Circulating vascular endothelial growth factor is not increased during relapses of steroid-sensitive nephrotic syndrome


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Background. An uncharacterized circulating factor that increases vascular permeability has previously been described in childhood steroid-sensitive nephrotic syndrome (SSNS). The aim of this study was to determine whether this factor is vascular endothelial growth factor (VEGF), the recently described endothelial cell mitogen and enhancer of vascular permeability.

Methods. Plasma and urine VEGF levels were measured in children with SSNS in both relapse and remission and in normal age- and sex-matched controls. Semiquantitative reverse transcriptase-polymerase chain reaction studies investigating VEGF mRNA expression were performed on peripheral blood mononuclear cells isolated from children with SSNS in relapse and controls. In two experimental models (one-hour and three-day follow-up postinfusion), Sprague-Dawley rats were intravenously administered 50 μg rVEGF to determine whether this induced either proteinuria or glomerular histologic change.

Results. Plasma VEGF levels and urine VEGF/creatinine ratios were not elevated in SSNS relapse compared with remission and control samples. Peripheral blood mononuclear cell VEGF mRNA expression was no different in SSNS patients compared with controls. The administration of VEGF to rats induced an acute reversible fall in systemic blood pressure but did not result in the development of either proteinuria or glomerular histologic change.

Conclusion. Increased circulating VEGF levels are not responsible for the proteinuria observed during relapses of SSNS. Further studies are warranted to investigate intrarenal VEGF expression.

Childhood steroid-sensitive nephrotic syndrome (SSNS) is characterized by the abrupt onset of proteinuria, which, if untreated, rapidly results in the development of generalized edema. Following successful treatment of the presenting episode, the majority of children follow a relapsing/remitting course, necessitating repeated courses of corticosteroids and other immunosuppressive therapies. Although there is considerable evidence supporting a central role for the immune system in the disease pathogenesis [1], the precise etiology is, as yet, undetermined, and histologic examination of renal biopsy tissue does not reveal evidence of a classic immunologically mediated inflammatory process involving either antibody and complement deposition or interstitial cell infiltration.

Over the past 20 years, a number of groups have investigated an uncharacterized factor with permeability-enhancing properties detected in the supernatant of peripheral blood mononuclear cells (PBMCs) isolated from patients with SSNS. PBMC supernatant from SSNS patients in relapse, when injected into guinea pig skin in the Miles assay [2] produced an increase in local capillary permeability that was not observed upon injection with supernatant from SSNS patients in remission and normal controls [3, 4]. CD4+ lymphocytes being later shown to be the predominant source of this factor [5]. Infusion of stimulated PBMC supernatant from patients with SSNS into the rat renal artery has been shown to produce a reduction in colloidal iron staining of the basement membrane, indicative of loss of anionic charge and foot process fusion [6], and also to increase urinary protein excretion in the first 12-hours postinfusion [7]. T-cell hybridomas from peripheral blood T cells isolated from SSNS patients in relapse have also been shown to induce proteinuria in the rat [8]. In a further model, Bakker et al cultured isolated Con A-stimulated monocytes from patients with SSNS and other proteinuric glomerulopathies with slices of rat kidney [9]. Colloidal iron staining was significantly reduced in a majority of SSNS cultures compared with a minority of those of other glomerulopathies.

Because of these aforementioned properties, this uncharacterized substance was termed vascular permeabil-

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Vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF), is a disulfide-linked homodimer of 34 to 42 kDa. It is an endothelial cell mitogen [11] that promotes angiogenesis and also has potent vascular permeability-enhancing properties [12]. Alternate splicing of mRNA results in the generation of four protein species of 121, 165, 189, and 206 amino acids. On the basis of protein structure, particularly the pattern of conserved cysteines and sequence homology, VEGF has been considered to be a member of the PDGF protein family [13].

Vascular endothelial growth factor mediates biological function by interaction with two specific tyrosine kinase receptors on endothelial cells: fms-like tyrosine kinase (flt-1) [14] and kinase insert domain containing receptor (KDR) [15], also known as VEGFR1 and VEGFR2, respectively. In an experimental model, a systemic injection of VEGF has been reported to induce proteinuria (abstract; Iijima et al, J Am Soc Nephrol 3:514, 1992).

