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Short title: Role of VEGF in rat crescentic glomerulonephritis

Blockade of VEGF accelerates proteinuria, via decrease in nephrin expression in rat crescentic glomerulonephritis.

Vascular endothelial growth factor (VEGF) is a potent angiogenic factor which maintains the glomerular and peritubular capillary network in the kidney. The soluble form of the VEGF receptor-1 (sFlt-1) is known to regulate VEGF activity by binding VEGF in the circulation. ~~In the present study, w~~We hypothesized that VEGF may be beneficial for maintaining glomerular filtration barrier and vascular network in rats with progressive glomerulonephritis (GN).

For blockade of VEGF activity in vivo, rats were transfected twice with plasmid DNA encoding the murine ~~soluble Flt-1 (~~sFlt-1~~)~~ gene into femoral muscle 3 days before and 2 weeks after the induction of anti-glomerular basement membrane (GBM) antibody-induced GN.

Inhibition of VEGF with sFlt-1 resulted in massive urinary protein excretion, concomitantly with down-regulated expression of nephrin in nephritic rats. Further, blockade of VEGF induced mild proteinuria ~~excretion of protein into the urines~~ in normal rats. Administration of sFlt-1 affected neither the infiltration of macrophages nor crescentic formation. In contrast, treatment of sFlt-1 accelerated ~~had impact on~~ the progression of glomerulosclerosis and interstitial fibrosis accompanied with renal

dysfunction and peritubular capillary loss at day 56.

VEGF may play a role in maintaining the podocyte function as well as renal vasculature, thereby protecting glomeruli and interstitium from progressive renal insults.

Key words: VEGF, proteinuria, podocyte, nephrin, anti-GBM disease

Introduction

Vascular endothelial growth factor (VEGF) is a potent angiogenic factor that induces endothelial cell migration, growth, differentiation and regeneration through its receptors, VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1) (1). In kidney, VEGF is abundantly expressed in glomerular epithelial cells (podocytes) and tubular epithelial cells, whereas the glomerular and peritubular capillary endothelial cells express cognate VEGF receptors (2, 3, 4). The importance of VEGF in kidney is evidenced by the fact that deficiency in VEGF selectively in the podocytes showed impaired glomerular capillary formation due to a loss of endothelial cells in mice (5), and antagonizing circulating VEGF caused glomerular endotheliosis in pregnant rats which is also noted in human preeclampsia (6).

In addition to the impacts of VEGF on endothelial cells, physiological levels of VEGF are pivotal for maintaining glomerular filtration barrier. In this regard, Sugimoto et al. reported that anti-VEGF antibodies and soluble VEGF receptor 1 (sFlt-1), which inhibit~~or~~ ~~of~~ VEGF activity by directly sequestering VEGF and by functioning as a dominant negative inhibitor against VEGF receptors (7) respectively, induced proteinuria associated with podocyte dysfunction in normal kidneys (8). Recent reports from human clinical cancer trials using anti-VEGF antibodies (Bevacizumab) suggest

that proteinuria may be associated with treatment protocols (9-11).

Therefore, we hypothesized in this study that VEGF may be responsible for maintaining the glomerular filtration barrier and vascular network in rats with progressive glomerulonephritis (GN). Molecular and pathological mechanisms involved in the increased levels of urinary protein, electron microscopic findings and the expression of slit diaphragm-associated molecules were examined. Further, the effect of inhibition of VEGF on podocyte-associated molecules was examined. The present study shows that blockade of VEGF activity by transfection of the plasmid DNA encoding sFlt-1 gene in femoral muscles resulted in massive urinary protein excretion, concomitantly with down-regulated expression of nephrin, which is one of the major glomerular slit diaphragm-associated molecules (12), in diseased glomeruli. Treatment with ~~of~~ sFlt-1 affected ~~had impact on~~ the progression of glomerulosclerosis and interstitial fibrosis, resulting in renal dysfunction. These results suggest that VEGF may play a role in maintaining the podocyte function as well as renal vasculature, thereby regulating urinary protein excretion and protecting glomeruli and interstitium from progressive insults.

Results

Expression of sFlt-1

Muscle fibers positive for sFlt-1 were observed in femoral muscles from the sFlt-1 gene-transfected rats 6 days after disease induction (Figure 1a). In contrast, sFlt-1 protein was not detected in the muscles from the empty plasmid-treated rats (Figure 1b). Further, RT-PCR analysis of muscle tissue for sFlt-1 mRNA showed that sFlt-1 mRNA was expressed at the injected sites in sFlt-1-treated rats (Figure 1c). Finally, urinary levels of sFlt-1 increased at day 6 in rats treated with sFlt-1 ~~as~~ compared with those from vehicle-treated rats (Figure 1d), and remained elevated ~~lasted significant elevation~~ for at least 14 days (data not shown).

