Renal vascular endothelial growth factor in neonatal obstructive nephropathy. II. Exogenous VEGF

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Burt LE, Forbes MS, Thornhill BA, Kiley SC, Minor JJ, Chevalier RL. Renal vascular endothelial growth factor in neonatal obstructive nephropathy. II. Exogenous VEGF. Am J Physiol Renal Physiol 292: F168–F174, 2007. First published June 20, 2006; doi:10.1152/ajprenal.00294.2005.—Chronic unilateral ureteral obstruction (UUO) in the neonatal rat causes delayed renal maturation, tubular apoptosis, and interstitial inflammation. Vascular endothelial growth factor (VEGF) acts as a survival factor for tubular cells and reduces renal injury in several models of renal disease. To determine whether exogenous VEGF attenuates renal injury from UUO, rats were subjected within the first 48 h of life to sham operation, partial UUO, or complete UUO. Saline vehicle or VEGF121 (50 mg/kg) was injected twice daily for 7 days, after which kidneys were harvested for histological study. The density of peritubular capillaries was measured with platelet-endothelial cell adhesion molecule-1 immunostaining, proliferating nuclei were detected by proliferating-cell nuclear antigen staining, apoptosis by the transferase-mediated dUTP nick end-labeling technique, macrophages by ED-1 immunostaining, and collagen by Sirius red staining. Glomerular number and maturation index were also determined in each group. Following chronic complete UUO in the neonatal rat, peritubular capillary density was significantly decreased. Cortical capillary density was further reduced by exogenous VEGF in the partially obstructed kidney. While UUO also decreased glomerular number and delayed glomerular maturation, exogenous VEGF exerted no additional effects. Cellular proliferation and tubular apoptosis increased in proportion to the severity of obstruction, but exogenous VEGF had no additional effects on proliferation, tubular apoptosis, or macrophage infiltration. However, VEGF reduced interstitial apoptosis in the kidney with partial UUO. We conclude that VEGF does not have salutary effects on the renal lesions caused by chronic UUO in the neonatal rat and may actually worsen obstructive nephropathy by aggravating the interstitial lesions.

CONGENITAL OBSTRUCTIVE NEPHROPATHY can bring about progressive renal injury and loss of renal function (10). A number of animal models have been utilized to better understand the pathophysiology of congenital obstructive nephropathy, including the neonatal rat, in which nephrogenesis continues during the early postnatal period. Chronic unilateral ureteral obstruction (UUO) in the neonatal rat results in delayed maturation of the kidney, including the vasculature, glomeruli, tubules, and interstitium (12, 14). In addition, chronic UUO leads to tubular apoptosis and tubular atrophy, interstitial inflammation and fibrosis, and decreased nephron number (9, 12). In the kidney, a variety of growth factors have been identified whose expression changes with maturation and is further altered by chronic UUO (5).

Renal vascular endothelial growth factor (VEGF) is produced by both glomeruli and tubules (25), and its expression is decreased following chronic complete UUO in the neonatal rat (2). Following chronic partial UUO, VEGF immunostaining can increase in the contralateral as well as the obstructed kidney, suggesting an adaptive-protective response (2). In addition to promoting vascular growth, VEGF has been shown to stimulate renal tubular proliferation and to inhibit tubular apoptosis (18, 29). We have previously demonstrated that the administration of exogenous epidermal growth factor or insulin-like growth factor-1 can significantly attenuate renal injury resulting from UUO in the neonatal rat (7, 8). These compounds act as survival factors, their administration resulting in decreased tubular apoptosis and interstitial fibrosis.

The administration of VEGF isoforms has been found to have a salutary effect in a number of models of renal injury. Thus the administration of VEGF protects against renal necrosis, accelerates recovery from thrombotic microangiopathy (22, 27), and accelerates glomerular recovery in necrotizing and crescentic glomerulonephritis (26). Inhibition of endogenous VEGF aggravates glomerular injury in rats with mesangioproliferative nephritis, suggesting an important role for this growth factor to be attenuation of vascular damage in this model (24). Moreover, exogenous VEGF also reduces renal tubulointerstitial injury resulting from renal ablation or cyclosporine nephropathy (19, 21). However, inhibition of VEGF attenuates glomerular lesions in models of type 1 or type 2 diabetes (25). Thus VEGF can have detrimental as well as beneficial effects in renal disorders.

