Kidney International, Vol. 60 (2001), pp. 1297–1308

Vascular endothelial growth factor ($VEGF_{121}$) protects rats from renal infarction in thrombotic microangiopathy

Shin-ichi Suga, Yoon-Goo Kim, Alison Joly, Ela Puchacz, Duk-Hee Kang, J. Ashley Jefferson, Judith A. Abraham, Jeremy Hughes, Richard J. Johnson,[1] and George F. Schreiner

Division of Nephrology, University of Washington Medical Center, Seattle, Washington; Scios Inc., Sunnyvale, California, USA; and Division of Nephrology, Samsung Medical Center, Sungkyunkwan University, Seoul, Korea

Vascular endothelial growth factor ($VEGF_{121}$) protects rats from renal infarction in thrombotic microangiopathy.

Background. Renal thrombotic microangiopathy, typified by the hemolytic uremic syndrome, is associated with endothelial cell injury in which the presence of cortical necrosis, extensive glomerular involvement, and arterial occlusive lesions correlates with a poor clinical outcome. We hypothesized that the endothelial survival factor vascular endothelial growth factor (VEGF) may provide protection.

Method. Severe, necrotizing, thrombotic microangiopathy was induced in rats by the renal artery perfusion of antiglomerular endothelial antibody, followed by the administration of VEGF or vehicle, and renal injury was evaluated.

Results. Control rats developed severe glomerular and tubulointerstitial injury with extensive renal necrosis. The administration of VEGF significantly reduced the necrosis, preserved the glomerular endothelium and arterioles, and reduced the number of apoptotic cells in glomeruli (at 4 hours) and in the tubulointerstitium (at 4 days). The prosurvival effect of VEGF for endothelium may relate in part to the ability of VEGF to protect endothelial cells from factor-induced apoptosis, as demonstrated for tumor necrosis factor-α (TNF-α), which was shown to be up-regulated through the course of this model of renal microangiopathy. Endothelial nitric oxide synthase expression was preserved in VEGF-treated rats compared with its marked decrease in the surviving glomeruli and interstitium of the antibody-treated rats that did not receive VEGF.

Conclusions. VEGF protects against renal necrosis in this model of thrombotic microangiopathy. This protection may be mediated by maintaining endothelial nitric oxide production and/or preventing endothelial cell death.

[1] Current address: Renal Section, Baylor College of Medicine, 6550 Fannin Street, Suite 1273, Houston, Texas 77030, USA.

Key words: hemolytic uremic syndrome, apoptosis, tumor necrosis factor-α, nitric oxide synthase, arterial occlusive lesions, renal injury.

Received for publication October 18, 2000
and in revised form April 13, 2001
Accepted for publication May 1, 2001

The hemolytic uremic syndrome (HUS), frequently associated with infection with verotoxin-producing *Escherichia coli*, is an example of thrombotic microangiopathy (TMA) affecting the renal microvasculature. It is characterized by acute renal failure, hemolytic anemia, and thrombocytopenia. It occurs predominantly in children, for whom it comprises one of the major causes of acute renal failure, but it can also affect adults in whom a more severe clinical course often is observed [1, 2]. Treatment has generally been only supportive, although plasma exchange has been used in uncontrolled studies. While most patients have complete functional renal recovery, the mortality rate is estimated to be as high as 5% and those with severe disease may develop end-stage renal failure [1]. In addition, as many as 20 to 40% will develop chronic renal sequelae ranging from mild proteinuria to renal insufficiency and/or hypertension after 5 to 10 years [3–5]. The typical renal histological feature of HUS consists of intraluminal fibrin/platelet thrombi, often with fragmented red blood cells, swelling, and detachment of endothelial cells from the basement membrane, with subendothelial widening and occlusion of capillaries in glomeruli and intimal swelling and thickening in arterioles. All of these are thought to be secondary to primary endothelial injury [1, 2]. Patchy areas of tubular injury and necrosis are observed as associated findings. Histological factors correlating with poor clinical outcome are the existence of cortical necrosis, extensive glomerular involvement and arterial occlusive lesions [4].

Insights into the pathogenesis and treatment of HUS have been hampered by the lack of relevant animal models. Verotoxins do not induce HUS-like diseases in rodents, due to the lack of the expression of globotriaosylceramide (G3b), the receptor for verotoxins, on endothelial cells. However, we have recently reported on a model of renal TMA with features of HUS, induced by the renal arterial infusion of an antiglomerular endothelial (GEN) anti-

body in rats [6]. Histologically and ultrastructurally, this model is characterized by renal microvascular injury with platelet aggregation, fibrin deposition, glomerular and arteriolar endothelial swelling and detachment, accumulation of electrolucent material in subendothelial spaces, and mesangiolysis. Tubular necrosis and interstitial disease are also observed. The ultrastructural and histological features of this model are similar to those of human HUS [7].

Because the clinical and experimental forms of HUS are primarily initiated by acute injury to endothelial cells, we have hypothesized that growth factors that promote endothelial cell survival or angiogenesis may provide benefit. One of the most important endothelial growth factors is vascular endothelial growth factor (VEGF) [8–11]. We have recently shown that the chronic administration of VEGF in a non-necrotizing model of renal microangopathy induced by low-dose administration of anti-GEN can stimulate angiogenesis and accelerate histologic and functional recovery [12]. While this latter study is of interest, it did not address whether VEGF can act as an endothelial cell survival factor because VEGF treatment was initiated after maximal glomerular injury [6, 12] and in a model lacking an acute necrotic component, which correlates with a poor clinical outcome in HUS [4]. Recent in vitro evidence has suggested an acute protective effect of VEGF on endothelial survival [8, 9]. To determine whether VEGF exerts a survival effect on acutely injured renal microvasculature in the absence of angiogenesis, we have modified the experimental model by administering a larger dose of anti-endothelial antibody eventuating in acute massive renal infarction. In this model, VEGF was administered one hour after induction of injury, and the effects on endothelial cell survival and renal necrosis were evaluated. The short observation time (up to 7 days) precluded any effects of VEGF on new capillary formation [13]. We report that VEGF is a potent endothelial cell survival factor in vivo. It is the first growth factor to be shown to reduce renal cortical and medullary necrosis in a model of thrombotic microangiopathy.

METHODS

Preparation of rh$VEGF_{121}$

To eliminate a glycosylation site in the recombinant product, the complementary DNA sequence for $VEGF_{121}$ was altered at codon 75 to encode glutamine rather than asparagine. The sequence was then placed under the control of the AOX1 promoter [14] in a Pichia pastoris host cell, and expression of recombinant $VEGF_{121}$ was initiated in fermentation culture by the addition of methanol. Conditioned medium from the fermentation was filtered and then fractionated sequentially on ion exchange, hydrophobic interaction, metal affinity, and reversed-phase chromatography columns. The purified product was lyophilized to dryness, resuspended in phosphate-buffered saline (PBS), and stored at −80°C until use. Although the rh$VEGF_{121}$ was fully active in an endothelial cell mitogenic assay, amino acid sequencing and mass spectrometry indicated that the product was missing four amino acids on the N terminus of the molecule and one amino acid from the C terminus. $VEGF_{121}$ is the only isoform of VEGF that lacks a heparin-binding region [10]. We reasoned that it could be injected and circulate to an affected organ such as the kidney, and further that it could be administered via subcutaneous injection, its lack of binding to the extracellular matrix allowing it to diffuse. This was proven by direct comparison of circulating plasma levels of subcutaneously administered $VEGF_{121}$ and its heparin-binding isoform, $VEGF_{165}$ (Fig. 1).

