 8 (Figure 12, E through H). However, the progression of glomerular sclerosis and renal dysfunction in the VEGF-treated group were delayed by VEGF165 treatment for 3 wk, and the degree of glomerular sclerosis, proteinuria, and renal function was better in the VEGF-treated group than in the vehicle-infused control group in week 8.

**Timing of VEGF165 Treatment in Anti-GBM GN**

We examined the appropriate timing of VEGF treatment in progressive GN (Figure 13). In rats with glomerular inflammation, administration of VEGF from day 2 (glomerular inflammation phase) prevented necrotizing and crescentic glomerular lesions, proteinuria, and renal dysfunction by day 9. When VEGF was started on day 7 (necrotizing glomerular phase), both proteinuria and renal dysfunction were almost completely recovered by week 2. Rats that received VEGF from week 2 (proliferative glomerular phase) showed partial but significant recovery of proteinuria and renal dysfunction by week 3. In the rats that were treated with VEGF from week 4 (sclerotic glomerular phase), administered VEGF could not enhance the angiogenic capillary repair in sclerotic glomerular lesions.
lesions, but VEGF tended to inhibit the progression of renal dysfunction and proteinuria by week 5.

**Discussion**

We have demonstrated in the present study that the progression of necrotizing and crescentic GN is accompanied by a decrease of VEGF expression in damaged glomeruli and impaired angiogenic capillary repair after the destruction of the glomerular capillary network. Systemic administration of VEGF<sub>165</sub> successfully induced glomerular healing, including glomerular capillary repair and resolution of extraglomerular crescent. In addition, VEGF<sub>165</sub> administration led to a decrease in ICAM-1 and MCP-1 expression in glomeruli, reduced glomerular infiltrating CD8<sup>+</sup> cells and macrophages, and inhibited acute inflammatory necrotizing and crescentic glomerular destruction. Importantly, VEGF significantly ameliorated renal damage when administration was initiated after appearance of necrotizing and crescentic lesions. In addition, VEGF tended to prevent progression of renal dysfunction when administration was started after development of glomerular sclerosis. These findings suggest a possible effect on established human renal diseases, and the therapeutic application of VEGF may be clinically useful for progressive GN accompanied by severe endothelial injury, capillary destruction, and glomerular inflammation.

In neonatal rodents, VEGF and its receptors play a critical role in glomerular capillary formation and endothelial cell differentiation in developing glomeruli, and VEGF has been known to be an essential molecule for kidney development, especially glomerulogenesis (13–15). In the recovery models of GN, glomerular capillary repair with endothelial cell proliferation is associated with upregulated expression of VEGF and its receptor, VEGFR-2 (8). In addition, a recent report using radiolabeled VEGF indicated that administered<sup>125</sup>I-VEGF accumulates in the kidney even in normal rats and that the major VEGF binding sites in kidney are in the glomeruli (26). In diseased glomeruli with upregulation of VEGFR-2, such as in the case of diabetic nephropathy, the binding of administered<sup>125</sup>I-VEGF in glomeruli is increased (26). In support of this evidence, systemic administration of VEGF<sub>121</sub> or VEGF<sub>165</sub> stimulates endothelial cell proliferation in several experimental models of renal diseases (6,18–20). Our previous studies dem-