Histopathological studies

Semiquantitative evaluation of deposition showed no significant difference in the deposition of rabbit IgG, rat IgG, or rat C3 between glomeruli from rats administered sFlt-1 gene or empty plasmid only (data not shown). These results suggest that induction of glomerulonephritis was equivalent in the two groups ~~achieved equally~~.

Glomerular lesions showed endocapillary proliferation, severe necrotizing lesions and crescentic formation at day 6~~, as previously reported~~. Numbers of ED-1-, PCNA-, and

CD8-positive cells did not significantly differ by the administration of sFlt-1 gene at day 6. Similarly, there was no significant difference between sFlt-1-treated and vehicle-treated rats in formation of crescentic lesions and the number of total glomerular cells at day 6 (Table 1).

Vehicle-treated nephritic animals showed a marked degree of glomerulosclerosis, as well as interstitial fibrosis at day 56 (Figure 2a, b, c, and d). ~~Two-time injection of~~ sFlt-1-treated rats showed ~~gene 3 days before renal insults and 14 days after beginning of inflammation in diseased kidneys~~ significantly more ~~increased~~ glomerulosclerosis and interstitial fibrosis ~~in this later phase~~ at day 56~~, suggesting the crucial role of VEGF in the pathogenesis of renal injury in crescentic GN (Figure 2c, d)~~.

Effects of VEGF blockade on ~~the~~ urinary protein excretion and renal function

Ten normal untreated rats excreted minute amounts of protein in the urine. ~~We determined whether VEGF blockade examined in this study induced urinary protein excretion in normal rats.~~ Blockade of VEGF induced mild proteinuria ~~excretion of protein into the urines~~ by day 6 in 6 normal rats without anti-GBM serum which was ~~and the levels of proteinuria were~~ maintained for 28 days, but ~~of disease course and~~ returned to nearly normal levels at day 56 (Figure 3~~a~~)~~, as previously reported in normal~~

~~mice (8)~~. In contrast, all nephritic rats injected with empty plasmid excreted markedly elevated amounts of protein in the urines at days 6, 14, and 56. The administration of sFlt-1 gene significantly increased ~~excreted amount of~~ proteinuria at days 14 and 56, compared with ~~those of~~ vehicle-treated rats ~~samples~~ (Figure 3~~b~~). Nephritic rats with inhibition of VEGF showed hypoproteinemia, evidenced by reduced serum total protein levels and ~~with~~ massive ascites at sacrifice (Figure 4a). At day 56, animals with empty plasmid developed renal dysfunction as evidenced by increased blood urea nitrogen levels. However, inhibition of VEGF by administration of sFlt-1 gene increased blood urea nitrogen levels (Figure 4b). Serum creatinine levels in sFlt-1-treated rats tended to increase compared with those in vehicle-treated rats at day 56, but the difference was not statistically significant. ~~These results suggest that neutralizing circulating VEGF by sFlt-1 gene administration exacerbates proteinuria, resulting in "nephrotic range" proteinuria, and chornic renal dysfunction.~~

Effects of VEGF blockade on glomerular epithelial cells and slit diaphragm-associated molecules

~~To further examine the molecular and pathological mechanisms involved in the increased levels of urinary protein, electron microscopic findings and the expression of~~

~~slit diaphragm-associated molecules were investigated.~~ Epithelial foot processes tended to fuse in all tufts~~, especially in tufts near infiltrated leukocytes~~ at day 6 in diseased glomeruli from rats treated with empty plasmid (Figure 5a; white arrow). In nephritic rats treated with sFlt-1, more severe fusion of epithelial foot processes occurred, and podocyte foot processes could be identified hardly (Figure 5b; black arrow). Podocytes in normal kidneys had faint desmin expression. In contrast, desmin was expressed by podocytes in nephritic rats and its expression was increased in sFlt-1-treated rats both at days 6 and 56 (Figure 5c and d)

~~Further, the impacts of inhibition of VEGF on podocyte-associated molecules were examined.~~ mRNA expression of nephrin was specifically decreased by the inhibition of VEGF (Figure 5e~~e~~). Nephrin protein, evaluated immunohistochemically in normal kidneys, was detected in a linear pattern along glomerular capillary walls (Figure 5f~~d~~). Compared with rats treated with empty plasmid (Figure 5g~~e~~), nephrin protein was reduced in sFlt-1-treated rats (Figure 5h~~f~~). Concomitantly, Western blot analysis ~~of the renal tissue~~ for nephrin protein revealed that nephrin was reduced in nephritic rats and that the reduction was more severe in rats treated with sFlt-1 (Figure 5i~~g~~). In addition, nephrin was faintly expressed in both sFlt-1-treated and empty plasmid-treated rats at day 56.