Experimental protocol

Twenty-one male Sprague-Dawley rats weighing 200 to 250 g were used. Experimental TMA was induced by selective perfusion of the right kidney through the superior mesenteric artery with 80 mg/kg purified IgG of goat anti-rat GEN antibody as previously described [6]. The nonperfused left kidney was left in situ. The rats were divided into two groups, and 50 μg/kg $VEGF_{121}$ ($N = 17$) or PBS ($N = 16$) were subcutaneously injected one hour after anti-GEN antibody perfusion, followed by twice-a-day injection at 12-hour intervals at different sites on the back for seven days. At four hours after the perfusion, six rats in each group were sacrificed to evaluate the presence of apoptosis in the kidney. For the remaining rats, a small renal biopsy was performed at 10 minutes and 4 days after the perfusion, and the right kidney was removed at day 7.

Renal histological studies and morphometry

Methyl Carnoy's fixed tissue was processed and paraffin embedded, and 4-μm sections were stained with the periodic acid-Schiff reagent (PAS). An indirect immunoperoxidase method was used to identify the following antigens as we reported [15]: endothelial cells with RECA-1, a monoclonal IgG_1 antibody specific for endothelial cells (Harlan Bioproducts, Indianapolis, IN, USA); platelets with PL-1, a mouse monoclonal antibody to rat platelets (generously provided by W.W. Baker, University of Groningen, Groningen, The Netherlands); α-smooth muscle actin (α-SMA) with 1A4, a mouse anti-α-SMA monoclonal IgG_{2a} antibody (Sigma Chemical Co., St. Louis, MO, USA); and endothelial nitric oxide synthase (eNOS; NOS III) with a mouse anti-eNOS monoclonal IgG_1 antibody (Transduction Labs, Lexington, KY, USA), as previously described [16]. To confirm the glomerular endothelial binding of the injected anti-GEN IgG, immunofluorescence was performed on 4-μm frozen tissue sections from the 10-minute biopsies using

rabbit anti-goat IgG (Cappel, Organ Teknika Corp., Durham, NC, USA) [6].

Morphometric analysis of the percentage of area occupied by necrotic tissue, RECA-1–positive glomeruli density, α-SMA positive vessels, and the percentage of area occupied by eNOS-positive tissue was performed using computer-assisted image analysis software (Optimas, version 6.2; Media Cybernetics, Silver Springs, MD, USA) and digitized images. The percentage of area occupied by necrotic tissue was measured at ×2.5 on whole cortex and medulla in sagittal sections of each biopsy. The combination of the central and peripheral zone of necrosis was evaluated. The number of RECA-1–positive glomeruli, PL-1–positive glomeruli, and α-SMA–positive vessels was quantified per 5× field in the entire cortical and juxtamedullary regions of sagittal sections, and mean number per mm^2 was calculated. The percentage of area occupied by eNOS-positive tissue was measured per 5× field and the mean percentage of area was calculated. For tubulointerstitial eNOS evaluation, non-necrotic cortical and juxtamedullary regions excluding glomeruli were examined. Semiquantification of glomerular eNOS immunostaining was performed as previously described [17]. Briefly, staining was graded (0–5) based on the % of eNOS positive area per non-necrotic glomerulus as follows: 0, no staining present; grade 1, <10% eNOS positive area; grade 2, 10 to 25% eNOS-positive area; grade 3, 25 to 50% ; grade 4, 50 to 75%; and grade 5, 75 to 100%.

Determination of renal TNF-α mRNA levels

Total RNA was isolated from dissected renal cortex in a separate group of rats at seven days after the initiation of injury using the Qiagen RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA); 100 ng was subjected to quantitative real-time PCR using an ABI Prism™ 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA, USA). The following primers and probes for rat TNF-α and 18S ribosomal RNA were designed using Primer Express software (PE Applied Biosystems): for TNF-α forward 5′-ACAAGGCTGCCC CGACTAT-3′, reverse 5′-CTCCTGGTATGATGGAA ATC-3′, and probe 5′-6FAM-TGCTCCTCACCCACAC CGTCAGC-TAMRA-3′; for 18S rRNA forward 5′-CGG CTACCACATCCAAGGAA-3′, reverse 5′-GCTGGA ATTACCGCGGCT-3′ and probe 5′-6FAM-TGCTGG CACCAGACTTGCCCTC-TAMRA-3′. Multiscribe reverse transcriptase and AmpliTaq Gold polymerase (PE Applied Biosystems) were used in all RT-PCR reactions. Relative quantitation of 18S rRNA and rat TNF-α mRNA was calculated using the comparative threshold cycle number for each sample fitted to a five point standard curve (ABI Prism 7700 User Bulletin #2; PE Applied Biosystems). TNF-α mRNA levels were normalized to 18S rRNA and related to samples derived from sham-operated controls. Data quoted are the mean of triplicate analysis.

Detection of apoptosis in vivo

Apoptotic cells were detected by two well-established methods. First, cells were identified as undergoing apoptosis on PAS-stained sections when they exhibited marked chromatin condensation, which is a classic morphological hallmark of apoptosis [18, 19]. Second, the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling (TUNEL) assay was used to identify apoptosis in the kidney [18]. Briefly, formalin-fixed sections were deparafined and rehydrated, followed by an antigen retrieval step involving boiling the tissues in citric acid (pH 6.0). Samples were then incubated with Proteinase K (6.2 μg/mL; Boehringer Mannheim, Indianapolis, IN, USA), followed by the mixture of TdT (300 U/mL; Amersham Pharmacia Biotech, Piscataway, NJ, USA) and Bio-14-dATP (0.94 nmol/L; GIBCO BRL, Grand Island, NY, USA). Biotinylated adenosine 5′-triphosphate (ATP) was detected by the ABC staining method (Vector Laboratories, Burlingame, CA, USA). Apoptosis of glomerular or tubulointerstitial cells was evaluated by counting the number of TUNEL-positive glomerular cells in 50 sequentially selected glomeruli or TUNEL-positive tubulointerstitial cells in 20 sequentially selected nonoverlapping fields of renal cortex at ×400 magnification, respectively, and expressed as the number of positive cells per glomerulus or per high power field (hpf) [20].

Cell culture and induction of apoptosis

Human umbilical vein endothelial cells (HUVECS) were placed in complete culture medium supplemented with 5% fetal bovine serum and allowed to grow to confluence. Recombinant human TNF-α (R&D Systems, Minneapolis, MN, USA) was added to the cultures for six hours, at which point the cells were assayed for caspase-3 activity, as an index of apoptosis [21, 22], using ApoAlert Caspase-3 Detection Assay (Clontech, Palo Alto, CA, USA). The effect of VEGF upon TNF-α–induced caspase activity was ascertained by coincubating the HUVECS with $rhVEGF_{121}$ at concentrations of either 5 or 50 ng/mL, reflecting plasma levels attained by rhVEGF when administered subcutaneously.

Statistics

Values are expressed as mean ± SE. Differences between the VEGF and vehicle-treated groups were evaluated with the unpaired Student *t* test except comparison of the number of apoptotic cells, which was performed with Mann-Whitney U test.