---

**Figure 11.** Glomerular sclerosis (A through C), proteinuria (D), blood urea nitrogen (E), and serum creatinine (F) levels in VEGF- or vehicle-treated groups by week 4. In week 4, glomerular sclerosis with type IV collagen deposition develops in vehicle-infused control groups (A), but minimal sclerosis is detected in adhesion area in the VEGF-treated group (B). (C through F) Values are expressed as mean ± SD. *P < 0.05, **P < 0.01, and ***P < 0.001 versus control. VEGF administration substantially inhibits glomerular sclerosis, reduces proteinuria, and prevents the loss of renal function. Magnification, ×200 in A and B (type IV collagen stain).
onstrated that glomerular capillary repair occurs through capillary regeneration from the remaining endothelial cells in the lesions, as well as from new capillary growth in the glomerular vascular pole (6,10). Recently, several investigators have established the presence of bone marrow–derived endothelial progenitor cells in the adult circulation and demonstrated that these cells contribute to glomerular healing, including glomerular capillary repair in experimental and human renal diseases (27–29). VEGF augments circulating endothelial progenitor cells and also induces engraftment of these cells into vasculature (30,31). In the present study, systemic administration of VEGF165 can stimulate angiogenic capillary repair in damaged glomeruli and improves renal function in necrotizing and crescentic GN. Further studies are ongoing to identify whether bone marrow–derived endothelial progenitor cells are essentially involved in capillary repair in this model and whether the number of these cells is increased by VEGF administration.

Importantly, VEGF mediates not only recovery of intraglomerular necrotizing lesions but also the resolution of extraglomerular cellular crescents. Glomerular crescents occupy the urinary space by covering and compressing the glomerular tuft, and crescent formation contributes directly to renal dysfunction through the reduction of glomerular filtration and urinary flow (11,12). The development of necrotizing lesions with capillary rupture may be essential in the process of crescent formation (11,12). In the present study, cellular crescent resolved in parallel with the recovery of necrotizing lesions after VEGF administration. Our results suggest that the acceleration of capillary repair by VEGF may reduce capillary wall injury and rupture leading to crescent formation, through the recovery of intraglomerular necrotizing lesions.

Infiltration by CD8-positive cells and macrophages in glomeruli through chemokines and adhesion molecules is a crucial event for the initiation and subsequent progression of anti-GBM GN (22,23). A key role for ICAM-1 and MCP-1 in leukocyte infiltration in this model has been indicated from studies using neutralizing antibodies against these chemokine and adhesion molecules (23–25). VEGF is known as a proin-
cytoprotective factor for endothelial cells and vascular protective factor in the adult vasculature and in disease (33–37). In these studies, VEGF can induce enhancement of endothelial functions that mediate the inhibition of vascular smooth muscle cell proliferation, prevention of endothelial cell apoptosis, prolongation of endothelial cell survival, suppression of coagulation system, and inhibition of inflammation. In addition, a recent study demonstrated that the administration of a single bolus dose of VEGF attenuates inflammation-induced leukocyte–endothelium interaction at both microvascular and macrovascular levels (38). In the present study, the expression of ICAM-1 and MCP-1 in glomeruli, particularly on the glomerular endothelial cells, which increased in the anti-GBM GN, was strongly suppressed by VEGF165 administration. This resulted in the resolution of glomerular inflammation and reduced the leukocyte-mediated necrotizing glomerular injury. These findings suggest that systemic VEGF165 administration can induce the resolution of glomerular inflammation in anti-GBM GN through the stabilization of glomerular endothelial cells. It also strongly supports the idea that VEGF could be a cytoprotective factor for endothelial cells and vascular protective factor for glomerular capillaries in anti-GBM GN.

During VEGF administration for 3 wk, we expected that a similar mechanism of accommodation in organ transplantation could develop and the intensity of anti-GBM GN, after stopping treatment with VEGF, could be reduced. However, after stopping treatment with VEGF, marked necrotizing and crescentic GN recurred. Our findings suggest that although the beneficial effect of VEGF cannot continue for a long period in this model after stopping of VEGF165 treatment, the resolution of glomerular inflammation in anti-GBM GN is dependent on VEGF165 administration.

