Renal vascular endothelial growth factor in neonatal obstructive nephropathy. I. Endogenous VEGF

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Burt LE, Forbes MS, Thornhill BA, Kiley SC, Chevalier RL. Renal vascular endothelial growth factor in neonatal obstructive nephropathy. I. Endogenous VEGF. Am J Physiol Renal Physiol 292: F158–F167, 2007. First published June 20, 2006; doi:10.1152/ajprenal.00293.2005.—Obstructive nephropathy constitutes a major cause of renal impairment in children. Chronic unilateral ureteral obstruction (UUO) impairs maturation of the developing kidney and leads to tubular apoptosis and interstitial inflammation. Vascular endothelial growth factor (VEGF) is involved in recovery from various forms of renal injury. We questioned whether the renal expression of endogenous VEGF and its receptor (VEGFR2/Flik-1) is modified by UUO in early development. Neonatal rats were subjected to partial or complete UUO or sham operation. The distribution of immunoreactive VEGF in each kidney was examined after 7, 14, or 28 days. Adult rats were also subjected to sham operation or complete UUO. Tubular VEGF increased between 14 and 28 days in sham-operated rats and in some partially obstructed neonatal rats but decreased with complete UUO. Parallel changes were found by Western blotting, but not by RT-PCR. Immunoreactive VEGF colocalized with mitochondria in proximal and distal tubules and also appeared in type A intercalated cells, glomerular vascular endothelium, and podocytes. While neonatal microvascular renal VEGFR2 receptor staining was strongly positive regardless of UUO, staining was weak in sham-operated adults but increased following UUO. Parallel changes in VEGF expression were verified by RT-PCR and Western blotting. We conclude that endogenous renal VEGF is developmentally regulated in the neonatal rat and is differentially regulated by partial and complete UUO. Following UUO in the adult, the VEGF receptor is upregulated. Endogenous VEGF may serve an adaptive role in responding to tubular injury caused by UUO and may modulate adaptation by the contralateral kidney.

mitochondria; renal maturation; VEGFR2

OBSTRUCTIVE NEPHROPATHY IS a major cause of renal insufficiency in infants and children, and a greater degree of kidney damage results from urinary tract obstruction in children compared with adults. Chronic ureteral obstruction brings about substantial alteration in the developing kidney, including progressive death of tubular epithelial cells and interstitial inflammation that leads to tubular atrophy and interstitial fibrosis (5). A number of growth factors play a role in the renal response to obstruction, including epidermal growth factor, transforming growth factor, hepatocyte growth factor, and platelet-derived growth factor (5).

Since its discovery, vascular endothelial growth factor (VEGF) has been extensively studied. VEGF is known to play multiple roles in normal renal physiology and renal disorders, but nevertheless it remains poorly understood (27). It promotes cellular proliferation, differentiation, and survival and contributes to interstitial matrix remodeling (27). In addition, VEGF may play a role in the enhancement of renal vascular and epithelial growth (15, 33, 34), and it also mitigates glomerular damage caused by inflammation (29). The present study was designed to examine the pattern of immunoreactive VEGF distribution, as well as that of VEGFR2 (Flk-1), its specific receptor. To make this evaluation, both neonatal and adult rats were examined after sham operation or complete unilateral ureteral obstruction (UUO). In addition, a new model of partial UUO was utilized that more closely approximates clinical congenital obstructive nephropathy (32).

MATERIALS AND METHODS

Animal model. Under isoflurane and oxygen anesthesia, newborn Sprague-Dawley rats were subjected to partial UUO (n = 25), complete UUO (n = 24), or sham operation (n = 26) within the first 48 h of life, as described previously (32). Animals were killed at 7, 14, or 28 days after operation. Kidneys were harvested and weighed. Animals were subjected to partial UUO with an 8-0 nylon ligature, using as a guide a wire template of 0.30-mm diameter, whereas complete UUO was created by ureteral ligation (32). Sham-operated pups underwent the same procedure, but without application of the ligature. At the time of death, kidneys and ureters were exposed and stained by heating in alkaline toluidine blue solution, whereas the left pelvic diameter and proximal ureteral diameter were measured. For partial UUO, ureteral patency was confirmed by distal transit of India ink injected into the left renal pelvis. Harvested kidneys were either fixed or snap-frozen. The experimental protocol was approved by the Animal Care and Use Committee of the University of Virginia.