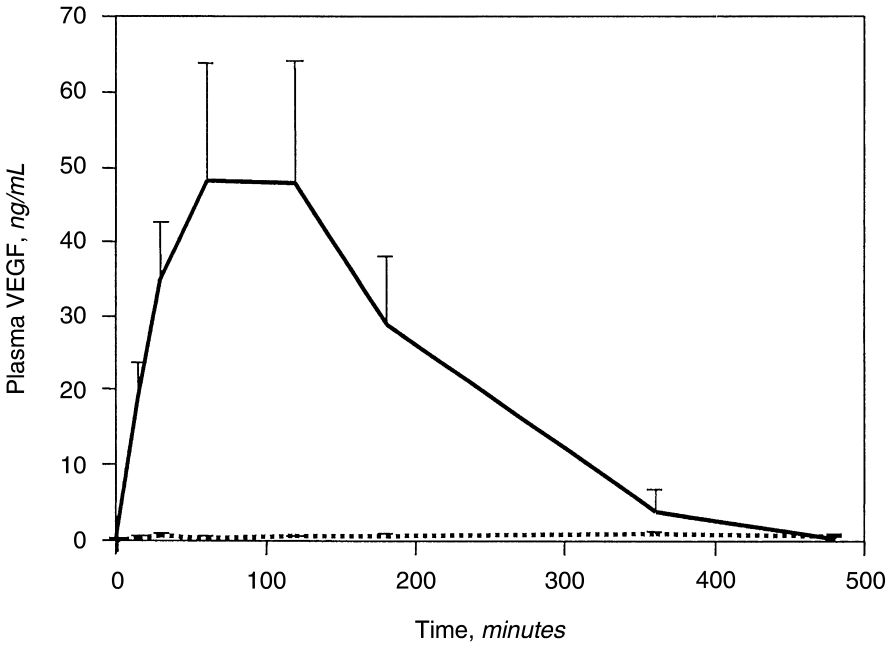

Fig. 1. Pharmacokinetics of $VEGF_{121}$ (solid line) and $VEGF_{165}$ (dotted line) after subcutaneous injection. The plasma levels of $VEGF_{121}$ and $VEGF_{165}$ in six rats after subcutaneous injection of 100 μg is shown. The injection of $VEGF_{121}$ resulted in significant plasma levels, peaking 100 minutes after injection.

RESULTS

$VEGF_{121}$ protects animal from renal necrosis in a hemolytic uremic syndrome-like model

The HUS-like model was induced in rats by selective perfusion of the right renal artery with anti-GEN antibody [6] and the non-perfused left kidney was left in situ. The lot and dose utilized (80 mg IgG/kg) induced a much more severe form of thrombotic microangiopathy than that reported previously [6] and resulted in large patchy areas of cortical and medullary necrosis. One hour after the injection of antibody, rats were randomized to receive either recombinant human $VEGF_{121}$ or vehicle. $VEGF_{121}$ was chosen because it is the only isoform of VEGF that has no heparin-binding ability and, therefore, can result in therapeutically effective plasma levels when administered subcutaneously (Fig. 1). The dose of $VEGF_{121}$ used (50 μg/kg twice a day) was selected as it has been found to be an effective dose for inducing angiogenesis when administered subcutaneously in an animal ischemic hind-limb model. These plasma levels of VEGF do not affect systemic blood pressure (R. Terjung and J. Abraham, unpublished observations).

At day 7, control rats perfused with anti-GEN antibody developed severe renal disease with macroscopic evidence of infarction (Fig. 2A). The perfused kidney of every animal in the control group was swollen and mottled, and the surface was covered with yellowish-white areas. The sectioned surface of every kidney demonstrated patchy yellowish-white areas distributed in the cortex as well as in the medulla consistent with infarction (Fig. 2B, C). Although cortical tissue just beneath the capsule was relatively preserved in some rats, tissue necrosis was extensive throughout the cortex and medulla (Fig. 2C). In marked contrast, systemic administration of $VEGF_{121}$ for seven days after anti-GEN antibody perfusion resulted in elimination of macroscopic evidence of cortical infarction (Fig. 2D) with preservation of renal architecture apparent upon sectioning (Fig. 2E), except for small patchy areas of necrosis in the medulla (Fig. 2F).

Necrotic areas in the control kidneys were widely distributed in the medulla and cortex. Light microscopic analysis revealed heterogeneous patches of necrosis (Fig. 3A, arrow), typically with a central zone composed of necrotic tissue with nuclear loss in glomeruli and tubules (Fig. 3B), a peripheral zone with polymorphonuclear cell infiltration, and a marginal zone with various degrees of necrosis, tubular regeneration, and fibrosis (Fig. 3C). Sagittal sections revealed that approximately 50% of cortex and more than 80% of the medulla had become necrotic by day 7. Between necrotic areas, marked atrophy or dilation of tubules, interstitial fibrosis, and mononuclear cell infiltration were observed. In contrast, the administration of VEGF beginning one hour after disease induction dramatically reduced the degree of necrosis. Quantification of the necrosis by computer image analysis demonstrated that 5 ± 3% of the cortex of VEGF-treated animals was necrotic compared to 46 ± 10% in controls ($P < 0.01$); similarly, VEGF-treated rats showed a more than 50% reduction in medullary necrosis (81 ± 19% in the controls versus 37 ± 11% in VEGF-treated rats, $P < 0.05$; Fig. 3D). In contrast to massive necrosis of the kidney in TMA rats, the most prominent histological feature in the kidneys of

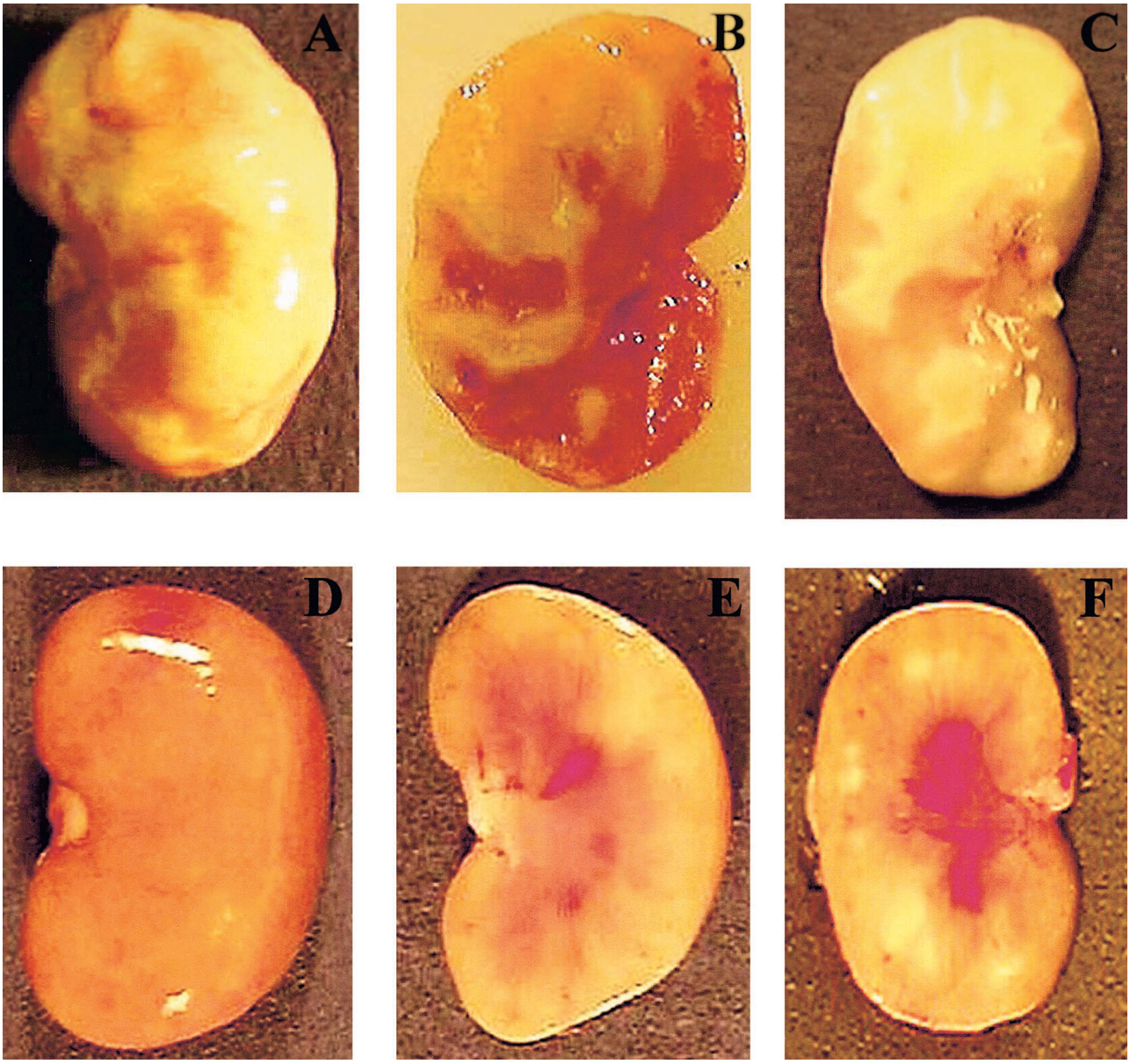

Fig. 2. Vascular endothelial growth factor (VEGF) reduces renal necrosis in a thrombotic microangiopathy (TMA) model. In control rats with TMA lesions, the kidneys at day 7 were covered with yellowish-white areas of necrosis (*A*), which were distributed in both cortex and medulla in sectioning (*B*). In some rats, the narrow rim of cortical tissue just underneath the capsule was relatively preserved but necrotic areas were distributed extensively in the cortex and medulla (*C*). In contrast, VEGF administration after anti-GEN antibody perfusion eliminated macroscopic evidence of infarction on the surface (*D*), although some residual necrosis was evident in the medulla (*E* and *F*).