In contrast to nephrin expression, expression of podocin, podoplanin, and podocalyxin did not change ~~differ~~ by the treatment with ~~of~~ sFlt-1 in either mRNA levels (Figure 6a, b, c) or protein levels (Figure 6d, e, f, g). ~~These findings suggested that inhibition of VEGF activity via sFlt-1 specifically downregulated the expression of nephrin, which may lead to large amount of proteinuria in rat crescentic GN.~~

Microvascular changes associated with blockade of VEGF

In normal kidneys, thrombomodulin (TM)-positive glomerular and peritubular capillary (PTC) endothelial cells were preserved (Figure 7a). Six days after disease induction, sFlt-1-treated rats (Figure 7c, d) had more severe TM-positive glomerular endothelial cell loss than that observed in rats injected with empty plasmid (Figure 7b, d). sFlt-1-treated rats tended to have decreased areas of TM-positive PTC endothelial cells compared with rats injected with empty plasmid at day 6 (Figure 7e). ~~The area of TM-positive glomerular and PTC endothelial cells of sFlt-1-treated rats was significantly reduced compared with that of rats with empty plasmid (Figure 7d and e). These results suggested that VEGF blockade promoted the progression of glomerular and peritubular capillary loss.~~

Expressions of VEGF and its receptors, Flk-1 and Flt-1, in normal and diseased kidneys

mRNA expressions of three isoforms of VEGF in diseased kidneys was were reduced in crescentic GN, and was which were further reduced by the inhibition of VEGF (Figure 8a, b). In addition, these total of those three isoforms of VEGF were only was faintly expressed in both sFlt-1-treated and rats with empty plasmid-treated rats at day 56 (Figure 8a, b). VEGF protein, evaluated immunohistochemically in normal kidneys, was detected on podocytes in normal kidneys by immunohistochemistry (Figure 8c). Compared with rats treated with empty plasmid (Figure 8d), sFlt-1-treated rats had reduced VEGF protein was reduced in sFlt-1-treated rats at day 6 (Figure 8e). In analyses of expression of VEGF receptor, Flk-1, was detected in all glomeruli and PTC endothelial cells in normal rat (Figure 8f). Six days after induction of nephritis, the percentage of positive area of glomerular and tubulointerstitial tissue Flk-1 expressions of Flk-1 was decreased compared with normal rats (Figure 8g, h, li, and mj). At day 56, Flk-1 expression in glomeruli and interstitium was reduced via VEGF blockade (Figure 8li, mj). Similarly, the expression of Flt-1 in diseased kidneys was barely detectable, while it was readily detected in podocytes in normal kidneys (Figure 8i, j, and k).

Discussion

This study demonstrates~~d~~ that administration of sFlt-1, a selective and potent inhibitor of VEGF, accelerates~~d~~ proteinuria with massive ascites, glomerulosclerosis and interstitial fibrosis in rat crescentic GN, and is associated with loss of a slit diaphragm-associated molecule, nephrin, and endothelium ~~in rat crescentic GN~~. In this study, ~~transfected~~ sFlt-1 gene transfected in femoral muscles was expressed at injected sites at mRNA and protein levels and ~~that transfected sFlt-1~~ was released to circulation, from which it ~~followed by~~ translocated~~ion~~ ~~from blood space~~ to urinary space beyond the GBM in diseased kidneys (7, 13~~25~~). This study ~~The results of this study, taken together,~~ suggests that VEGF in rats with crescentic GN may be important for maintaining renal vasculature and preventing proteinuria ~~urinary protein excretion~~ via regulation of ~~integrity and function of~~ nephrin, VEGF appears to protect the kidney ~~which may result in the protection~~ from renal insults.

Blockade of VEGF accelerated proteinuria ~~excretion of urinary protein in the urine~~ and reduced specifically nephrin. Nephrin, a product of the NPHS1 gene whose mutations cause congenital nephrotic syndrome of the Finnish type, is exclusively expressed by glomerular podocytes within the kidney and is localized to the podocyte slit diaphragm (12, 14~~26~~). Recently, it has been reported that nephrin not only is a key slit diaphragm

component but also ~~mediates the action through which it~~ prevents podocyte apoptosis (15~~27~~). ~~Even though detailed molecular mechanisms involved in nephrin expression via VEGF remain to be determined,~~ M~~m~~ice injected with anti-VEGF antibody or sFlt-1 developed proteinuria accompanied by disruption/loss of slit diaphragm and specific nephrin downregulation (8). Therefore, one explanation for decreased expression of podocyte-associated molecules is that as a result of anti-GBM antibodies induced GN, podocyte damage occurred and progressed, followed by decreased expression of nephrin. Moreover, treatment ~~of~~ with VEGF results in decreased ~~in~~ urinary protein excretion and renal dysfunction~~, associated with renal pathology~~ (16~~28~~). Until now, the beneficial effect of VEGF on podocytes was thought to ~~may~~ be mediated indirectly by improvement of glomerular endothelial cell survival ~~function due to its survival signals from VEGF~~, because podocytes were not known ~~has not been considered~~ to express VEGF receptors. However, in addition to this paracrine role of VEGF in the glomerulus, it is possible that VEGF has an autocrine function through its tyrosine-kinase receptor that is required for podocyte survival in vitro (15~~27~~, 17~~29~~). The ~~Taken together, previous and our experimental~~ data suggest that VEGF maintains ~~the~~ podocyte function and survival by regulation of nephrin, possibly acting in an autocrine and/or paracrine fashion in the progressive disease.