Topical application of VEGF to the rat cremaster muscle [16] and infusion of VEGF into the parietooccipital cortex of mice [17] have been shown to increase capillary permeability within 30 minutes. Coincident with these local changes in capillary endothelium is the dynamic, temporal appearance of interendothelial gaps and the formation of fenestrae-like openings.

Vascular endothelial growth factor is known to be constitutively expressed by a wide range of normal adult tissues [18], including glomerular epithelial cells [19], and PBMCs have been reported to both express VEGF mRNA [20] and to secrete VEGF protein (abstract; Watson et al, J Am Soc Nephrol 7:1725, 1996). Glomerular endothelial cells have been shown to express mRNA for both VEGF receptors, flt-1 and KDR [19]. Dysregulated VEGF expression has previously been implicated in a number of pathological situations, including tumor angiogenesis/metastasis [21], rheumatoid arthritis [22, 23], diabetic retinopathy [24, 25], ovarian hyperstimulation syndrome [26], and glomerular disease associated with mesangial proliferation (abstract; Iijima et al, J Am Soc Nephrol 6:923, 1995).

In view of the seemingly considerable body of evidence to support a potential role for VEGF in the pathogenesis of SSNS, we undertook the following series of investigations. Our hypothesis was that exposure of glomerular capillary cells to circulating VEGF or VEGF produced locally in the kidney would modulate endothelial permeability through a noninflammatory mechanism, thus inducing albuminuria. The specific aims of the study were to determine whether (a) circulating (plasma) or urinary VEGF levels were increased in children during relapses of SSNS compared with levels in children in remission and healthy age- and sex-matched controls, (b) PBMC VEGF mRNA expression was different in children with SSNS compared with healthy controls, and (c) the administration of rVEGF resulted in the development of proteinuria in the Sprague-Dawley rat.

**METHODS**

Vascular endothelial growth factor enzyme-linked immunosorbent assay kits were purchased from R&D Systems (Abingdon, UK). rVEGF for the animal studies was a kind gift from Dr. D. Ogilvie of Zeneca Pharmaceuticals (Alderley Edge, UK). Lymphocyte separation medium (Optiprep) was purchased from Robins Scientific (Knowle, UK). Sprague-Dawley rats were purchased from Charles Rivers (Margate, UK). Taq DNA polymerase, Superscript reverse transcriptase, RNase inhibitor, oligo (dT)12-18, 100 bp DNA ladder, and dNTP solutions were all purchased from Life Technologies (Paisley, UK).

**Plasma and urine VEGF levels in childhood SSNS**

Plasma and urine samples were collected from children with relapsing SSNS during disease relapse and remission and from age- and sex-matched normal controls. Children receiving regular immunomodulatory therapy and those who had received corticosteroids within six weeks or alkylating agents (cyclophosphamide or chlorambucil) within six months were excluded from the study. Ethical approval was granted by the Salford Area Health Authority Ethical Committee. Relapse samples were collected early during disease relapse. Parents were asked to contact the department as soon as possible after the development of proteinuria, allowing samples to be collected before the children became clinically nephrotic (as part of routine clinical care, families of children with SSNS are instructed to test their child’s early morning urine for proteinuria on a daily basis using Albustix™). Remission samples were collected during periods of stable remission. Control samples were collected from otherwise healthy children undergoing minor elective surgical procedures under general anesthesia. Samples were collected within two minutes of induction of anesthesia prior to the commencement of surgery. A separate venipuncture site was chosen rather than using the intravenous cannula to obtain samples in order to avoid any contamination by intravenous anesthetic agents. Blood was collected into Becton and Dickenson ethylenediaminetetraacetic acid (EDTA) blood tubes (0.072 ml 7.5% EDTA per 3 ml sample), and centrifugation of samples (2000 r.p.m. for 10 min) and separation of plasma was performed within two hours of venipuncture. Plasma was used in preference to serum, as we have...
previously shown that a significant increase in measured VEGF levels occurs upon clotting of blood due to platelet VEGF release [27]. Early morning urine samples were collected into universal containers. Plasma and urine samples were stored in multiple aliquots at −80°C prior to assaying.