VEGF-treated rats consisted of dilation and atrophy of tubules in the outer medulla and, to a lesser extent, in the cortex (Fig. 3E).

By light microscopy, the viable glomeruli in control rats demonstrated endothelial injury, duplication of glomerular basement membrane with subendothelial widening (Fig. 4A), and intravascular thromboses. Glomeruli from VEGF-infused rats displayed a more normal architecture, with less cellular damage and thromboses (Fig. 4B).

There was no difference in deposition of the pathogenic goat IgG between two groups at 10 minutes after perfusion (data not shown).

$VEGF_{121}$ administration preserves glomerular endothelium and vasculature

We next evaluated the effects of VEGF on the glomerular and arteriolar injury induced by anti-GEN antibody using immunohistochemical analysis. The glomerular endothelial lining was examined by immunostaining with RECA-1, a monoclonal antibody to an endothelial cell-

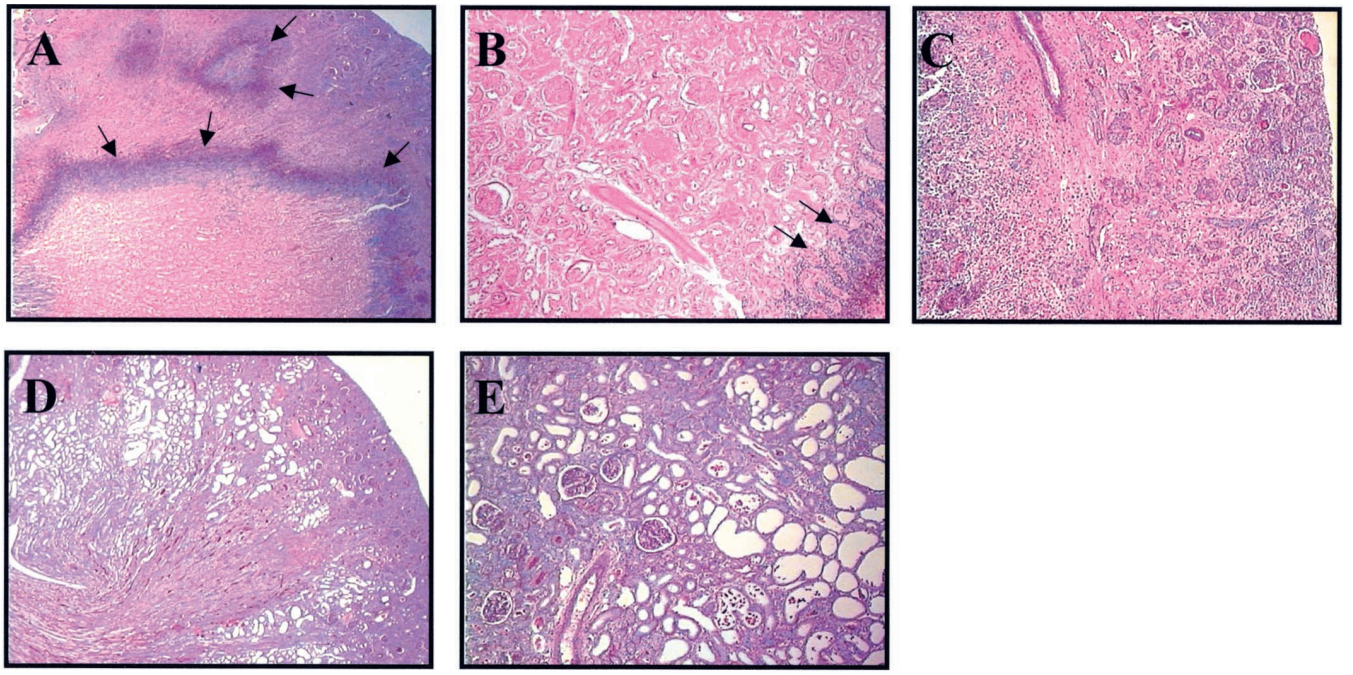

Fig. 3. VEGF reduces cortical and medullary necrosis induced by anti-GEN antibody. Renal histology was examined at day 7 by periodic acid-Schiff (PAS) staining. Perfusion of anti-GEN IgG resulted in massive cortical and medullary necrosis (*A*, ×25). A central dead zone composed of necrotic tissues with nuclear loss in glomeruli and tubules (*B*, ×100), peripheral dead zone with polymorphonuclear cell infiltration (B, arrow), and marginal zone with various degrees of necrosis, tubular regeneration and fibrosis (*C*, ×100) are also shown. Although there was tubulointerstitial injury with tubular dilatation, interstitial widening, and patchy areas of necrosis particularly in the medulla, VEGF treatment caused dramatic reduction in necrotic area in the cortex and medulla (*D,* ×25, and *E*, ×100).

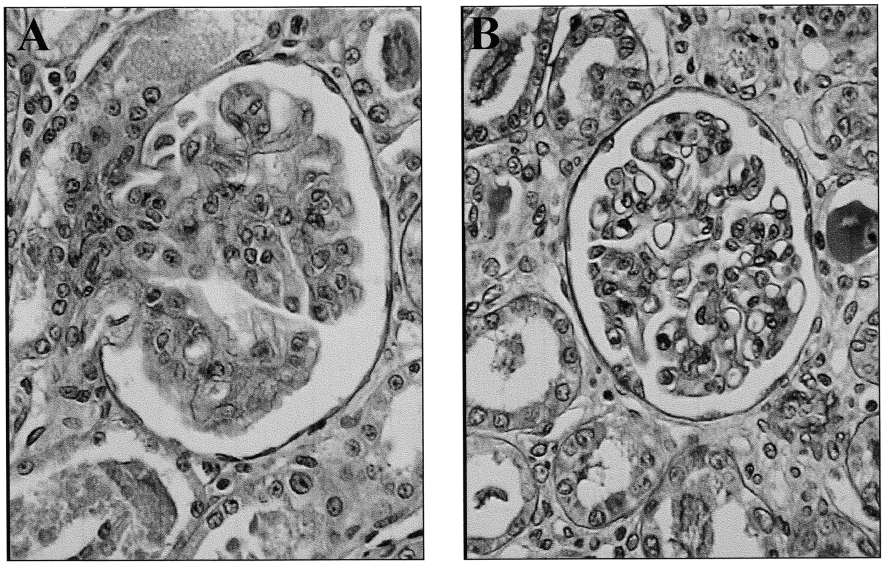

Fig. 4. Renal injury is lessened by VEGF treatment. (*A*) Glomerulus of control rat demonstrating features of thrombotic microangiopathy, with endothelial swelling and detachment, and duplication of glomerular basement membrane with subendothelial widening (day 4, ×600). (*B*) Glomerulus from a VEGF-treated rat showing preservation of the glomerular architecture, day 4 (×600).