VEGF expression was downregulated via sFlt-1 in this particular model. A r~~R~~ecent report demonstrated that signaling through ~~the~~ extracellular matrix proteins, in particular, laminin and its receptor $\alpha 3 \beta 1$ integrin which is highly expressed in podocytes regulates VEGF production in cultured podocytes (18~~30~~). Hence, downregulated VEGF in this model, at least in part, is likely ~~may be probably~~ due to the disturbance of the GBM matrix-podocyte interaction by anti-GBM antibodies. Further decrease in VEGF expression was observed by VEGF blockade via sFlt-1 during the course of disease~~d course~~. ~~Considering possible roles of VEGF for maintaining podocyte function,~~ T~~t~~his might be explained by podocyte damage being ~~which was~~ augmented by inhibition of VEGF activity (5, 8, 15~~27~~, 17~~29~~). Therefore, ~~once~~ downregulated VEGF ~~is downregulated~~ in this model~~, which in turn,~~ might perpetuate further podocyte loss and endothelial injury, thereby leading to ~~eventually developing~~ glomerulosclerosis.

VEGF binds two related receptors, Flt-1 and KDR/Flk-1 (1, 3). In rat kidney, Flk-1 has been shown to be expressed in glomerular and peritubular endothelial cells (3). However ~~On the other hands~~, Flt-1 expression is controversial~~ and was hardly expressed in diseased kidneys (data not shown)~~. In the present study, Flk-1 was locally

expressed in glomerular and peritubular endothelial cells and was diminished during the disease course, concomitantly with progressive glomerular and PTC loss. Flt-1 was detected on podocytes in normal glomeruli but was barely detected in diseased kidneys in this model. Therefore, Flk-1 appears to be the major mediator of endothelial cell mitogenesis and survival as well as angiogenesis in a crescentic GN, as reported previously (19~~31~~, 20~~32~~). PTC loss contributes to the etiology of the interstitial fibrosis by playing an essential role in impaired blood flow in tubular cells and interstitial cells. PTC loss may result from downregulated expression of VEGF in progressive renal diseases directly affecting PTC loss via its prosurvival effect on endothelial cells (19, 21). PTC loss may be also caused by filtered urinary proteins that lead to parenchymal damage and, eventually, renal fibrosis and dysfunction (22). Therefore, PTC loss observed in sFlt-1-treated rats may play a crucial role in deterioration of renal function. ~~However, Flt-1 has been reported to play a role in stimulation of angiogenesis in ischemic tissues and that anti-Flt-1 treatment inhibited prototypic angiogenic disorders such as cancer, retinal ischemia, arthritis and atherosclerosis in mice (33).~~ Recently, Flt-1 has been shown to be ~~is also~~ expressed on mesangial cells (23~~34~~) and conditionally immortalized human podocyte cell line (17~~29~~). In addition, it is upregulated in certain disease~~d~~ models ~~conditions~~ such as diabetic, passive Heymann nephritis, and

puromycin aminonucleoside nephrosis ~~rat models~~ (3, 24~~35~~). Therefore, VEGF blockade via sFlt-1 accelerated the injury of endothelial cells as well as podocytes, mainly through the inhibition of Flk-1~~ and, if any, Flt-1~~.

Monocytes/macrophages participate in inflammatory processes in ~~a~~ crescentic GN. VEGF promotes monocyte chemotaxis via a primary effect on the receptor Flt-1 (25~~36~~). In the present study, however, no significant effect of VEGF inhibition on crescentic formation, ED-1 positive macrophages, CD8 positive T lymphocytes, or PCNA positive cells in the glomeruli was seen at day 6. These findings were consistent with the previous report that VEGF had no effect on infiltration of neutrophils, CD3-T lymphocytes, and ED-1 positive macrophages in the Thy-1/habu-snake venom GN model (26~~37~~). One plausible reason ~~may be explained as follows.~~ is that ~~D~~downregulated VEGF could not ~~in this model did not have chemotactic effect enough to~~ induce migration and activation of monocytes/macrophages, because VEGF-mediated chemotaxis for monocytes/macrophages ~~is~~ depends~~ent~~ ~~of~~ on the dose and gradient of VEGF (25~~36~~). Therefore, although VEGF affects migration and activation of monocytes/macrophages ~~at its upregulated circumstances~~ when upregulated such as in aortic and coronary vascular inflammation (7, 1325, 27~~38~~), the residual VEGF

~~particularly~~ in this model might have no significant ~~minor~~ impact on monocytes/macrophages compared with ~~those~~ its effects on endothelial and glomerular epithelial cells.