Plasma and urine VEGF levels were measured using a monoclonal antibody (mAb)-mediated capture ELISA (R&D Systems). Urinary protein levels were measured using a benzethonium chloride-binding assay (Hitachi 911 autoanalyzer), and urinary creatinine levels were measured using a kinetic Jaffé method (Hitachi 911 autoanalyzer). Urine VEGF levels were expressed as a VEGF/creatinine ratio to correct for variation in urine concentration between samples.

**Semiquantitative VEGF mRNA expression in PBMCs in childhood SSNS**

At the time of venous sampling for obtaining plasma samples, a further sample of blood was collected from children with SSNS in relapse into Becton and Dickenson EDTA tubes for isolation of PBMCs. These were isolated from the whole blood samples by the centrifugation of cells through lymphocyte separation medium (Flow Laboratories, Irvine, UK). Total RNA was extracted from PBMC cell pellets using a modification of the guanidinium thiocyanate-phenol-chloroform method [28]. cDNA was synthesized from 5 μg of total RNA using Superscript™ reverse transcriptase and oligo (dT)12–18 according to the manufacturer’s instructions. Semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using previously described primers known to amplify all reported VEGF splice variants [20]. In addition, primers designed to amplify β-actin [29] were used to control for variation in cDNA template concentration, and competitive DNA fragments were constructed for both PCR reactions to control for inter-PCR amplification variation.

A VEGF competitive DNA fragment of 269 bp was generated using the primers: forward 5’CGAAGTGCTGAAGTCACTGGGATAGAGCAAGACAAGA AATCCCTG3’ and reverse, 5’TCTTGTATCGTCTTCCCCTCCATCGAAGTACC CCATGACGAGAGG3’ (sequences in bold denote primers used in VEGF cDNA amplification, and the underscored sequence corresponds to nucleotide 460–484 GenBank accession no. M32977). A β-actin competitive DNA fragment of 225 bp was generated using the primers: forward 5’GCCGTCCTCCCCCTCCATCGAAGTGACC CCATGACGAGAGG3’ and reverse 5’TAGCAACGT ACAACTGGCTGGG3’ (sequences in bold denote primers used in β-actin cDNA amplification, and underlined sequence corresponds to nucleotide 242–262 GenBank accession no. X00351). Titration experiments were conducted (data not shown) to determine the optimum number of amplification cycles and the optimum amounts of cDNA template and competitive fragment DNA for the VEGF and β-actin RT-PCR amplifications. RT-PCR of the samples was carried out in a 20 μl reaction overlaid with paraffin oil containing 1/25th volume (4%) of the cDNA reaction; 10 pmol of each primer (with the forward primer 5’ fluorescently end labeled with a CY5™-tag); 0.75 mm of each dNTP; 10% dimethyl sulfoxide; 3.5 mM MgCl₂; 16.6 mM (NH₄)₂SO₄; 67 mM Tris-HCl, pH 8.0; 85 μg/ml bovine serum albumin and 0.5 units of Taq DNA polymerase; and either 10 fg of VEGF or 1 pg of β-actin competitive DNA fragment as appropriate. Thermal cycling conditions consisted of an initial denaturation step at 94°C for three minutes followed by 30 cycles for VEGF and 25 cycles for β-actin at 94°C for one minute; 55°C for one minute and 65°C for one minute for VEGF and β-actin, respectively; and 72°C for one minute, with a final extension step at 72°C for five minutes. VEGF transcript expression was quantitated for each isoform using the ALFexpress DNA analysis system (Pharchem Biotech, Quebec, Canada). One microliter of PCR product was electrophoresed in duplicate through a 6% 39:1 acrylamide/bis-acrylamide 1 × TBE gel, and average values of peak fluorescence were determined. Transcript values were divided by competitive fragment values to correct for inter-PCR variation and were then expressed as a ratio of the peak area fluorescence of the transcript of interest over the β-actin transcript.