Podocytes express VEGF in normal glomeruli. In Gn, both resident glomerular cells, including podocytes, endothelial cells, activated mesangial cells, and infiltrating leukocytes, can release glomerular VEGF (6,8,18,19). Our results in the present study showed that the expression of VEGF on podocytes was markedly reduced in necrotizing and crescentic GN. In addition, VEGF expression was not upregulated in both resident glomerular cells and infiltrating leukocytes during the progression of crescentic GN. A possible explanation for the loss of VEGF expression in damaged glomeruli could be through the loss of or severe injury to glomerular podocytes and mesangial and endothelial cells by necrotizing glomerular destruction and crescent formation. In addition, a variety of cytokines and growth factors are involved in the initiation and progression of anti-GBM GN (11,18,25), and several of these, such as, IL-1α, IL-6, and TNF-α, downregulate VEGF production (18). In the present study, the mechanisms of this decrease of VEGF production in glomeruli was not fully investigated, but it may seem that impaired capillary repair and capillary regression in this model is paralleled by a progressive loss in glomerular VEGF expression. We therefore presume that impaired glomerular capillary regeneration and capillary regression leading to the progression of anti-GBM GN and irreversible glomerular scarring may be associated with VEGF depletion.

Recently, the therapeutic effects of angiogenic growth fac-

![Figure 13. Various timing of VEGF (●) or vehicle (○) treatment and the morphologic alterations (A through D) or changes of renal functions (E and F). Systemic VEGF administration was initiated on day 2 (graph: 2d to 9d), day 7 (graph: 1w to 2w), week 2 (graph: 2w to 3w), and week 4 (graph: 4w to 5w) and continued for 1 wk. Values are expressed as mean ± SD. *P < 0.05, **P < 0.01, and ***P < 0.001 versus control. In the rats that were treated with VEGF from the early phase of GN, necrotizing and crescentic lesions are prevented (A, control on day 9; B, VEGF-treated group on day 9), and urinary protein and blood urea nitrogen levels seem to improve 1 wk after VEGF treatment. In the rats that were treated with VEGF from the late phase of GN (after development of glomerular sclerosis), VEGF does not mediate enough angiogenic capillary repair in sclerotic lesions (C, control in week 5; D, VEGF-treated group in week 5). However, VEGF tends to inhibit the development of renal dysfunction and proteinuria. Magnification, ×200 in A through D (PAM stain).](image-url)
tors have been investigated clinically and in animal models. The administration of VEGF, basic fibroblast growth factor (FGF-2), hepatocyte growth factor (HGF), or vectors encoding these proteins results in the improvement of hemodynamics and increased capillary density in ischemic tissues (39,40). In renal disease, these angiogenic growth factors are known to mediate endothelial cell proliferation in glomeruli (6,8,13–15,18–20,41,42). However, intravenous injection of FGF-2 results in glomerular podocyte injury, promoting glomerular sclerosis (43). Although systemic administration of HGF can stimulate endothelial cell proliferation and capillary repair in glomeruli (42), our preliminary study using anti-GBM GN showed no or minimal effects of administered HGF on resolution of glomerular inflammation (A.S. and T.M., personal communication). On the basis of this background, we examined the beneficial effects of VEGF165 and conclude that systemic administration of VEGF165 resolves glomerular inflammation and accelerates glomerular repair in the progressive necrotizing and crescentic GN. Systemic administration of VEGF165 may be therapeutically effective in GN accompanied by extensive endothelial damage and severe glomerular inflammation.

Acknowledgments

We express special thanks to Dr. David Stern (Columbia University, New York, NY) and Dr. Yukio Yuzawa (School of Medicine, Nagoya University, Nagoya, Japan) for providing the anti-TM antibody; Dr. Yasuhiro Natori and Dr. Naoyuki Nakao (Research Institute, International Medical Center of Japan, Tokyo, Japan) for providing the anti-GBM antibody; Dr. Clive Patience (Immerge Biotherapeutics, Cambridge, MA) and John M. Lavelle (Transplantation Biology Research Center, Massachusetts General Hospital, Boston, MA) for excellent advice and critical review of the manuscript. We are also grateful to Dr. Toshiyuki Ishiwata (Department of Pathology, Nippon Medical School) for advice and Mr. Takashi Arai, Ms. Mitsue Kataoka, Ms. Arimi Ishikawa, and Ms. Naomi Tamura for expert technical assistance.

References