In conclusion, our study showsed that VEGF plays a role in maintaining ~~the~~ podocyte function as well as renal vasculature, thereby protecting glomeruli and interstitium from progressive renal insults.

Methods

Animals

Inbred male WKY rats, purchased from Charles River Japan Inc. (Atsugi, Kanagawa, Japan), aged 12 wk were fed standard rat chow and given free access to water under 24h light control. All the procedures used in the animal experiments complied with the standards set out in the Guideline for the Care and Use of Laboratory Animals in Takara-machi Campus of Kanazawa University.

Expression Vector

The 3.3-kb mouse soluble Flt-1 gene, originally obtained from the mouse lung library (28~~12~~), was cloned into the BamH1 (5') and Not1 (3') sites of the eukaryotic expression vector plasmid cDNA3 (7).

Preparation of Anti-Rat Glomerular Basement Membrane Antibodies

~~The preparation of anti-rat glomerular basement membrane antibodies was performed as described previously (13, 14).~~ Rat glomerular basement membrane (GBM) was prepared using the method of Krakower and Greenspon (29). The preparation of anti-rat GBM antibodies was described previously (30). Specificity was confirmed by in vitro indirect immunofluorescence assays, using FITC-conjugated anti-rabbit IgG, on frozen sections of normal Wistar rat kidneys. Sharp linear immunofluorescence was observed only along the glomerular basement membrane.

Experimental Design

Twenty male WKY rats were injected intravenously with 0.1ml of nephrotoxic serum at day 0. Nine of 20 rats received an intramuscular injection of sFlt-1 plasmid (500 μg/150 μl phosphate buffered saline) into the femoral muscles with a 26-gauge needle 3 days before injection of nephrotoxic serum, and five of 9 rats received another ~~further~~

injection of the plasmid 2 weeks thereafter. To enhance transgene expression, electroporation was performed at the injected site immediately after injection as previously described (31~~15~~, 32~~16~~). sFlt-1-treated rats were killed at days 6 (4 rats) or 56 (5 rats), and blood samples were collected. Muscle samples were obtained by partial excision of the sFlt-1-injected sites at days 6 and 56. Urine was collected 0, 6, 14, and 56 days after the nephrotoxic serum injection. The remaining 11 nephritic rats injected ~~transferred~~ with empty plasmid with electroporation were also killed at days 6 (5 rats) or 56 (6 rats)~~, as nephritic rats treated with sFlt-1 plasmid~~. As a control, urinary excretion of protein was measured at days 6, 14, 21, 28, and 56 in 6 normal rats transfected with sFlt-1 plasmid with electroporation. Levels of urinary protein excretion were determined at days 6, 14, and 56 in 10 normal untreated rats to establish normal values.

Expression of sFlt-1

The expression of sFlt-1 in skeletal muscle, which was fixed in 10% formalin followed by embedding in paraffin, was detected by immunohistochemistry~~cal~~ ~~method~~ using peroxidase-labeled polymer conjugated to goat anti-rabbit IgG (Envision System; DAKO, Carpinteria, CA) and anti-Flt-1 antibodies (C-17; Santa Cruz Biotechnology, Santa Cruz,

CA) 6 days after disease induction (31). Moreover, Flt-1 mRNA in femoral muscle was detected using reverse transcription-polymerase chain reaction (RT-PCR). ~~method~~. In brief, the complementary DNA (cDNA) was reverse-transcribed from 1 μg total RNA using a reverse transcription-PCR kit (Takara Shuzo, Tokyo, Japan). The cDNA product was amplified by PCR. Primers for mouse sFlt-1 (5'-GGTGCCCGCTCTTTG-3' [sense]; 5'-TGTCTCAGTGGGGATTGC-3' [antisense]) were used to detect sFlt-1 (33~~17~~). The housekeeping gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was used for PCR controls.

Urinary sFlt-1 measurements

The commercially available enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN) were used to measure mouse urinary sFlt-1 according to manufacturer's instructions.

Histopathological studies

A portion of renal tissue was prepared as previously reported (30~~15~~). The total number of cells and number of glomeruli with crescentic formation were measured and expressed as described previously (30~~14~~). The extent of glomerular sclerosis was

expressed as a percentage of the PAM-positive area per whole glomerular area as described previously (34~~18~~). Each area was measured by a computer-aided manipulation using Mac Scope version 6.02 (Mitani Shoji Co., Ltd., Fukui, Japan). The extent of interstitial fibrosis, expressed as blue in Mallory-azan staining, was evaluated as previously reported (32~~16~~, 35~~19~~).