Microscopic examination. For the majority of histological observations, kidneys were removed and fixed by immersion in 10% phosphate-buffered formalin, dehydrated in alcohol, and embedded in paraffin. Sections ranging from 2 to 4 μm in thickness were prepared on a Leica RM 2155 microtome (Leica Microsystems).

Kidneys intended for plastic embedding were fixed by vascular perfusion with the same phosphate-buffered formalin used for immersion fixation, but with a phosphate buffer preswab. Following formaldehyde perfusion, kidneys were removed and immersed in fresh fixative solution, then cut into three coronal segments with a razor blade before additional overnight fixation at 4°C, and washed subsequently in phosphate buffer. The perfused kidneys were cut into 50-μm sections with an automatic vibrating microtome (DSK Microslicer DTK-3000, Ted Pella, Redding, CA). Some of these free-floating sections were immunostained for VEGF localization. The protocol for processing immunostained free-floating sections intended for plastic embedment has been previously described in detail (13).

For detailed microscopic observation, the 50-μm sections were flattened on glass slides and covered with 60°C for 48 h. Areas of interest were cut from the embedment and mounted on plastic stubs, and semithin plastic sections (0.25–1 μm) were cut with glass knives on a Sorvall MT-2B ultramicrotome and stained by heating in alkaline toluidine blue solution. Digital micrographs of areas of interest in both

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paraffin and plastic sections were captured with a Q-Imaging MicroPublisher camera mounted on a Leica DMLS compound brightfield light microscope.

**Immunohistochemistry.** The presence of VEGF was assessed using a monoclonal antibody against VEGF<sub>121</sub> [1:100 dilution, Oncogene VEGF (Ab-5) monocalonal, San Diego, CA] and/or another monoclonal anti-VEGF antibody (1:200 dilution, Clone JH121, Upstate Biotechnology, Lake Placid, NY). As a check on specificity, serial sections of several 14- and 28-day sham as well as 14-day partial UUO kidneys were compared using the Oncogene and Upstate antibodies, as well as the Lab Vision/Neomarkers monoclonal antibody Ab-4 used for Western blotting. Although Lab Vision lists this antibody as “Not suitable” for immunohistochemistry, it resulted in patterns of tubular staining that were fundamentally identical to those obtained with the two antibodies recommended for paraffin sections.

Colocalization of VEGF and H<sup>+</sup>-ATPase in the intercalated cells of the collecting ducts was confirmed using 2-μm serial sections alternately stained with monoclonal VEGF<sub>121</sub> and polyclonal H<sup>+</sup>-ATPase (sc-20943, Santa Cruz Biotechnology, Santa Cruz, CA). Immunohistochemical staining was carried out primarily on paraffin sections, with additional observations made on free-floating tissue sections that were immunostained with monoclonal VEGF<sub>121</sub> and polyclonal H<sup>+</sup>-ATPase (sc-20943, Santa Cruz Biotechnology, Santa Cruz, CA). Immunohistochemical protocols incorporated regimens to quench endogenous peroxidase (hydrogen peroxidase in methanol) and to block endogenous biotin (Avidin/Biotin Blocking Kit, Vector Laboratories, Burlingame, CA), which is particularly concentrated in kidney tissue.