We elected to determine whether the administration of exogenous VEGF attenuates the renal injury resulting from UUO in the neonatal rat. In view of its documented actions on the renal microvasculature, we examined the effects of VEGF on peritubular capillaries, glomerular maturation, and glomerular number. Because VEGF has been shown to act as a survival factor for tubular epithelial cells, we also measured its effects on tubular cell proliferation and apoptosis. Since most clinical obstructive nephropathy involves partial rather than complete urinary tract obstruction, rats with partial UUO were also studied.

MATERIALS AND METHODS

Animal model. Under isoflurane and oxygen anesthesia, Sprague-Dawley rats were subjected to sham operation (n = 15), partial UUO (0.3-mm luminal diameter, n = 12), or complete UUO (n = 11) within the first 2 days of life, as described previously (2). Higher-weight splices

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variants of VEGF-A such as VEGF165 have the property of becoming bound to heparin-containing sites such as those of the extracellular matrix and thus may fail to reach the circulation (27). Of the various splice variants of VEGF, VEGF121 is both abundant and readily diffusible. Recombinant human VEGF121 (50 μg/kg body wt, R&D Systems, Minneapolis, MN) or saline vehicle was therefore injected subcutaneously twice daily for the first 7 days after surgery, and the animals were killed 12 h after the final dose. This dose and duration have been demonstrated to protect rats from renal infarction in a model of thrombotic microangiopathy (27). To confirm that the protein was maintained in the circulation, 50 μg/kg body wt VEGF121 was injected subcutaneously in a 14-day-old rat, and serum was collected after 2 h. ELISA analysis demonstrated an increase in circulating VEGF relative to a vehicle-injected control (11,129 vs. 6 pg/ml, R&D Quantiglo human VEGF chemiluminescent sandwich ELISA assay, R&D Systems). The experimental protocol was approved by the Animal Care and Use Committee of the University of Virginia.

Cell proliferation assays. Human umbilical vein endothelial cells (Cambrex BioScience Walkersville, Walkersville, MD) suspended 5,000 cells/ml in assay medium (medium-199 with 1× Earle’s salts and supplemented with 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% vol/vol heat-inactivated fetal bovine serum) were dispensed to collagen type I-coated 96-well cell culture plates, 0.1 ml cell suspension/well. Recombinant VEGF-121 (R&D Systems) was diluted in assay medium and added to quadruplicate wells at final concentrations varying from 0.1 to 100 ng/ml. The assay was incubated at 37°C in a humid 5% CO2 atmosphere for 72 h according to the R&D Systems’ protocol. Cell proliferation was monitored by two nonradioactive methods: 5-bromo-2-deoxyuridine (BrdU) incorporation with chemiluminescence detection (Roche Applied Science ELISA kit, Penzberg, Germany) and ATP quantitation with a CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI). For BrdU incorporation, BrdU was added to wells at 48 h and assay incubation continued to the 72-h end point. BrdU and ATP ELISA assays were performed according to the manufacturers’ instructions, and chemiluminescence was recorded using a Bio-Tek FLX-800i plate reader (Bio-Tek Instruments, Winooski, VT).

Fig. 1. VEGF121-induced endothelial cell proliferation. Human umbilical vein endothelial cells were incubated with increasing concentrations of VEGF121 for 72 h. Cell proliferation was detected by measuring cellular ATP (A) or 5-bromo-2-deoxyuridine (BrdU) incorporation (B). Values are means ± SE for quadruplicate wells. Both experiments were performed twice with similar results.

Fig. 2. Representative photomicrographs of renal microvasculature identified by platelet-endothelial cell adhesion molecule-1 (PECAM-1) immunoreactivity (brown staining) in neonatal rats receiving injections of saline vehicle. A: renal cortex from sham-operated rat. B: renal medulla from sham-operated rat. C: renal cortex from obstructed kidney following partial unilateral ureteral obstruction (UUO). D: renal medulla from obstructed kidney following partial UUO (PUUO). E: renal cortex from obstructed kidney following complete UUO. F: renal medulla from obstructed kidney following complete UUO. Scale bar = 100 μm.
Data are shown as means ± SE relative luminescence units from quadruplicate wells.