In an indirect immunoperoxidase staining, endothelial cells were detected with mouse anti-thrombomodulin monoclonal antibody (141C01; NeoMarkers, Fremont, CA), injured podocytes with mouse anti-desmin monoclonal antibody (D33; DAKO Co.), VEGFR-1 with goat anti-Flt-1 polyclonal antibodies (C-17; Santa Cruz Biotechnology), and VEGFR-2 with mouse anti-Flk-1 monoclonal antibody (A-3; Santa Cruz Biotechnology). In each biopsy, thrombomodulin-, and Flk-1-positive areas in glomeruli and tubulointerstitium were identified and expressed in similar fashion~~s~~ as described above, respectively (34~~18~~, 35~~19~~).

Another portion of renal tissue was frozen rapidly and immunostained directly with fluorescein isothiocyanate (FITC)-conjugated, anti-proliferating cell nuclear antigen (PCNA; Leinco Technologies Inc., St. Louis, Mo.; No. 033L245) or indirectly with a mouse monoclonal antibody against rat tissue monocytes and macrophages, ED1 (IgG1, BMA Biomedicals Ltd.,Augst, Switzerland) or a mouse monoclonal antibody against ~~the~~

rat CD8 ~~molecules~~ (IgG1, Cedarlane, Hornby, Ontario, Canada; No. 0412). Positive cells were counted on at least 50 randomly chosen glomeruli. Renal tissues obtained from four normal WKY rats were used as negative controls. To evaluate ~~that~~ the induction of glomerulonephritis ~~was achieved equally~~, FITC-conjugated anti-rabbit IgG (No. 38236; Organon Teknika Corporation), FITC-conjugated anti-rat C3 (No. 38810; Organon Teknika Corporation), and FITC-conjugated anti-rat IgG (No. 38731; Organon Teknika Corporation) were used (30~~14~~).

Detection of VEGF protein and its mRNA

To examine the production of VEGF in kidney before and after disease induction, immunohistochemical analysis was performed on~~in~~ paraffin embedded tissue specimens with mouse anti-VEGF monoclonal antibody (C-1; Santa Cruz Biotechnology). In addition, to determine transcripts of VEGF, RT-PCR was performed using primers for VEGF (5'-GACCCTGGTGGACATCTTCCAGGA-3' [sense]; 5'-GGTGAGAGGTCTAGTTCCCGA-3' [antisense]) with expected sizes of 514, 462, 330 bp for amplification of VEGF188, VEGF 164, and VEGF 120, respectively (36~~20~~).

Detection of the glomerular epithelial slit diaphragm-associated molecules and extent of

foot process effacement.

Morphological changes of podocytes were examined by ~~under an~~ electron microscopy as previously described (Hitachi H-600, Tokyo, Japan) (30~~14~~). Next, the presence of nephrin was demonstrated immunohistochemically in frozen tissue specimens with mouse anti-rat nephrin monoclonal antibody 5-1-6, and that of podocalyxin with mouse anti-podocalyxin monoclonal antibody (4D5; gift from Dr. Masanori Hara).

mRNA expression of nephrin, podocin, and podocalyxin ~~podoplanin, and podocalyxin~~ was analyzed by ~~the~~ real-time ~~semiquantitative reverse transption-polymerase chain reaction (~~RT-PCR~~)~~ on isolated cortices of kidneys from each group of rats. The ~~complementary~~ cDNA was reverse-transcribed~~pted~~ from 1 μ g total RNA using ~~a~~ SuperScript II RNase H- reverse transcriptase (Invitrogen, Carlsbad, CA). ~~reverse transcription-PCR kit (Takara Shuzo, Tokyo, Japan).~~ Reverse transcription was performed using the following parameters: 10 minutes at 25℃, 30 minutes at 48℃, and 5 minutes at 95℃. In real-time PCR experiments, the Sequence Detection System (7900HT, Applied Biosystems) was used. Primers for nephrin (Rn00575235, Nphs1, Applied Biosystems) ~~(21)~~, podocin (Rn00709834, Nphs2, Applied Biosystems) ~~(22)~~, ~~podoplanin (21)~~, and podocalyxin (Rn00593804, Podxl, Applied Biosystems) (~~21~~) were used. The housekeeping gene, actin beta (Rn00667869, Actb, Applied Biosystems)

~~glyceraldehydes-3-phosphate dehydrogenase (GAPDH)~~ was used for PCR controls. The reactions were incubated at 95℃ for 10 min, followed by 40 cycles of 15s at 95℃, 10s at 55℃, and 20s at 72℃. mRNA expression of nephrin, podocin and podocalyxin in each sample was finally described after correction with actin beta expression. In addition, expression of podoplanin mRNA was analyzed by RT-PCR. cDNA was reverse-transcribed from 1 μ g total RNA using a reverse transcription-PCR kit (Takara Shuzo). Primers for podoplanin (forward: 5'-GAGCGTTTGGTTTCTGGGACTCA-3', reverse: 5'-GGTGAGAGGTCTAGTTCCCGA-3') (37) were used. The housekeeping gene, GAPDH was used for PCR control. The reactions were incubated at 94℃ for 3 min, followed by 35 cycles of 30s at 94℃, 30s at 58℃, and 60s at 72℃ (37). Scanner analysis was examined as previously described (32~~16~~, 35~~19~~).