The following antibodies were also used: prohibitin (ab1836, Abcam, Cambridge MA), antigen retrieval in heated citrate buffer, pH 6.0, 1:100 primary antibody dilution, biotinylated secondary antibody developed with Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) and DAB; VEGFR2 (Fli-k) (sc-505, Santa Cruz Biotechnology), antigen retrieval with 0.1% pepsin; 1:1,000 primary antibody dilution, secondary antibody and streptavidin peroxidase treatment with Dako LSAB-2 kit (Dako, Carpinteria, CA); H<sup>+</sup>-ATPase (Santa Cruz sc-20943), no antigen retrieval, 1:800 primary antibody dilution, biotinylated secondary antibody, developed with ABC reagent (see above) followed by VIP reagent (Vector SK-4000); and pimonidazole hydrochloride (Hypoxprobe-1, Chemicon, Temecula, CA), 0.06 mg/g body wt 1 h before death, 1:50 pimonidazole-adduct antibody, followed by 10 min in biotin-SP-conjugated fragment rabbit anti-mouse IgG (Accurate Chemical and Scientific, Westbury, NY) and 10 min in streptavidin peroxidase (Dako), followed by DAB development. Biotinylated lectins were used to identify proximal tubules (*Phaseolus vulgaris*: 1:500 dilution, Vector Laboratories) and collecting ducts (*Dolichos biflorus*: 1:400 dilution, Vector Laboratories). These were applied in the manner of biotinylated antibodies and developed by the ABC-DAAB method.

**Semiquantitative analysis of VEGF and VEGFR2 (Fli-k) mRNA expression.** RNA was extracted from frozen kidney tissue with TRIzol reagent, and 1 μg total RNA was used for first-strand cDNA synthesis. RNA was annealed to 0.5 μg oligo(dT)<sub>15</sub> primer (Promega, Madison, WI) in a volume of 12 μl at 65°C for 5 min, then chilled on ice for 2 min. First-strand cDNA was synthesized by reverse transcription in a 10-μl mixture containing the 12 μRNA annealed to oligo(dT) primer mixture, plus 50 mM Tris·HCl, pH 8.3, 75 mM KCl, 3 mM MgCl<sub>2</sub>, 0.5 mM of each dNTP, 10 mM DTT, 40 U RNaseOUT; and 200 U Moloney murine leukemia virus RT. All reagents for reverse transcription were purchased from Invitrogen (Carlsbad, CA) unless stated otherwise. The reaction mixture was incubated at 37°C for 50 min and was then heated to 70°C for 15 min to inactivate RT.

Amplification of VEGF, VEGFR2 (Fli-k), and GAPDH cDNAs was achieved using 2 μl inactivated RT reaction mixture and 10 μM each gene-specific primer (forward and reverse) in a 50-μl reaction volume containing 200 μM each dNTP, 1.5 mM MgCl<sub>2</sub> and 2.5 U of Taq DNA polymerase in a proprietary reaction buffer, pH 8.5 (PCR Master Mix, Promega). Sequence-specific primers for VEGF span the exons 1–8 of rat VEGF-A to facilitate amplification of the major VEGF splice variants: VEGF<sub>121</sub>, VEGF<sub>165</sub>, and VEGF<sub>189</sub>. The predicted lengths of the PCR products were 360 bp for VEGF<sub>121</sub>, 492 bp for VEGF<sub>165</sub>, and 564 bp for VEGF<sub>189</sub>. The oligonucleotide sequences of each rat VEGF primer were 5′-TGC ACC CAC GAC AGA AGG GGA-3′ (forward) and 5′-TCA CCG CCT TGG CTT GTC ACA T-3′ (reverse) (1). Sequence-specific primers for mouse VEGFR2 (Fli-k) span the NH<sub>2</sub>-terminal half of the conserved intracellular tyrosine kinase domain and kinase insert region of the rat Flk-1 receptor. The predicted length of the PCR product was 537 bp, and the oligonucleotide sequences of each primer were 5′-GCC AAT GAA GGG GAA CTG AAG ACA-3′ (forward) and 5′-CTG GCT GCT GGT TAT GCT GTC-3′ (reverse) (35). GAPDH was amplified as a control housekeeping gene, and its predicted PCR product was 309 bp. GAPDH-specific primers were 5′-TCC CTC AAG ATT GTC AGC AA-3′ (forward) and 5′-AGA TCC ACA ACG GAT ACA TT-3′ (reverse) (31). The PCR conditions consisted of an initial denaturation at 94°C for 1 min followed by 32 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min, with these cycles followed by a final 6-min extension at 72°C. RT-PCR products were separated on 2% agarose gels by electrophoresis and identified with ethidium bromide staining. Gel images were collected with an Alpha Innotech gel imager (Alpha Innotech, San Leandro, CA).