Renal histological analysis. Kidneys were weighed and processed for histological analysis. Kidneys were placed in ice-cold saline, decapsulated, blotted dry, and weighed before being placed in 10% buffered formalin fixative for 24 h. Kidneys were then dehydrated through graded alcohols and xylene, embedded in paraffin, and sectioned (serial and nonserial) at 4 μm for immunohistochemical study. Peritubular capillaries were identified using CD-31 [platelet-endothelial cell adhesion molecule-1 (PECAM-1), Santa Cruz Biotechnology, sc-1506, Santa Cruz, CA] at 1:1,000 primary dilution, followed with a biotinylated secondary antibody and ABC-DAB development. Cellular proliferation was detected using the transferase-mediated dUTP nick end-labeling (TUNEL) technique (Apoptag in situ Apoptosis Detection Kit, Chemicon International, Temecula, CA) at 1:400 dilution, with ABC-DAB development. Macrophages were stained with monoclonal ED-1 (CD68: Serologicals, Oxford, UK) at 1:400 primary dilution, following proteinase K antigen retrieval with ABC-DAB development. Apoptosis was detected using the transferase-mediated dUTP nick end-labeling (TUNEL) technique (Apoptag in situ Apoptosis Detection Kit, Chemicon International, Temecula, CA) as previously described (3).

Morphometric measurements. Glomerular numbers were determined using renal sagittal sections stained with periodic-acid Schiff (PAS), and glomerular maturation was assessed on the basis of an index maturation score as described previously (12). Remaining morphometric analysis was done by light microscopy at a total magnification of ×400, examining 10 fields from each kidney or kidney region (i.e., to establish cortical vs. medullary values). For each parameter, the same observer made all measurements, blinded as to treatment group. For measurement of peritubular capillaries, an eyepiece-mounted graticle with 121 intersections was used, and the number of blood vessel profiles overlapping intersections was measured in each field. The results were expressed as vascular volume fraction (VV), which is the percentage of renal volume occupied by blood vessels. These were further subcategorized to extraglomerular fractions in the renal cortex and medulla. A similar counting procedure was used in the same PECAM-stained sections to establish the volume fraction of extraglomerular cortical interstitium. In this way, the vascular component, much of which lies in the spaces between the tubules, already was clearly marked, and thus overestimation of interstitium was avoided. Apoptotic nuclei were counted separately for tubular and interstitial compartments. The PCNA-stained nuclei were counted with a computerized image-analysis program (ImagePro Plus 4.5.1, Media Cybernetics, Silver Spring, MD), and macrophage populations were measured in ED-1-stained sections as a total area percentage using ImagePro software.

Statistical analysis. Data are presented as means ± SE. Comparisons between groups were made with one-way ANOVA followed by the Student-Newman-Keuls test. Comparisons between left and right kidneys were made using Student’s t-test for paired data. Statistical significance was defined as P < 0.05.

RESULTS

As shown in Table 1, there were no differences in body weight or kidney weight between treatment groups. VEGF121

### Table 1. Characteristics of rats

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>VEGF</th>
<th>Partial UUO</th>
<th>VEGF</th>
<th>Complete UUO</th>
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<tbody>
<tr>
<td>No. of rats</td>
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<td>10</td>
<td>6</td>
<td>6</td>
<td>5</td>
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<tr>
<td>Body wt, g</td>
<td>19.0 ± 0.7</td>
<td>19.5 ± 0.6</td>
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<td>18.1 ± 0.6</td>
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<td>Left kidney wt, mg</td>
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<td>91.7 ± 3.1</td>
<td>81.9 ± 1.9</td>
<td>88.5 ± 2.7</td>
<td>96.6 ± 5.2</td>
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<tr>
<td>Right kidney wt, mg</td>
<td>90.5 ± 3.3</td>
<td>92.3 ± 2.9</td>
<td>91.2 ± 2.4</td>
<td>100.0 ± 3.8</td>
<td>103.0 ± 2.7</td>
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Values are means ± SE. VEGF, vascular endothelial growth factor; UUO, unilateral ureteral obstruction.