Western blot analysis

Isolated cortical portions of kidneys from each group were prepared as described elsewhere. The protein concentration of each sample was measured by Bradford Protein Assay kit (Bio-Rad Laboratories, Inc., Hercules, CA). The homogenate was diluted with Laemmli sample buffer (Bio-Rad Laboratories, Inc.) and was boiled for 5 min and cooled on ice. Ten micrograms of protein were subjected to sodium dodecyl sulfate

polyacrylamide gel electrophoresis and electroblotted onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Inc.). The membrane was blocked with bovine serum albumin-containing TBST buffer (20 mM Tris, pH 7.6, 140 mM NaCl, and 0.1% Tween 20), and then incubated with goat anti-nephrin polyclonal antibodies (N-20; Santa Cruz Biotechnology) (38) or rabbit anti-podocalyxin polyclonal antibodies (KR064; Transgenic Co., Hyogo, Japan). To visualize the signals, the membrane was incubated with biotinylated rabbit anti-goat immunoglobulins (DAKO Co.) followed by streptoavidin-HRP complex (DAKO Co.) for nephrin or with peroxidase-labeled polymer conjugated to goat anti-rabbit IgG (Envision System; DAKO Co.) for podocalyxin. ~~Western blot analysis was examined using goat anti-nephrin polyclonal antibodies (N-20; Santa Cruz Biotechnology) as previously described (23).~~

Determination of urinary protein, blood urea nitrogen, serum creatinine, and serum total protein concentrations

Urinary protein concentrations were determined as previously reported (39~~24~~). Urinary protein excretion was expressed as the ratio of urinary protein to urinary creatinine. Blood urea nitrogen, serum and urinary creatinine, and serum total protein levels were measured using an automated analyzer (Hitachi, Tokyo, Japan) according to

the manufacturer's instructions.

Statistical analyses

The mean and standard error were calculated on all parameters determined in this study. Statistical analyses were performed using the unpaired *t* test and Kruskal-Wallis test. Values of $p < 0.05$ were considered statistically significant.

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Table 1 Effects of inhibition of VEGF activity via sFlt-1 on histopathological changes on day 6[a]

	Normal	Vehicle	sFlt-1	p value[c]
Crescentic formation, %	0	37.3±2.7	36.4±5.4	0.88
Total cell number[b]	55.3±1.1	109.2±1.5	113.5±1.6	0.09
PCNA-positive cells[b]	1.8±0.5	7.0±0.2	7.1±0.4	0.88
ED-1-positive cells[b]	0.8±0.1	13.9±0.5	13.4±0.7	0.52
CD8-positive cells[b]	0.2±0.02	1.9±0.1	1.9±0.1	0.89

[a] Values are given as mean±SEM. n=4 in normal group, n=5 in vehicle group, and n=4 in sFlt-1 group.

[b] Number of cells per glomerular cross section.

[c] Statistical analyses are based on unpaired *t* test and Kruskal-Wallis test.

Figure legends

Figure 1. sFlt-1 expression in femoral muscle.

The expression of sFlt-1 in skeletal muscles, where gene transfer was performed with electroporation, was detected by an immunohistochemical method. Fibers positive for sFlt-1 were observed in the femoral muscle from the plasmid DNA encoding sFlt-1 transfected rats 6 days after induction of GN (a). There was no staining for sFlt-1 in the muscles from rats transfected with empty plasmid (b). sFlt-1 mRNA in muscles was determined by RT-PCR analysis. sFlt-1 mRNA was detected in muscles 6 days after induction of GN, and barely ~~hardly~~ detected 56 days after induction of GN (c). Urinary levels of sFlt-1 increased 6 days after disease induction in sFlt-1-treated rats (d). Magnification, ×100. *, $p<0.05$ compared with rats transfected with empty plasmid.

Figure 2. VEGF inhibition accelerated glomerulosclerosis and interstitial fibrosis.

Progressive renal lesions exhibited glomerulosclerosis (a, c) and interstitial fibrosis (c, d) in rats transfected with empty plasmid at day 56. In contrast, sFlt-1 treatment accelerated renal pathology. Glomerulosclerosis (c) and interstitial fibrosis (d) were more prominent. Original magnification, × 200. *, $p<0.05$ compared with rats

transfected with empty plasmid.

Figure 3. Increased urinary protein excretion by inhibition of VEGF activity.