**Kidney homogenates and Western blotting.** Homogenates were prepared on ice with a Polytron homogenizer using 150–200 mg frozen kidney tissue/ml homogenization buffer [20 mM Tris·HCl (pH 7.4) containing 0.25 M sucrose and 5 mM EDTA] including all protease and phosphatase inhibitors previously described for cell lysis buffer (19). Homogenate samples were normalized by protein concentration (100 μg protein/lane) and solubilized with boiling Laemmli buffer. Proteins were separated on 7.5% (for Fli-k) or 12.5% (for VEGF) acrylamide gels by SDS-PAGE, transferred to nitrocellulose, and prepared for immunoblot analysis as previously described (19). Immunoblots were incubated with primary antibodies diluted in 20 mM Tris·HCl, pH 7.4, 0.5 M NaCl, 1% wt/vol BSA and 0.02% wt/vol sodium azide for 2 h at room temperature on a rocking platform: Lab Vision (MS-351) mouse anti-VEGF monoclonal antibody-4 (2 μg/ml); Upstate Biotechnology (clone JH121) mouse anti-VEGF monoclonal antibody (1:1,000); or Santa Cruz (sc-315) rabbit anti-Flk-1 antibody C-20 (1:1,000). Immunoreactive bands were visualized with enhanced chemiluminescence (ECL) reagents according to the manufacturer’s recommendations, and film exposure times ranged from 1 to 30 min, depending on the primary antibody.

**RESULTS**

Immunocytochemical staining for endogenous VEGF in rat kidney showed it to be located primarily in tubular epithelial cells. The intensity and patterns of this distribution varied, however, according to the age of the animals, and the type and duration of surgical ureteral obstruction. In kidneys from sham-operated animals, VEGF staining was weak at both 7 (data not shown) and 14 days of age (Fig. 1A) but became substantially more pronounced at 28 days (Fig. 1B). Following 14 days of partial UUO, VEGF staining often appeared particularly intense in the proximal and distal tubules of both kidneys (Fig. 1, C and E) and intensified into a distinctly granular pattern in the proximal tubules after 28 days (Fig. 1, D and F). In contrast, following 14 or 28 days of complete UUO, VEGF immunostaining was relatively diminished in both kidneys (Figs. 1, G–J).

RT-PCR from VEGF mRNA indicates changes in VEGF expression between day 14 and day 28 sham kidneys, or day 14 sham and partial UUO kidneys shown in Fig. 1 are not
transcriptionally regulated (Fig. 2A). No differences in VEGF or GAPDH mRNA levels are observed for any condition examined. In contrast, immunoblots of whole kidney homogenates stained with anti-VEGF antibodies support most of the immunohistochemical observations shown in Fig. 1. Figure 2B shows 19-to 20-kDa VEGF protein expression on duplicate blots stained with two different anti-VEGF antibodies: a Lab Vision antibody recommended for Western blotting, but not immunohistochemistry; and an Upstate Biotechnology antibody recommended for immunohistochemistry, but not Western blotting. Both antibodies recognize the change in VEGF expression between day 14 and day 28 sham kidneys, as well as the loss of VEGF expression in completely obstructed kidneys at either time point. Differences between day 14 sham and partial UUO kidneys were not apparent among kidneys from the individual animals shown; however, VEGF immunohistochemical staining varied from weak to intense (intense staining shown in Figs. 1, C and E) among different animals of this age subjected to partial UUO.

Compared with tubules, VEGF immunostaining of glomeruli was minimal in paraffin sections. In partial UUO, however, through the use of plastic sections treated by preembedding...
immunocytochemistry, immunoreactive VEGF could be detected in podocytes and capillary endothelium; this was the case in both contralateral and obstructed kidneys (Fig. 3, A and B). In paraffin sections of 28-day partial UUO kidneys, VEGF was not detectable in glomeruli from contralateral kidneys (Figs. 1D and 3C) but was prominent in the glomerular capillaries of the partially obstructed kidney (Figs. 1F and 3D).