specific antigen [15]. At day 7, the number of glomeruli with intact endothelium in entire cortical and juxtamedullary areas of sagittal sections was significantly greater in the VEGF$_{121}$-treated group than in the control group with anti-GEN antibody perfusion (3.3 ± 1.3 per mm^2 in the controls vs. 5.9 ± 0.3 per mm^2 in VEGF-treated rats, $P < 0.05$; Fig. 5A, B). In addition, the number of glomeruli with platelet aggregation tended to be smaller in VEGF$_{121}$-treated rats, although the difference was not statistically significant (1.64 ± 0.65 in the controls vs. 0.38 ± 0.20 per mm^2 in VEGF-treated rats, $P = 0.072$). The number of α-SMA–positive vessels in sagittal sec-

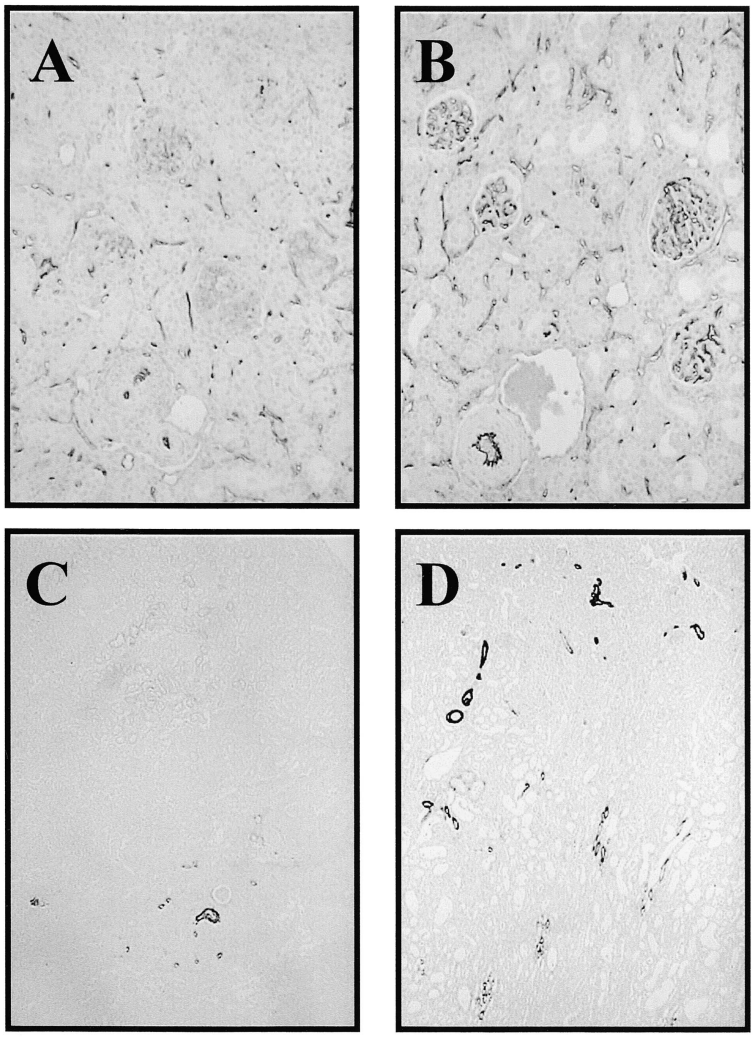

Fig. 5. VEGF preserves glomerular endothelial lining and α-smooth muscle actin (α-SMA)–positive arterioles. The glomerular endothelial cells were stained with RECA-1, a monoclonal antibody to endothelial cell-specific antigen. At day 7, the number of glomeruli with intact endothelial lining was significantly greater in the VEGF-treated group (*B*, ×100) than in the injured control group (*A*, ×100). α-SMA–positive vessels, characteristic of arteries and arterioles, were rarely observed in the medulla of controls (*C*, ×25). In contrast, there was a preservation of interlobular arteries, arterioles and vascular bundles (vasa rectae) in VEGF-treated rats (*D*, ×25). Some α-SMA–positive vessels were preserved in superficial cortex of control rats (C, upper left), but there was a significant decrease in the number of α-SMA–positive vessels, when compared with VEGF-treated rats (D).

tions also was examined. α-SMA–positive vessels, characteristic of arteries and arterioles, were rarely observed in the medulla in control animals (Fig. 5C). However, preservation of interlobular arteries, arterioles, and vascular bundles (vasa rectae) was readily apparent in VEGF-treated rats (Fig. 5D). Some α-SMA–positive vessels were preserved in the superficial cortex of control rats, but there was a significant decrease in the number of α-SMA–positive vessels, when compared to the VEGF-treated group (10 ± 1 in the controls vs. $15 \pm 2/mm^2$ in VEGF-treated rats, $P < 0.05$).

The preservation of glomeruli with intact endothelium and α-SMA–positive vessels suggested that VEGF might be acting as a survival factor for endothelial cells. VEGF is known to exert at least two biological effects that could moderate vascular susceptibility to injury that would otherwise cause infarction of dependent tissue: (*1*) induction and maintenance of eNOS expression and (*2*) inhibition of endothelial apoptosis.

VEGF preserves eNOS expression

Endothelial nitric oxide synthase is normally expressed in glomerular and peritubular capillary endothelial cells and in tubular epithelial cells of the outer medulla [16]. In control rats with severe TMA, eNOS expression was markedly diminished at day 7 with minimal expression in the endothelium of arteries, arterioles, and glomeruli in the cortex and virtually no staining of eNOS in the medulla except capillary endothelial cells in the areas surrounding infarction (Fig. 6A, C). In VEGF-treated rats, eNOS expression was preserved and remained widely distributed in glomerular and vascular endothelial cells as well as in vascular bundles and tubular epithelial cells in the medulla (Fig. 6B, D). Semiquantitative analysis revealed that the intensity of glomerular eNOS staining was stronger in VEGF-treated rats than in controls in non-necrotic areas (1.2 ± 0.2 in the controls vs. 2.0 ± 0.2 in VEGF-treated rats, $P < 0.05$). Computer image analysis in noninfarcted renal tissue also revealed better