Fig. 3. Effects of UUO and of exogenous VEGF on renal peritubular capillaries in neonatal rats. A: extraglomerular cortical vasculature, for neonatal rats treated with vehicle (filled bars) or VEGF (open bars). B: medullary vasculature. C: extraglomerular cortical interstitial area measurements in partially and completely obstructed kidneys. Values are means ± SE for each group. CUUO, complete unilateral ureteral obstruction; contra., contralateral; obst., obstructed. *P < 0.05 vs. sham-operated animal. †P < 0.05 vs. vehicle-treated group.
bioactivity was confirmed using two different cell proliferation assays: BrdU incorporation and ATP content. VEGF121-induced cell proliferation was dose dependent in human umbilical vein endothelial cell cultures, as shown in Fig. 1. Both nonradioactive proliferation assays yielded similar results with respect to VEGF121 bioactivity; however, the ATP assay was more consistent among replicates, resulting in greater statistical significance at each dose of VEGF relative to control values. As shown in Figs. 2 and 3, there was a progressive decrease in the relative microvascular area, with increasing severity of ureteral obstruction in both cortex and medulla. In rats receiving exogenous VEGF, cortical peritubular capillaries were further reduced following partial UUO (Fig. 3B). As shown in Figs. 6, A–C, and 7A, renal cellular proliferation increased following 7 days of UUO in the neonatal rat and was related to the severity of obstruction. Exogenous VEGF had no additional effect on cellular proliferation (Fig. 7A). As shown in Figs. 6, D–I, and 7B, renal tubular apoptosis also increased proportionally to the severity of obstruction, without an additional effect of VEGF. However, VEGF reduced renal interstitial apoptosis in rats with partial (but not complete) UUO (Fig. 7C). As noted above (Fig. 3C), interstitial volume fraction values are not significantly different between partially obstructed kidneys treated with vehicle or VEGF. There was no effect of partial or complete UUO or of VEGF on cellular bioactivity was confirmed using two different cell proliferation assays: BrdU incorporation and ATP content. VEGF121-induced cell proliferation was dose dependent in human umbilical vein endothelial cell cultures, as shown in Fig. 1. Both nonradioactive proliferation assays yielded similar results with respect to VEGF121 bioactivity; however, the ATP assay was more consistent among replicates, resulting in greater statistical significance at each dose of VEGF relative to control values. As shown in Figs. 2 and 3, there was a progressive decrease in the relative microvascular area, with increasing severity of ureteral obstruction in both cortex and medulla. In rats receiving exogenous VEGF, cortical peritubular capillaries were further reduced following partial UUO (Fig. 3A).

There was no additional effect of VEGF on the medullary vasculature (Fig. 3B). Furthermore, there was no effect of partial or complete UUO or of VEGF on microvascular area in the contralateral kidney (Fig. 3, A and B). Measurements of interstitial area in obstructed kidneys revealed no significant differences between vehicle- and VEGF-treated animals (Fig. 3C). The medullary vascular population was significantly reduced in the obstructed-side kidneys in animals subjected to complete UUO (Fig. 3B). The decrease in vascular volume fraction in this region compared with sham or partially obstructed kidneys is attributable to medullary fibrosis, in which the remaining medullary space becomes filled with connective tissue which is virtually devoid of any vasculature (Fig. 4).

There was a progressive decrease in the number of glomeruli with increasing severity of ureteral obstruction in neonatal rats (Fig. 5A). However, there was no additional effect of exogenous VEGF administration. Similarly, there was a reduction in glomerular maturation with increasing severity of ureteral obstruction, but there was no additional effect of exogenous VEGF administration (Fig. 5B). There was no effect of partial or complete UUO or of VEGF on glomerular number in the contralateral kidney (Fig. 5A).

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proliferation or apoptosis in the contralateral kidney (Fig. 7). Comparison of macrophage populations in sham, partially obstructed, and completely obstructed kidneys showed no statistically significant effect of VEGF administration (Fig. 7D).

DISCUSSION

The major findings in this study are that chronic UUO in the neonatal rat reduces the density of peritubular capillaries and that exogenous VEGF can aggravate, rather than ameliorate, this vascular loss in the renal cortex. While chronic UUO reduces the number of glomeruli and delays glomerular maturation in the neonatal rat, exogenous VEGF does not exert any additional effects. Similarly, exogenous VEGF has no effect on the increases in renal cellular proliferation, tubular apoptosis, and macrophage infiltration that are induced by chronic UUO. However, the increase in interstitial apoptosis stimulated by chronic partial UO is attenuated by exogenous VEGF.