The specificity of the immunohistochemical staining in these preparations was determined by comparing serial sections to which primary antibody was applied (Fig. 4A) or omitted (Fig. 4B); omission of the primary antibody resulted in a loss of specific staining. We also compared three different monoclonal anti-VEGF antibodies and obtained similar immunohistochemical staining patterns with all three (data not shown). To examine the association of VEGF localization with tubular cell organelles, serial consecutive sections were correlated for VEGF staining (Fig. 4C) and immunolocalization of prohibitin, a mitochondrion-specific protein (Fig. 4D). A distinct correlation was seen between the distributions of VEGF and prohibitin in both proximal and distal tubules; in collecting ducts, however, this correlation was lost (Fig. 4E and F). Prohibitin also stained cell structures in regions not usually positive for VEGF, e.g., the various cell types within glomeruli (Fig. 4D), as well as vascular smooth muscle and endothelial cells of larger vessels.

As pointed out above, VEGF staining can be especially intense in both contralateral and obstructed kidneys of rats subjected to 14 days of partial UUO. The intracellular pattern in tubules, furthermore, is segment specific: coarse in the cytoplasm of proximal tubule cells, finer in distal tubules, and

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**Fig. 2.** VEGF mRNA and protein expression in the developing rat kidney: variation with age and surgical treatment. A: messenger RNA for 3 splice variants of VEGF and GAPDH was uniformly expressed in all kidneys analyzed. Left (L) and right (R) kidneys of sham-operated animals at 14 and 28 days of age as well as obstructed (Ob) and contralateral (CL) kidneys from animals subjected to PUUO for 14 days are shown. B: VEGF protein expression detected by immunoblot changes with age of developing kidney and surgical manipulation. Shown are kidneys from sham (Sh), PUUO, and complete UUO. Duplicate kidneys for each condition were analyzed, and representative samples are shown from individual gels or blots. VEGF (LV), stained with Lab Vision anti-VEGF antibody; VEGF (UBI), stained with Upstate Biotechnology anti-VEGF antibody.

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**Fig. 3.** Glomerular VEGF immunostaining. A: in a semithin plastic section of a kidney contralateral to 14 days PUUO, both podocytes (P) and blood vessels (BV) are positive for VEGF. B: similar staining in plastic sections was seen in glomeruli of the obstructed kidney; a high-magnification detail shows typical podocyte structure in VEGF-positive cells. Scale bar = 10 μm. C: tubular, but not glomerular, VEGF staining is present in paraffin sections of the kidney contralateral to 28 days of PUUO. D: glomerular VEGF staining is prominent in the obstructed kidney following 28 days of PUUO. As shown by the immunostaining surrounding an erythrocyte (arrow), VEGF is present in glomerular capillaries. Scale bar = 10 μm and applies also to A and C.
concentrated within the apices of certain cells of collecting duct walls (Fig. 5A). A similarly intense and segment-associated pattern is present in sham-operated animals at 28 days of age (Fig. 5B); in some tubules there appear scattered larger particles that also stain positively for VEGF (Fig. 5B, inset). In 28-day animals subjected to partial UUO, this pattern is more pronounced in selected tubules (Fig. 5C and D) but not in the majority (Fig. 5E and F).

A distinctly focal pattern of VEGF staining appeared in both cortical and medullary collecting ducts, identified on the basis of their lectin specificity (Fig. 6A and B). In contrast to proximal tubules, collecting ducts exhibited little evidence of hypoxia as indicated by pimonidazole staining (Fig. 6C). Based on their domelike shapes and the presence of vacuolar ATPase, it was determined that the cells containing immunoreactive VEGF within their apical cytoplasm were type A intercalated cells (Fig. 6D and E). Immunoreactive VEGF was also present in semithin plastic sections (Fig. 6F); comparison in serial consecutive sections of VEGF and prohibitin staining, however, revealed that VEGF immunoreactivity was restricted to the cytoplasm immediately beneath the apical membranes of intercalated cells, whereas mitochondrion-specific staining was found throughout the cells’ cytoplasm (Fig. 6G and H).