**Experiments to determine whether the administration of rVEGF induces proteinuria in the Sprague-Dawley rat**

**Short-term follow-up model.** All animal care was in accordance with United Kingdom Home Office guidelines. Ten male Sprague-Dawley rats (mean weight 244.9 ± 27.7 g; Charles Rivers, UK) were anesthetized by the administration of a bolus of 100 mg/kg of intraperitoneal thiopentone; further intraperitoneal doses of 2 mg/kg were given if necessary until surgical anesthesia was attained. Following shaving of the neck and abdomen, animals were placed in the prone position on a heated operating table. The neck vessels were exposed and following ligation of the vessel distally, the left external jugular vein was cannulated using PP10 tubing (Orme Scientific, Manchester, UK). A bolus of 1 ml 0.9% saline warmed to 37°C was administered over a two-minute period, followed by a continuous infusion of 200 μl/min to ensure that a good urine output was maintained. The right internal carotid artery was then exposed and, following ligation of the distal vessel, was cannulated using PP50 tubing and connected via a Statham pressure transducer (P23 Dc) to a Grass Polygraph (model 79C) to provide systemic blood-pressure monitoring. Only animals in which the mean arterial blood pressure (BP) was sustained above 80 mm Hg for the entire duration of the
experiment were accepted for inclusion into the study. A tracheotomy was performed using a diathermy loop to ensure a clear airway.

A midline laparotomy incision was made, and the left ureter was catheterized using PP10 tubing to allow collection of urine from the left kidney. A baseline urine sample from the left kidney was then collected over a 30 minute period into a preweighed eppendorf. At this point, 50 μg of rVEGF_{165} in a total volume of 500 μl 0.9% saline or an identical volume of 0.9% saline alone (5 animals in each group) were infused into the animal over a 10-minute period via the external jugular vein using a Graseby syringe pump. A dose of 50 μg was chosen on the basis of a previous report (abstract; Iijima et al, J Am Soc Nephrol 3:514, 1992), suggesting that this dose induced proteinuria. Human rVEGF_{165} has previously been shown to be biologically active in rat models [16], and physiological responses relating to altered vascular wall function have been reported to occur rapidly in both the Miles assay [2] and a rat cremaster muscle model [16]. The biological activity of the rVEGF_{165} used had previously been confirmed in endothelial cell mitogenesis assays (A. Canfield, University of Manchester, UK, personal communication). Following completion of the 10-minute period of intravenous infusion, a further four 15-minute timed collections were made, after which the left kidney was instantly removed and divided into two portions. One (for light microscopy studies) was placed in 10% neutral-buffered formalin, and the other (for electron microscopy studies) was placed in 2.5% glutaraldehyde in cacodylate buffer. Animals were then sacrificed by overdosing with intravenous anesthetic. A terminal blood sample was collected into an EDTA blood tube for the measurement of plasma VEGF levels using the mAb-mediated capture assay. Tissues were processed for light microscopy and electron microscopy by the Department of Pathological Sciences, University of Manchester, UK. The diameter of endothelial fenestrations was measured in electron micrographs (magnification ×39,000). Capillary loops were selected randomly for measurement and with a minimum of 10 glomeruli being examined in each kidney. The mean (range) number of fenestrations measured in each kidney was 93 (73 to 114).

Urine volumes were calculated by reweighing the preweighed eppendorfs and subtracting the pre-experimental from the post-experimental weights. Urine samples were frozen and batched for measurement of total protein concentration using a colorimetric assay (BioRad, Hemel Hempstead, UK) for protein concentration based on the Lowry assay [30]. Protein excretion from the left kidney was expressed as milligrams per hour, calculated using the product of the protein concentration and the volume of urine passed.

**Longer term follow-up model.** Eight male Sprague-Dawley rats (mean weight 238.3 g ± sd 15.1 g; Charles Rivers, London, UK) were placed in metabolic cages for a two-day period prior to commencement of the experimental period to allow complete acclimatization. Throughout the entire period of experimentation, animals were allowed free access to water and were fed standard chow in powdered form to prevent food being dragged from the feed hoppers into the main body of the cage (thus avoiding possible contamination of collected urine with protein-containing feed). Room temperature remained constant, and standard 12-hour light and dark cycles were used. At 0700 hours on experimental day 1, the metabolic cages were washed thoroughly, and the first 12-hour baseline urine collection commenced. At 1900 hours on the same day, using an aseptic technique, animals were administered either 50 μg rVEGF_{165} in 1 ml 0.9% saline or 1 ml 0.9% saline by slow intravenous injection into a tail vein using a 23G cannula.