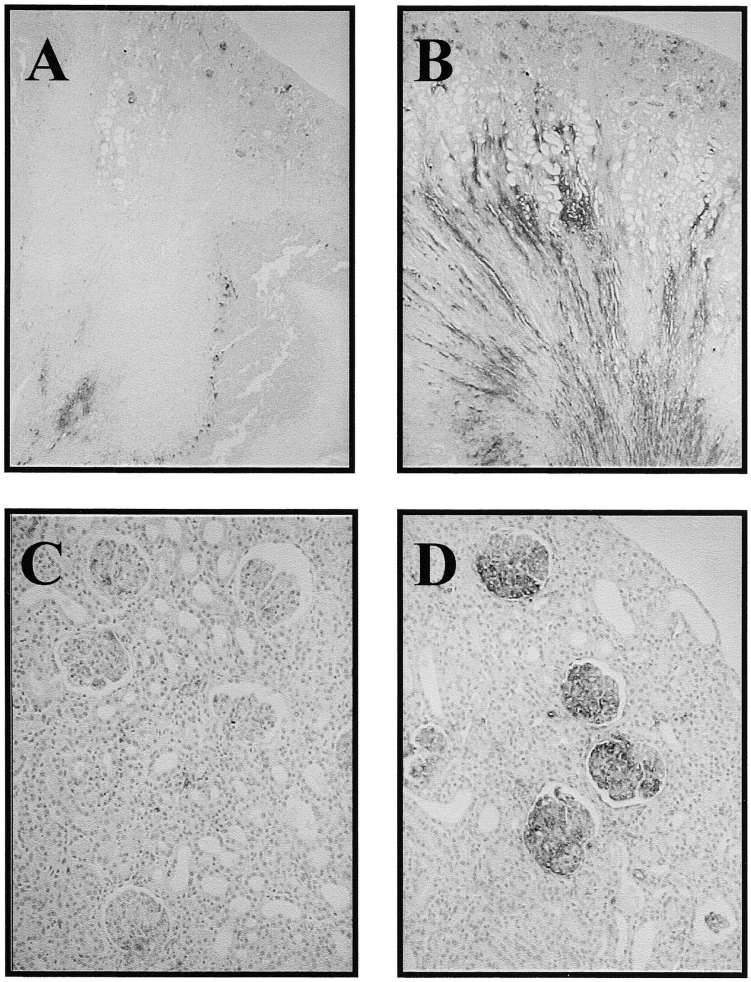

Fig. 6. VEGF treatment preserves endothelial nitric oxide synthase (eNOS) expression in rats with HUS-like lesions. In control rats, the endothelial isoform of eNOS was markedly diminished with residual staining of the endothelium of some arteries, arterioles, and glomeruli in the cortex and with little staining in the medulla except for occasional capillary endothelial cells in the areas surrounding infarction (marginal zone of infarction; *A*, ×50). In VEGF-treated rats, eNOS was widely preserved in glomerular and vascular endothelial cells as well as in tubular epithelial cells and vascular bundles in the medulla (*B*, ×50). Glomerular eNOS staining was stronger in VEGF-treated rats than in controls in surviving non-necrotic areas (*C*, controls ×200; *D*, VEGF-treated rats ×200).

preservation of eNOS staining in tubulointerstitium (0.7 ± 0.1 in the controls vs. 1.4 ± 0.2 in VEGF-treated rats, % of noninfarcted area, $P < 0.01$).

$VEGF_{121}$ as an endothelial survival factor

Vascular endothelial growth factor can serve as an in vitro survival factor for vascular endothelium [8, 9]. We therefore examined the effect of VEGF on apoptosis in our model. At four hours after the induction of the injury, significant numbers of apoptotic cells were observed within capillary lumina of the glomeruli, consistent with the early glomerular endothelial cell apoptosis that is observed in this model [20]. These intraluminal cells within glomerular capillaries showed typical nuclear chromatin condensation that is the morphological hallmark of apoptosis by PAS staining (Fig. 7A) as well as positive TUNEL staining (Fig. 7B). The administration of VEGF resulted in a significant reduction of the number of TUNEL-positive cells in glomeruli of TMA kidneys at four hours (Fig. 7C). Apoptosis of tubulointerstitial cells also was prominent in rats with experimental TMA. While VEGF did not reduce the number of apoptotic cells in the tubulointerstitium at four hours, VEGF-infused rats did have a threefold decrease in the number of TUNEL-positive tubulointerstitial cells at day 4 (Fig. 7D).

To clarify the possible mechanisms by which VEGF could reduce apoptosis, we examined the in vitro effect of VEGF on endothelial apoptosis induced by TNF-α, an inflammatory cytokine known to be a potent inducer of endothelial cell death. VEGF significantly protected HUVEC from TNF-α–mediated apoptosis (Fig. 8). At 50 ng/mL of VEGF, a 100% inhibition of TNF-α–induced caspase activation (a surrogate marker of apoptosis) in HUVEC was observed, in agreement with previous observations [23]. Since HUVEC may differ from glomerular endothelial cells in several characters, the use of HUVEC must be regarded as a model system. Nonetheless, this observation that VEGF reduced TNF-α–mediated apoptosis of cultured endothelial cells may be of relevance to the progression of renal necrosis in this model of HUS. Quantitative real-time PCR analysis revealed a 8.4 ± 1.2-fold increase in TNF-α mRNA in control TMA rats

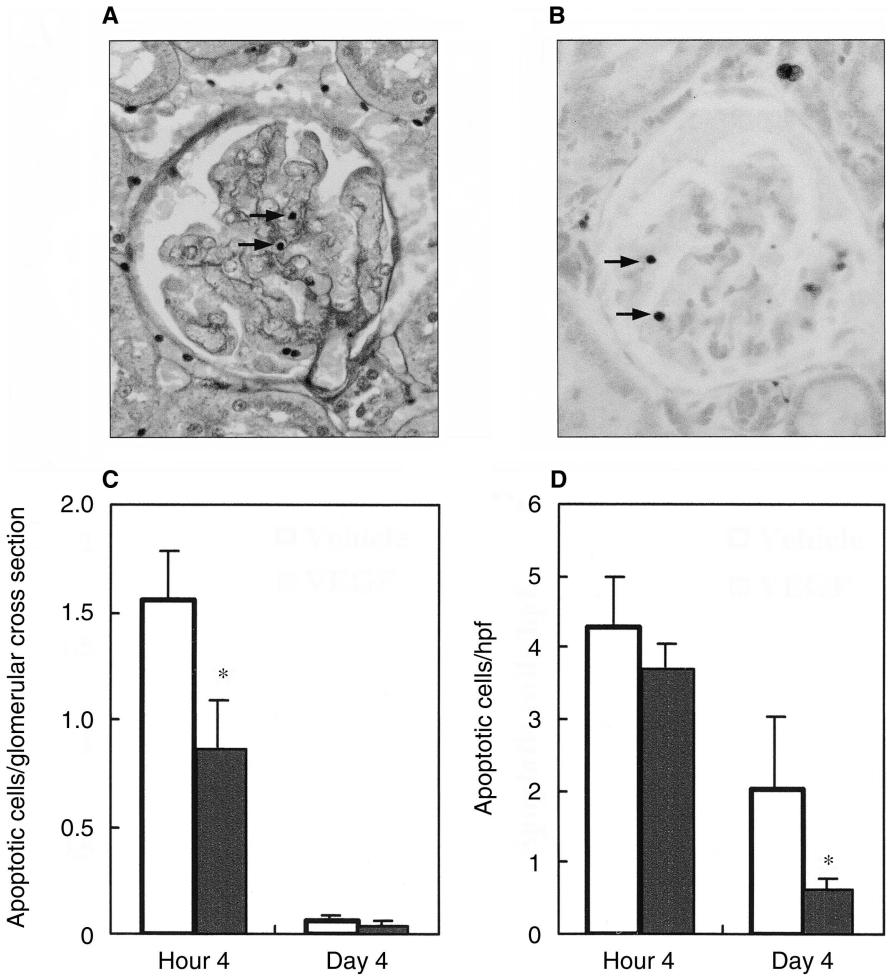

Fig. 7. Glomerular and tubulointerstitial cell apoptosis is markedly reduced in VEGF-treated rats. Glomerular endothelial cell apoptosis was demonstrated at four hours by PAS (*A*), and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling (TUNEL; *B*). Note the marked condensation of nuclear chromatin that is the classical morphological marker of apoptosis. VEGF-treatment protects glomerular cells (at 4 hours; *C*) and tubulointerstitial cells (at day 4; *D*) from undergoing apoptosis in rat thrombotic microangiopathy (TMA) kidney. Symbols are: (□) vehicle; (■) VEGF; $*P < 0.05$ vs. TMA kidney treated with vehicle.

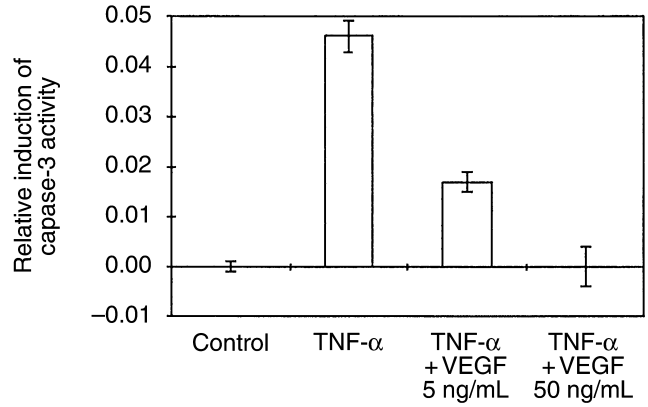

Fig. 8. VEGF inhibits TNF-α–mediated apoptosis in HUVECS. Caspace-3 activity was determined as a measure of apoptosis in HUVECs following a six-hour stimulation with 1 ng/mL of TNF-α with 0, 5, or 50 ng/mL of $VEGF_{121}$. All experiments were performed in triplicate.

at day 7 compared with sham controls. VEGF treatment did not affect the level of TNF-α mRNA expression (6.6 ± 0.4-fold increase), indicating its protective effects may be more related to the effector functions of TNF-α rather than modulation of its expression.