Treatment of normal rats with sFlt-1 induced mild proteinuria, and its levels were maintained for days 28 and were decreased by day 56 (■~~a~~). *, p<0.01 compared with a level of proteinuria at day 0. Results from rats administered with empty plasmid (○) and sFlt-1 (●). Administration of sFlt-1 markedly increased proteinuria ~~(b)~~. #, p<0.05 compared with rats transfected with empty plasmid

Figure 4. Decreased serum total protein levels and deterioration of renal function by sFlt-1 treatment.

Serum total protein levels were decreased in sFlt-1-treated rats at day 56 (a). *, p<0.05 compared with normal rats. #, p<0.05 compared with vehicle-treated rats. Rats treated with sFlt-1 developed renal dysfunction, evidenced by increased blood urea nitrogen levels at day 56 (b). *, p<0.001 compared with normal rats. #, p<0.05~~1~~ compared with vehicle-treated rats.

Figure 5. Effects of anti-GBM GN and sFlt-1 treatment on podocytes and nephrin.

Podocyte foot processes fused in all tufts at day 6 in diseased glomeruli from rats transfected with empty plasmid (a). sFlt-1 treatment accelerated fusion of podocyte foot processes (b). Original magnification, ×3000. Expression of desmin at day 6 in diseased glomeruli from rats with empty plasmid (c). Increased desmin expression in podocytes was observed in sFlt-1-treated rats at days 6 and 56 (d). Original magnification, ×400. mRNA expression of nephrin in normal and diseased kidneys (ee). Expression of nephrin mRNA was decreased in anti-GBM GN, and further downregulated by sFlt-1 treatment. *, $p<0.05$ compared with normal rats. #, $p<0.051$ compared with vehicle-treated rats. Immunofluorescent microscopic findings of nephrin in normal rats (fd) and rats with empty plasmid (ge). Nephrin expression was reduced at day 6. Nephrin expression was further reduced in sFlt-1-treated rats compared with rats with empty plasmid at day 6 (hf). Original magnification, ×200. sFlt-1 treatment markedly remarkably downregulated nephrin in diseased glomeruli as shown revealed by Western blot analyses (ig). Molecular markers are shown in the left lane. Data are representative of 3 experiments.

Figure 6. Effects of anti-GBM GN and sFlt-1 treatment on podocyte-associated molecules.

mRNA expressions of podocin (a), podocalyxin (b), and podoplanin (c) was were decreased by anti-GBM GN, and sFlt-1 treatment did not have impact on mRNA expressions of these three podocyte-associated molecules. *, $p<0.05$ compared with normal rats. Immunofluorescent microscopic findings of podocalyxin in normal rats (d). Podocalyxin expression was reduced at day 6 in nephritic rats transfected with empty plasmid (e), which was not different from that in sFlt-1-treated rats (f). Original magnification, ×200. sFlt-1 treatment did not alter the podocalyxin expression in diseased glomeruli revealed by Western blot analyses (g). Molecular markers are shown in the left lane. Data are representative of 3 experiments.

Figure 7. VEGF inhibition accelerated glomerular and peritubular endothelial loss. Thrombomodulin (TM)-positive endothelial cells were detected ubiquitously along with glomerular and peritubular capillaries in normal rats (a). TM-positive endothelial cells were reduced in rats transfected with empty plasmid at day 6 (b). sFlt-1 treatment accelerated reduction of TM-positive endothelial cells at day 6 (c). Original magnification, ×400. Loss of TM-positive glomerular and peritubular endothelial cells was prominent by sFlt-1 treatment (d, e). *, $p<0.05$ compared with normal rats. #, $p<0.05$ compared with vehicle-treated rats.

Figure 8. Effects of anti-GBM GN and sFlt-1 treatment on expression of VEGF and VEGF receptors, Flt-1 and Flk-1.

mRNA expressions of VEGF in normal and diseased kidneys. Expression of VEGF mRNA was decreased in anti-GBM GN, and further downregulated by sFlt-1 treatment (a). Densitometric analysis of data in (a) (b). Total expression of three isoforms of VEGF mRNA in each sample is presented as a ratio to GAPDH mRNA expression in that sample. *, $p<0.001$ compared with normal rats. #, $p<0.001$ compared with vehicle-treated rats. VEGF was detected in podocytes in normal kidneys (c). In nephritic rats, VEGF expression was reduced (d), and was further decreased by sFlt-1 treatment at day 6 (e). Flk-1 was expressed in glomerular and peritubular endothelial cells (f). Flk-1 expression was reduced in diseased kidneys, especially in glomeruli involved with crescentic lesions at day 6 (g, li, and mj). Flk-1 expression was more severely decreased in sFlt-1-treated rats (h, li, and mj). Flt-1 was detected on podocytes in normal glomeruli (i) and was hardly expressed in diseased kidneys of both vehicle-treated (j) and sFlt-1-treated rats (k). Original magnification, ×400. *, $p<0.05$ compared with normal rats. #, $p<0.05$ compared with vehicle-treated rats.