Following the infusion of either VEGF or saline, urine was collected every 12 hours for a total of 72 hours (six 12-hour samples). Urine was collected into preweighed containers. After repeat weighing to calculate the volume of urine passed, the urine was microfuged to remove debris and was stored in multiple aliquots at −80°C prior to assaying. At the end of the 72 hour period of urine collection, the animals were sacrificed, and the left kidney was instantly removed and placed in 10% neutral-buffered formalin. Tissues were processed for light microscopy by the Department of Pathological Sciences, University of Manchester, UK.

Urine total protein concentrations were measured as described earlier here. Total protein excretion was expressed as mg per 12-hour period, calculated using the product of the protein concentration and the volume of urine passed.

**Statistical analysis**

Plasma VEGF levels and urine VEGF/creatinine ratios were shown not to be normally distributed, and the Kruskal–Wallis test was used to compare relapse, remission, and control values. The Mann–Whitney test was used to compare VEGF mRNA levels. Calculations were performed using GraphPad Prism™ (GraphPad Software Inc., USA).

**RESULTS**

**Plasma and urine VEGF levels in childhood SSNS**

Twenty-two children (16 boys and 6 girls) with SSNS were studied in relapse, and 13 (11 boys, 2 girls) were studied in remission. The mean (sem) age of the relapse group was 8.7 (±0.08) years, and the remission group’s mean age was 9.3 (±1.40) years. All children in both relapse and remission groups were not on maintenance immunomodulatory therapy and had not received oral
corticosteroids in the preceding six weeks or cyclophosphamide in the preceding six months. Control subjects ($N = 24$) had a mean age of 7.2 ($\pm 0.94$) years. There was no significant difference in the ages or male:female ratios between the three groups.

All children had their urinary protein excretion quantitated by measurement of an early morning protein/creatinine ratio (normal range less than 25 mg/mmol, nephrotic proportion proteinuria more than 250 mg/mmol). This confirmed the presence of nephrotic proportion proteinuria in the relapse group and the absence of proteinuria in the remission and control groups.

Plasma VEGF levels were not significantly increased in children with SSNS in relapse ($N = 22$, median 50.6 pg/ml, interquartile range 31.2 to 97.3 pg/ml) compared with remission ($N = 13$, 31.2 pg/ml, 31.2 to 39.1 pg/ml) or normal age- and sex-matched controls ($N = 24$, 61.1 pg/ml, 31.2 to 104.3 pg/ml; $P = 0.295$, Kruskal–Wallis test; Fig. 1A). Urine VEGF/creatinine ratios (Fig. 1B) were also not significantly higher in relapse ($N = 15$, 34.2 ng/mmol, 18.1 to 45.6 ng/mmol) compared with remission ($N = 10$, 20.1 ng/mmol, 19.2 to 30.2 ng/mmol) or control samples ($N = 17$, 23.7 ng/mmol, 18.2 to 38.0 ng/mmol; $P = 0.632$, Kruskal–Wallis test).

VEGF mRNA expression in PBMCs in childhood SSNS

Nine children with SSNS in relapse and nine normal control children were studied. Semiquantitative RT-PCR studies of VEGF mRNA expression in unstimulated PBMCs did not show any significant difference in levels of VEGF$_{121}$, VEGF$_{165}$, or total VEGF mRNA between the two groups (Table 1).

Experiments to determine whether the administration of rVEGF induces proteinuria in the Sprague-Dawley rat

Short-term follow-up model. Both VEGF and control groups of animals were of similar weight [VEGF group mean ($\pm \text{sd}$) weight 250.2 g ($\pm 16.5$ g), control group 239.6 g ($\pm 7.7$ g), $P = \text{NS}$, unpaired $t$-test]. There was no significant difference between the BPs of the two groups of animals in the 30-minute run-in period prior to the commencement of the infusion. In animals administered VEGF, there was an acute and precipitous fall in systemic BP immediately upon commencement of the VEGF infusion (Fig. 2A). The mean BP at two-minutes after commencement of the VEGF infusion was significantly lower than baseline (10) values ($P = 0.003$, paired $t$-test). This fall in BP was transient, with recovery to close to preinfusion levels occurring by 30-minutes post-infusion. No similar effect was seen in the control animals administered a 1 ml bolus of 0.9% saline.