Immunostaining for VEGFR2/Flk-1 was intense in neonatal kidneys and was localized in the vasculature, including glomerular capillaries. This staining pattern was similar in both sham-operated (Fig. 7, top left) and 7-day obstructed neonatal kidneys (Fig. 7, bottom left). In contrast, vascular VEGFR2 immunostaining was minimal in kidneys of sham-operated adult animals (Fig. 7, top right). Following obstruction of the adult kidney, however, VEGFR2 staining became more intense, resembling the pattern seen in the neonate (Fig. 7, bottom right). Semiquantitative RT-PCR and immunoblots support the immunohistochemical observations shown in Fig. 7 and indicate that the changes in VEGFR2/Flk-1 expression are transcriptionally regulated (Fig. 8). VEGFR2/Flk-1 mRNA (Fig. 8A) and protein (Fig. 8B) expression is the same in 7-day sham or obstructed neonatal rat kidneys. VEGFR2/Flk-1 is not expressed in adult rat kidneys (sham), but expression was induced by 7 days of complete obstruction.
DISCUSSION

In the developing rat, immunoreactive renal VEGF is not detected by embryonic day 14 but appears in late fetal life (33). In rat metanephric organ culture, exogenous VEGF induces proliferation of tubular epithelial cells (33). In neonatal mice treated with blocking antibody to endogenous VEGF, nephrogenesis is impaired, with formation of glomeruli lacking capillary tufts (21), while homozygous deletion of VEGF-A in mouse glomeruli results in perinatal lethality (10). These observations underscore the importance of endogenous VEGF in both glomerular and tubular development. The major findings in this study are that VEGF expression increased between 14 and 28 days of age, a period in which nephrogenesis has been completed and nephrons are continuing to mature. The intracellular distribution of staining is similar to that of mitochondria and lysosomes. VEGF expression in kidneys of animals subjected to chronic partial UUO for 14 days varied from faint to intense staining in both the contralateral and obstructed

Fig. 5. Age-related shifts in VEGF staining pattern. A: immunoreactive VEGF in cortex of obstructed kidney following 14 days of PUUO, demonstrating segment-specific tubular distribution: coarse in proximal tubules (PT), finer in distal tubules (DT), and concentrated in the apices of collecting-duct intercalated cells (arrows). Scale bar = 25 μm. B: 28-eight day sham-operated animal. The VEGF immunostaining pattern is virtually identical to that of the 14-day partially obstructed animal, with the exception that larger positive-staining bodies (inset, arrows) are beginning to appear among the smaller positive mitochondria. Scale bars = 50 and 20 μm in panel and inset, respectively. C: partially obstructed kidney at 28 days. VEGF is primarily limited to large coarse bodies. D: semithin section of 28-day partially obstructed kidney treated with preembedding immunocytochemical staining for VEGF, which is limited to profiles resembling lysosomes. Scale bar = 20 μm. E and F: serial consecutive sections of 28-day partially obstructed kidney stained for VEGF and prohibitin, respectively. VEGF staining is now most evident only in isolated tubules (*), whereas mitochondrion-specific immunostaining is consistently present throughout the field. Scale bar = 50 μm.
kidneys, but the intracellular pattern was constant. Chronic complete UUO for 14 or 28 days decreased VEGF staining in both kidneys relative to sham-control kidneys.

In a previous report on adult rats subjected to complete UUO, tubular VEGF immunostaining was found to increase following 1 wk of obstruction (redistributed to the basolateral aspect of tubular cells) but then proceeded to decrease markedly over the following 2 wk (23). Redistribution of VEGF to the basolateral surface of tubular cells has also been reported following ischemia-reperfusion injury (16). This phenomenon is reversed following 40–80 min of reperfusion and could be duplicated by subjecting cultured tubular epithelial cells to temporary hypoxia (16). Chronic partial UUO results in reduced renal blood flow to the obstructed kidney, but increased flow to the contralateral kidney (4). In the present study, however, VEGF was located in punctate bodies throughout the