DISCUSSION

In the present study, severe renal injury was induced in rats by the perfusion of an anti-endothelial cell antibody. While the renal injury in this model is not initiated by verotoxin-induced endothelial injury as in classical HUS, the endothelial injury does result in features characteristic of TMA, with platelet aggregation, fibrin deposition, and glomerular and arteriolar endothelial injury. Indeed, the dose of anti-endothelial cell antibody used in this study resulted in a severe form, characterized by extensive glomerular involvement, arterial occlusive lesions and renal necrosis, all histopathological features of severe HUS associated with a poor clinical outcome [4].

Since endothelial cell injury induced by various insults is regarded as a major pathogenic pathway of human HUS and TMA [2, 7], we hypothesized that the administration of a growth factor, VEGF, which preferentially targets the endothelium, might provide protection in this model. At one hour after injection of the necrotizing antibody, $VEGF_{121}$ administration was initiated, resulting in reduced glomerular endothelial cell apoptosis, preserved microvascular endothelium, and less cortical and medullary necrosis.

The major beneficial effect of VEGF appeared to be the reduction in cortical and medullary infarction. There are likely multiple beneficial effects of VEGF that converge to prevent the progressive loss of vascular integrity that underlies the consequent infarction of dependent renal tissue. VEGF is known to stimulate endothelial NO release as well as induce and maintain eNOS expression and activity [24, 25]. Local NO may then act to maintain vascular patency by causing smooth muscle cell relaxation and vasodilation and also by inhibiting platelet adhesion and aggregation [26]. Indeed, VEGF induces NO-dependent vasodilation in vivo [27], and VEGF-induced NO release from microvascular endothelial cells inhibits leukocyte-endothelial interaction and preserves vascular patency [28]. The observation in the present study that TMA rats displayed a marked loss of eNOS expression, which was prevented by daily VEGF administration, supports this as one of the potential mechanisms for protection.

Vascular endothelial growth factor also is known to be an endothelial cell survival factor [9, 23], inhibiting endothelial apoptosis induced in vitro by serum withdrawal and irradiation. Apoptotic inhibition is consistent with our finding that VEGF-treated rats had fewer intraluminal apoptotic cells in endothelial locations within glomeruli at four hours and better preservation of both glomerular staining though the course of the injury. Because there is no clear relevance of observations based on in vitro serum withdrawal to in vivo observations on decreased endothelial cell death, we examined our model for the presence of injurious factors that could induce apoptosis in endothelial cells. The inflammatory cytokine TNF-α has been shown to mediate apoptosis of cultured endothelial cells [23]. We demonstrated, to our knowledge for the first time, that our model of HUS is associated with a sustained increase in the expression of mRNA for TNF-α. We further demonstrated, as has been shown previously for another isoform of VEGF [23], that $VEGF_{121}$ is highly protective in vitro against apoptosis induced in human umbilical vein endothelial cells by TNF-α, a protection observed at tissue culture concentrations equivalent to plasma concentrations attained in vivo after our injections of VEGF. Activation of the anti-apoptotic phosphatidylinositol 3-kinase/Akt signal transduction pathway by VEGF has been associated with endothelial survival in cultured endothelial cells [29] and in a hind-limb ischemic model in vivo [30], as has elevation of Bcl-2 levels [31], induction of anti-apoptotic proteins [32], and activation of cellular adhesion [33]. It is beyond the scope of this article to ascribe anti-apoptotic effects of VEGF in a complex model of in vivo angiopathy and necrosis to any or all of these mechanisms largely derived from in vitro observations. However, they do support inhibition of apoptosis as a plausible mechanism underlying the protective effects of VEGF in this model of renal disease. The lack of VEGF therapeutic effect on expression of TNF in our model supports modulation of the effector functions of TNF rather than modification of the induction of TNF as a mechanism for endothelial protection conferred by VEGF. It is highly likely that there are numerous stimuli other than TNF contributing to endothelial cell death in this complex in vivo model. The expression of TNF associated with angiopathy and moderation of its impact upon a model system of cultured endothelium should be considered as a representative of potential multiple protective effects conferred by VEGF in vivo, as should the known inductive effects of VEGF on NO synthase expression.

We have previously observed an initial increase in glomerular VEGF expression in the earliest phases of this model of HUS, followed by a rapid decrease to nearly undetectable levels within 48 hours after initiation of the lesion [6]. We speculate that the initial increase in glomerular VEGF may comprise as an early renal protective response to vascular injury, a response whose attenuation could be associated with the subsequent progression to endothelial injury and renal infarction. Thus, administered VEGF could plausibly be considered an exogenous amplification of an endogenous renal microvascular defense mechanism that was inadequate to protect against progressive vascular injury. It is important to recognize, however, that protection by VEGF was only partial, and identifying other ways to protect against endothelial injury are needed.

Vascular endothelial growth factor also is a potent angiogenic factor that can stimulate endothelial cell proliferation and new capillary growth. However, the current study was designed to evaluate the role of VEGF to prevent necrosis as opposed to accelerating vascular regrowth. Specifically, VEGF was administered before the maximal endothelial cell injury in order to determine whether it could block endothelial apoptosis and renal infarction, and the major endpoints were preservation of glomerular architecture and arteriolar structures, which are not products of acute angiogenesis. Furthermore, capillary formation in response to VEGF usually takes at least 10 to 14 days [13] and could not account for the protection against infarction. However, we have recently studied the ability of chronic VEGF administration to stimulate capillary repair in a milder noninfarction form of this model [12]. In this case, the VEGF was not initiated until after 24 hours when endothelial injury was maximal, and then the VEGF was continued for 14 days in order to give adequate time for capillary repair and angiogenesis to occur. VEGF resulted in significant increases in peritubular capillary density, less interstitial fibrosis, and better preservation of renal function [12]. Thus, VEGF appears to benefit the diseased kidney in at least two ways: preservation of the renal microvasculature

against acute injury and replacement, at least in the interstitium, of the microvasculature by stimulated new growth.

In conclusion, the administration of exogenous VEGF significantly reduced the severity of renal parenchymal infarction and necrosis in an experimental model of TMA in rats. To our knowledge, this is the first demonstration of in vivo infarct prevention by VEGF in any model of necrotizing vascular injury, although VEGF has been shown to prevent sinusoidal damage in liver tissue subjected to cold preservation [33]. The observation that VEGF administration after the initiation of injury could reduce tissue necrosis in the kidney may be relevant to other diseases associated with cortical or renal infarction, such as eclampsia. Since intrarenal endothelial cell injury takes place in several forms of experimental glomerular diseases, including the remnant kidney model and glomerulonephritis [34, 35], where glomerular endothelial cells are not the primary sites of injury, prevention of endothelial injury by VEGF also may be relevant to therapeutic intervention in various forms of glomerular disease.

ACKNOWLEDGMENTS

Support for this study was provided by United States Public Health Science grants DK-52121, HL-68607, DK-47659 and a small grant from Scios Inc. Dr. Shin-ichi Suga was supported by Banyu Fellowships Awards in Lipid Metabolism and Atherosclerosis, which are sponsored by Banyu Pharmaceutical Co., Ltd., and the Merck Company Foundation, and Uehara Memorial Foundation Research Fellowship. Dr. Ashley Jefferson was supported by a fellowship grant from the National Kidney Foundation.