In the control animals, urine output increased steadily throughout the period of experimentation secondary to the relative volume expansion produced by the continuous saline infusion (Fig. 2B). In the VEGF-administered animals that received an identical volume of 0.9% saline during the period of experimentation, there was a fall in urine output to below baseline levels for the first 45-minutes after completion of the VEGF infusion. Urinary protein excretion similarly fell in the VEGF-treated group, with excretion remaining constant in the saline control animals (Fig. 2C). Terminal plasma VEGF levels in control animals were all below the background detection limit of the mAb-mediated capture assay, whereas in VEGF-treated animals, despite 1 in 10 dilution of samples, plasma levels were in gross excess of the upper detection limit of the assay, indicating circulating VEGF levels to be in excess of 20 ng/ml. Light and electron microscopy studies on renal tissue submitted from three
VEGF-administered and three control animals revealed no abnormality in either group. In particular, electron microscopy studies showed no evidence of podocyte foot process abnormality in VEGF-treated animals, and the diameter of endothelial cell fenestrations was not significantly greater in the VEGF-treated group than the saline control group [median (interquartile range) diameter 103 nm (77 to 128 nm) and 120 (85 to 154 nm), respectively, P = 0.17, Mann–Whitney test].

**Longer term recovery model**

Both VEGF-treated and saline control animals were of similar weights [VEGF group mean (±sd) weight 235.5 g (±7.9 g), control group 241.0 g (±8.1 g), P = NS, unpaired t-test]. All animals were inspected on a twice-daily basis throughout the entire experimental period and remained well, with no adverse clinical signs. Urine output showed significant diurnal variation in both VEGF and saline control-treated animals, with increased urine production during the overnight 12-hour period (1900 to 0700 hr) compared with the daytime periods (0700 to 1900 hr) (Fig. 3A). There was no significant difference in urine output between the two groups. Protein excretion showed similar diurnal variation in both groups (Fig. 3B). No difference in protein excretion was detected between the VEGF-treated and saline control animals.

Light microscopy studies on renal tissue submitted from both VEGF-administered and control animals revealed no abnormality in either group.

**DISCUSSION**

We have clearly shown that circulating VEGF levels, as measured using a mAb-mediated capture assay, are not elevated during relapses of childhood SSNS compared with both remission and normal controls. This mAb-mediated capture assay is known to measure both free VEGF₁₂₁ and VEGF₁₆₅, and, therefore, it is not possible to determine which is the predominant protein variant being measured, although we have previously shown that this assay detects only free VEGF and not VEGF–flt-1 complexes [27]. Semiquantitative RT-PCR studies showed no difference in PBMC VEGF mRNA expression between SSNS relapse and control groups.