Fig. 6. Representative photomicrographs of kidneys from neonatal rats injected either with saline vehicle (A–F) or with VEGF (G–L). A–C: proliferating cells identified by proliferating-cell nuclear antigen (PCNA) staining (brown). A: sham-operated rat. B: PUUO. C: CUUO. D–I: apoptotic cells identified by the transferase-mediated dUTP nick end-labeling technique (brown staining). D and G: sham-operated rat (arrows indicate apoptotic nuclei). E and H: PUUO. F and J: CUUO. There is an overall increase in the abundance of proliferating and apoptotic nuclei with increasing severity of obstruction. J–L: macrophage (ED-1) staining in sham, PUUO, and UUO, respectively. Scale bar = 100 μm.
Chronic complete UUO in the adult rat leads to a reduction in peritubular capillary density (23), and similar observations have been made in kidneys from patients with interstitial inflammatory disorders (13). Following renal ablation, there is an early increase in peritubular capillary proliferation, followed by a decrease in tubular VEGF production and a decrease in capillary proliferation to a level below that of sham-operated controls (20). The administration of exogenous VEGF in this model resulted in a threefold reduction in peritubular capillary rarefaction (19). Consistent with a recent review (25), the results of the present study show that the renal vascular effects of exogenous VEGF differ markedly with the nature of renal injury. The differences are not the result of a paucity of VEGF receptors in the developing kidney, since VEGFR2 receptors are more abundant in the neonatal than the adult rat (2). Since the action of VEGF promotes both proliferation and survival of endothelial cells (15), it is likely that the VEGF-induced aggravation of peritubular capillary rarefaction in the present study is a secondary effect. While VEGF has been shown to induce human monocyte migration in vitro (1), renal interstitial macrophage infiltration was not affected by VEGF in the present study.

We have shown previously that chronic UUO reduces the number of glomeruli in neonatal rats, even when obstruction is initiated following the completion of nephrogenesis (12). Recombinant VEGF induces nephrogenesis in rat metanephric culture (28). While exogenous VEGF protects glomeruli from injury in a model of thrombotic microangiopathy (27), glomerular lesions do not develop in neonatal rats subjected to UUO until late in adulthood (11). Although endogenous VEGF expression is increased in glomeruli of neonatal rats subjected to UUO (2), it appears that pharmacological doses of VEGF have no additional effects that act to preserve glomeruli. In this regard, endogenous VEGF appears to play a significant role in preserving damaged glomerular capillaries, but it does not act on normal glomeruli (24). The index of glomerular maturation is based on the number of capillary loops per glomerulus, and the administration of exogenous VEGF in the present study also failed to have any effect on glomerular maturation. Thus, while endogenous VEGF contributes to glomerular hypertrophy in diabetes and the response to unilateral nephrectomy (16, 17), pharmacological doses of VEGF do not accelerate glomerular maturation once nephrogenesis is complete.

As shown in Fig. 6, partial or complete UUO leads to marked tubular dilatation and apoptosis. Since VEGF acts as a survival factor for rat tubular cells exposed to hydrogen peroxide or serum starvation (18, 29), we reasoned that exogenous VEGF might inhibit apoptosis in neonatal UO. However, the results failed to show any effect of VEGF on tubular apoptosis or proliferation in the hydronephrotic rat kidney. It is likely that the stimuli for apoptosis of renal tubular cells attributable to chronic UUO are very different from the extreme stresses of hydrogen peroxide or serum starvation. Thus, in contrast to epidermal growth factor or insulin-like growth factor-1 (7, 8), VEGF is unlikely to be a candidate therapeutic agent to prevent the tubular consequences of obstructive nephropathy.

In the present study, interstitial apoptosis was significantly reduced by VEGF administration to rats with partial UUO. Since the accumulation of renal interstitial cells (macrophages, fibroblasts) following chronic UUO generally contributes to progressive injury, this response may actually aggravate the
lesion. This process may, in fact, contribute indirectly to the greater rarefaction of cortical peritubular capillaries in VEGF-treated animals. In preliminary studies, we have found that administration of enalapril to neonatal rats with partial UUO also worsens the renal interstitial lesions, leading to greater interstitial fibrosis in the obstructed kidney (4). It is possible that as with angiotensin, either too little or too much VEGF can interfere with normal renal maturation and can compound the injury (6). Although apoptosis was not measured in the study, exogenous VEGF was shown by others to reduce tubulointerstitial injury in a model of experimental thrombotic microangiopathy (22). This again highlights the importance of the unique aspects of each experimental model.

In summary, chronic UUO in the neonatal rat leads to a decreased density of peritubular capillaries, which is further aggravated by exogenous VEGF. Chronic UUO also leads to loss of glomeruli and delay in glomerular maturation, but exogenous VEGF exerts no additional effects. Following 7 days of UUO, proliferation and tubular apoptosis were increased in proportion to the severity of obstruction. Although exogenous VEGF had no additional effects on tubular apoptosis, interstitial apoptosis in the kidney with partial UUO was reduced by VEGF administration. We conclude that unlike other growth factors tested previously, VEGF does not have salutary effects on the renal lesions caused by chronic UUO in the neonatal period. Pharmacological administration of VEGF may in fact worsen obstructive nephropathy by aggravating the interstitial lesions.

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REFERENCES