Fig. 6. Collecting duct VEGF immunostaining. A–C: consecutive serial sections of renal cortex from kidney contralateral to 14-day PUUO. A: VEGF staining is localized in scattered CD cells. B: Dolichos biflorus lectin affinity is specifically associated with these tubules, confirming their identity as CD. C: pimonidazole (Hypoxprobe-1) staining identifies hypoxic adducts in nearby PT, but there is a lack of any staining that would denote hypoxic adducts in the collecting ducts. Scale bar = 50 μm. D and E: consecutive 2-μm serial sections of CD, showing correlation between VEGF-staining localization (D, arrows) in the cell apices of epithelial cells and the H+–ATPase localization in the same cells (E, arrows), which identifies them as being type A intercalated cells. Scale bar = 50 μm. F: semithin plastic section of kidney from obstructed kidney following 14 days of PUUO. Immunoreactive VEGF appears within the apex of a type A intercalated cell. Scale bar = 10 μm. G and H: serial consecutive 2-μm sections stained for VEGF and prohibitin, respectively. Type A cells are positive in their apical cytoplasm for VEGF (arrows); however, in these CD, mitochondrion-specific staining is present throughout the cytoplasm of both type A cells and principal cells, indicating that VEGF is not located in the mitochondria of CD. Scale bar = 10 μm.
cytoplasm of tubular epithelial cells in both obstructed and contralateral kidneys, suggesting that hypoxia is not driving renal VEGF distribution following neonatal UUO. Moreover, in 14-day-old rats with partial UUO, intense VEGF immunostaining appeared in type A intercalated cells of the collecting ducts, whereas uptake of the hypoxia marker pimonidazole was negative for these cells. In the adult rat, chronic hypoxia does not alter VEGF expression (28). The lack of change in VEGF mRNA demonstrated by RT-PCR indicates that regulation of renal VEGF expression is not occurring at the transcriptional level.

In human studies, expression of VEGF mRNA and protein has been localized within glomerular epithelia and collecting duct cells (14, 30). While podocytes are the renal cells that normally express VEGF in the human adult, patients with tubulointerstitial injury exhibit decreased expression of VEGF in sclerotic glomeruli and increased tubular VEGF expression (8). Podocyte VEGF immunostaining is variable, however, and may depend in part on tissue processing (16, 24). This is also the case in the present study, in which podocyte staining is detectable in plastic sections, but less apparent in paraffin sections. However, 28 days of partial UUO in the neonatal rat resulted in increased VEGF immunostaining of glomerular capillaries. Localization of VEGF to glomerular capillaries has been reported previously in the developing rat (33). Persistent localization of VEGF to glomerular capillaries in the neonatal rat with chronic UUO may reflect delayed glomerular maturation, which is an established consequence of neonatal UUO (6).

In the present study, changes in VEGF immunostaining resulting from UUO developed in both obstructed and contralateral kidneys. Following chronic UUO, the contralateral kidney undergoes finely regulated compensatory growth that is dependent on the duration and severity of obstruction (7, 36). While increased VEGF expression may be an adaptive response that counters renal tubular and interstitial injury in the obstructed kidney (15, 18), the response of the contralateral kidney may be necessary for compensatory nephron growth (12). Partial UUO in the neonatal guinea pig increases the renin content of both obstructed and contralateral kidneys (3), while complete UUO in the neonatal rat increases renin content of the obstructed kidney and decreases that of the contralateral kidney (9). Following 12 days of complete UUO in the neonatal mouse, cellular proliferation is increased and glomerular maturation is delayed in the contralateral kidney (although not as severely as that of the obstructed kidney) (2). In a recent report, kielin/chordin-like protein (an endogenous enhancer of renal
bone morphogenetic protein signaling, which attenuates renal fibrosis through Smad1) was found to be activated in both kidneys of mice subjected to UUO (22). Clearly, there are multiple factors that distinguish the kidney contralateral to UUO from that in a normal (sham-operated) animal: these differences are related to the alterations in VEGF immunostaining of the contralateral kidney.