In addition to failing to detect increased circulating VEGF levels in human subjects with SSNS, it was also not possible to induce proteinuria in the Sprague-Dawley rat by the administration of recombinant human VEGF using both a short-term and a longer term model. The choice of dose of VEGF, mode of administration, and time courses studied warrant further discussion. As previously outlined, a dose of 50 μg was chosen because a previous report suggested that this would induce proteinuria (abstract; Iijima et al, *J Am Soc Nephrol* 3:514, 1992); if proteinuria had occurred, further experiments would have been performed using lower, more physiological VEGF doses. VEGF was administered systemically rather than directly into the renal artery or descending aorta to avoid any possible renal hypoxia associated with vessel cannulation, hypoxia being well recognized as a potent stimulus for up-regulation of VEGF mRNA expression [31–33]. Widespread binding of administered VEGF to receptors on endothelium throughout the entire vasculature as a potential cause of the absence of any observed effect was excluded by the detection of high circulating levels (more than 20 ng/ml) in VEGF-treated animals at the end of the experimental period. Although we did not formally test whether an even higher VEGF dose would result in the development of proteinuria, this would appear unlikely in view of this finding. A 60-minute preliminary follow-up period was chosen, as it was anticipated that any VEGF-induced changes might occur within this time frame in view of the rapidity of biological action detected in both the Miles assay [2] and the rat cremaster muscle model [16]. In addition to a complete absence of any evidence of a VEGF-induced functional change in the form of proteinuria, there was no histologic change noted at either light or electron microscopy levels, in particular no evidence of foot process fusion or alteration in endothelial fenestration. The rationale for including the transmission electron microscopy analysis, in particular the quantitation of the size of glomerular endothelial cell fenestrae, was that VEGF binding to endothelium, both *in vivo* [16, 17] and *in vitro* [34], clearly modulates the number and structure of fenestrae as part of the mechanism controlling capillary permeability. If structural change in the endothelium had occurred in the kidney subsequent to...
VEGF administration, this may not have automatically led to functional proteinuria through compensatory mechanisms operating at the podocyte filtration slits.

When the duration of follow-up was increased, necessitating the use of metabolic cages, again, neither proteinuria nor morphological change was induced by the systemic administration of VEGF.

Rats injected with VEGF in the short-term (60-min follow-up) model underwent an acute fall in systemic BP observed immediately upon commencement of the VEGF infusion, with the mean BP two-minutes postcommencement of the infusion being significantly lower than that at commencement of the infusion. An identical effect has previously been reported following the infusion of fibroblast growth factor, which appears to be related to increased nitric oxide (NO) synthesis and resultant vasodilation [35]. *In vitro* studies on vessels isolated from rabbits have shown that VEGF produces a dose-dependent rise in NO [36], and the *in vivo* infusion of VEGF into the coronary circulation has been shown to stimulate NO-dependent dilation of coronary microvessels [37]. Therefore, it appears likely that the observed hemodynamic changes were secondary to NO-mediated vasodilation, and it confirms that the injected rVEGF preparation had significant biological activity at the dose used.

These data refute our hypothesis that high circulating VEGF levels mediate the development of proteinuria in SSNS and that the circulating factor previously detected by others [3–9] was VEGF.

These rat studies clearly demonstrate that the glomerular capillary endothelium is insensitive to the effects of VEGF circulating in blood. The fact that VEGF was detected at levels in excess of 20 ng/ml at the end of the experiment is important, as it rules out the possibility of neutralization of VEGF by high circulating levels of flt-1 acting as a natural antagonist; the assay does not detect VEGF in the form of such complexes. The glomerular endothelium is reported to express both VEGF receptors, flt-1 and KDR [19], and ought therefore to be sensitive to the biological action of VEGF similar to other endothelial beds. One potential explanation for the disparity between the results of this study and of those demonstrating increased permeability in other endothelial beds [2, 16] is the direction of presentation of VEGF to the endothelium. We presented VEGF via

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**Fig. 2.** (A) Blood pressure (mean ± SEM) in Sprague-Dawley rats prior to, during, and for 60 minutes after the administration of rVEGF165 (50 µg; N = 5) or 0.9% saline (N = 5). Symbols are: (■) VEGF-administered animals; (▲) saline-administered animals. (B) Urine output (mean ± SEM) in Sprague-Dawley rats prior to and following administration of rVEGF165 (50 µg; N = 5) or 0.9% saline (N = 5). (C) Urinary protein excretion (mean ± SEM) by Sprague-Dawley rats following administration of rVEGF165 (50 µg; N = 5) or 0.9% saline (N = 5).
crete VEGF into the glomerular basement membrane, and in this scenario, glomerular endothelial cells would also naturally encounter VEGF from an abluminal direction. We have previously reported preliminary data suggesting that podocyte VEGF mRNA expression is increased in minimal change disease (abstract; *Nephrol Dial Transplant*, in press), and this is currently under further investigation. Although these studies have clearly shown that VEGF is not the circulating factor previously described in SSNS, a more indirect role for podocyte-derived VEGF in the disease pathogenesis cannot be excluded and warrants further study.

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