Reprint requests to George F. Schreiner, M.D., Scios Inc., 820 West Maude Avenue, Sunnyvale, California 94086, USA.
E-mail: schreiner@sciosinc.com

REFERENCES

1. Gordjani N, Sutor AH, Zimmerhackl LB, *et al*: Hemolytic uremic syndromes in childhood. *Semin Thromb Hemost* 23:281–293, 1997
2. Remuzzi G, Ruggenenti P: The hemolytic uremic syndrome. *Kidney Int* 48:2–19, 1995
3. Tonshoff B, Sammet A, Sanden I, *et al*: Outcome and prognostic determinants in the hemolytic uremic syndrome of children. *Nephron* 68:63–70, 1994
4. Gagnadoux MF, Habib R, Gubler MC, *et al*: Long-term (15–25 years) outcome of childhood hemolytic-uremic syndrome. *Clin Nephrol* 46:39–41, 1996
5. Van Dyck M, Proesmans W, Depraetere M: Hemolytic uremic syndrome in childhood: renal function ten years later. *Clin Nephrol* 29:109–112, 1988
6. Nangaku M, Alpers CE, Pippin J, *et al*: A new model of renal microvascular endothelial injury. *Kidney Int* 52:182–194, 1997
7. Laszik Z, Silva FG: Hemolytic-uremic syndrome, thombotic thrombocytopenic purpura, and systemic sclerosis (systemic scleroderma), in *Heptinstall's Pathology of the Kidney* (5th ed), edited by Jennette JC, Olson JL, Schwartz MM, Silva FG, Philadelphia, Lippincott-Raven Publishers, 1998, pp 1003–1057
8. Benjamin LE, Golijanin D, Itin A, *et al*: Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal. *J Clin Invest* 103:159–165, 1999
9. Alon T, Hemo I, Itin A, *et al*: Vascular endothelial growth factor acts as a survival factor for newly formed retinal vessels and has implications for retinopathy of prematurity. *Nat Med* 1:1024–1028, 1995
10. Ferrara N, Davis-Smyth T: The biology of vascular endothelial growth factor. *Endocr Rev* 18:4–25, 1997
11. Tuder RM, Flook BE, Voelkel NF: Increased gene expression for VEGF and the VEGF receptors KDR/Flk and Flt in lungs exposed to acute or to chronic hypoxia: Modulation of gene expression by nitric oxide. *J Clin Invest* 95:1798–1807, 1995
12. Kim YG, Suga SI, Kang DH, *et al*: Vascular endothelial growth factor accelerates renal recovery in experimental thrombotic microangiopathy. *Kidney Int* 58:2390–2399, 2000
13. Couffinhal T, Silver M, Zheng LP, *et al*: Mouse model of angiogenesis. *Am J Pathol* 152:1667–1679, 1998
14. Cregg JM, Vedvick TS, Raschke WC: Recent advances in the expression of foreign genes in Pichia pastoris. *Biotechnology* 11: 905–910, 1993
15. Thomas SE, Anderson S, Gordon KL, *et al*: Tubulointerstitial disease in aging: evidence for underlying peritubular capillary damage, a potential role for renal ischemia. *J Am Soc Nephrol* 9:231–242, 1998
16. Lombardi D, Gordon KL, Polinsky P, *et al*: Salt-sensitive hypertension develops after short-term exposure to angiotensin II. *Hypertension* 33:1013–1019, 1999
17. Hugo C, Pichler R, Gordon K, *et al*: The cytoskeletal linking proteins, moesin and radixin, are upregulated by platelet-derived growth factor, but not basic fibroblast growth factor in experimental mesangial proliferative glomerulonephritis. *J Clin Invest* 97: 2499–2508, 1996
18. Baker AJ, Mooney A, Hughes J, *et al*: Mesangial cell apoptosis: The major mechanism for resolution of glomerular hypercellularity in experimental mesangial proliferative nephritis. *J Clin Invest* 94:2105–2116, 1994
19. Kerr JF, Wyllie AH, Currie AR: Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 26:239–257, 1972
20. Hughes J, Nangaku M, Alpers CE, *et al*: C5b-9 membrane attack complex mediates endothelial cell apoptosis in experimental glomerulonephritis. *Am J Physiol (Renal Physiol)* 278:F747–F757, 2000
21. Casciola-Rosen LA, Miller DK, Anhalt GJ, *et al*: Specific cleavage of the 70-kDa protein component of the U1 small nuclear ribonucleoprotein is a characteristic biochemical feature of apoptotic cell death. *J Biol Chem* 269:30757–30760, 1994
22. Lazebnik YA, Kaufmann SH, Desnoyers S, *et al*: Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature* 371:346–347, 1994
23. Spyridopoulos I, Brogi E, Kearney M, *et al*: Vascular endothelial growth factor inhibits endothelial cell apoptosis induced by tumor necrosis factor-alpha: Balance between growth and death signals. *J Mol Cell Cardiol* 29:1321–1330, 1997
24. Papapetropoulos A, Garcia-Cardena G, Madri JA, *et al*: Nitric oxide production contributes to the angiogenic properties of vascular endothelial growth factor in human endothelial cells. *J Clin Invest* 100:3131–3139, 1997
25. van der Zee R, Murohara T, Luo Z, *et al*: Vascular endothelial growth factor/vascular permeability factor augments nitric oxide release from quiescent rabbit and human vascular endothelium. *Circulation* 95:1030–1037, 1997
26. Moncada S, Palmer RM, Higgs EA: The discovery of nitric oxide as the endogenous nitrovasodilator. *Hypertension* 12:365–372, 1988
27. Horowitz JR, Rivard A, van der Zee R, *et al*: Vascular endothelial growth factor/vascular permeability factor produces nitric oxide-dependent hypotension. Evidence for a maintenance role in quiescent adult endothelium. *Arterioscler Thromb Vasc Biol* 17:2793–2800, 1997
28. Scalia R, Booth G, Lefer DJ: Vascular endothelial growth factor attenuates leukocyte-endothelium interaction during acute endothelial dysfunction: Essential role of endothelium-derived nitric oxide. *FASEB J* 13:1039–1046, 1999
29. Kureishi Y, Luo Z, Shiojima I, *et al*: The HMG-CoA reductase inhibitor simvastatin activates the protein kinase Akt and promotes angiogenesis in normocholesterolemic animals. *Nat Med* 6:1004–1010, 2000
30. Gerber HP, McMurtrey A, Kowalski J, *et al*: Vascular endothelial growth factor regulates endothelial cell survival through the

phosphatidylinositol 3′-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. *J Biol Chem* 273:30336–30343, 1998
31. Zhang Y, Ikejima K, Honda H, *et al*: Glycine prevents apoptosis of rat sinusoidal endothelial cells caused by deprivation of vascular endothelial growth factor. *Hepatology* 32:542–546, 2000
32. Tran J, Rak J, Sheehan C, *et al*: Marked induction of the IAP family antiapoptotic proteins survivin and XIAP by VEGF in vascular endothelial cells. *Biochem Biophys Res Commun* 264:781–788, 1999
33. Moriga T, Arii S, Takeda Y, *et al*: Protection by vascular endothelial growth factor against sinusoidal endothelial damage and apoptosis induced by cold preservation. *Transplantation* 69:141–147, 2000
34. Kitamura H, Shimizu A, Masuda Y, *et al*: Apoptosis in glomerular endothelial cells during the development of glomerulosclerosis in the remnant-kidney model. *Exp Nephrol* 6:328–336, 1998
35. Iruela-Arispe L, Gordon K, Hugo C, *et al*: Participation of glomerular endothelial cells in the capillary repair of glomerulonephritis. *Am J Pathol* 147:1715–1727, 1995