To our knowledge, the present study is the first to identify VEGF within the type A intercalated cells of the collecting duct. A previous study of the effects of renal ablation on the peritubular microcirculation concluded that VEGF immunostaining is specific to isolated dome-shaped cells in distal tubules: it is likely that these actually represent intercalated cells in collecting ducts (25). In the present study, VEGF staining appeared localized to the mitochondria in proximal and distal tubules. Proximal tubule mitochondria are typically large and elongated, whereas distal tubular mitochondria are smaller and more rounded. When VEGF staining patterns in these tubule segments are compared with mitochondrion-specific ones, there is good agreement. Consistent with these observations, fibrotic lesions in bleomycin-treated rat lungs were found to contain type II pneumocytes and Clara cells that express VEGF, which after labeling with immunogold, was identified by electron microscopy as existing within the mitochondria (11). However, it should be noted that VEGF staining in the apical cytoplasm of intercalated cells of kidney collecting ducts does not correspond to the mitochondrial distribution in these cells.

In addition to the association of VEGF with mitochondria in the proximal and distal tubules, we observed the appearance of intensely opacified bodies in these tubules in obstructed neonates (28 days), as well as in sham-operated and obstructed adults. This is consistent with an intracellular process that depends on lysosomal degradation of aged organelles. Proximal tubules have a well-developed lysosomal system, by which spent mitochondria are sequestered in so-called secondary lysosomes, thereby creating “secondary” lysosomes that eventually become lipofuscin deposits (20). This may explain in part the increased VEGF tubular immunostaining following UUO. Over 28 days of partial UUO in the neonatal rat, the tubular epithelium may prematurely develop activation of subcellular machinery such as the lysosomal system, which in unobstructed animals would not occur until adulthood.

Immunoreactive VEGFR2 receptors become localized to glomerular and peritubular capillaries in fetal development, and receptor mRNA expression normally decreases with maturation from the neonate to the adult (33). In the present study, chronic UUO in the neonatal rat had no demonstrable effect on the distribution or intensity of VEGFR2 staining. While immunoreactive VEGFR2 was minimally expressed in the sham-operated adult rat, renal vascular expression increased following UUO. Changes in renal VEGFR2 mRNA and protein paralleled those for VEGFR2/Flk-1 localization by immunohistochemistry. A transient increase in VEGFR2 peritubular capillary immunostaining was reported previously in adult rats subjected to complete UUO (23). Others have demonstrated VEGFR2 staining in renal tubular cells in the adult rat, particularly those of distal tubules and collecting ducts (15). The same investigators reported increased microvascular VEGFR2 receptor immunostaining in rats subjected to ischemia-reperfusion (17). It therefore appears that UUO or ischemic renal injury in the adult induces reversion of the vascular phenotype, with increased expression of VEGFR2, while there is no additional increase in receptor expression following neonatal UUO. This is consistent with other effects of renal injury that induce the recapitulation of earlier developmental programs (26).

In summary, chronic UUO in the neonatal rat leads to alterations in the intrarenal distribution of immunoreactive VEGF. Following prolonged partial UUO, there was an increase in VEGF staining of glomeruli as well as of the tubules in some kidneys. The tubular localization of immunoreactive VEGF corresponded to that of mitochondria in proximal and distal tubules, suggesting a relationship between VEGF localization and metabolically active intracellular regions. VEGF was also localized in the apical cytoplasm of type A intercalated cells but was not present in principal cells of the collecting duct. Of note, increased tubular (but not glomerular) VEGF staining in the neonate also developed in the kidney contralateral to partial UUO. This is consistent with an adaptive response, the function of which is possibly to contribute to cell survival in the obstructed kidney, and to adaptive nephron growth of the contralateral kidney. Stimulation of VEGF protein accumulation is not transcriptionally regulated and is not driven by chronic local hypoxia. Complete UUO, in contrast, leads to suppression of VEGF in both kidneys. In contrast to the ligand, the distribution and intensity of renal VEGFR2 in neonatal rats was not altered by UUO, while obstruction in the adult animal caused reversion of the faint, mature VEGFR2 staining back to the intense pattern characteristic of the neonate. This is consistent with an adaptive response to injury. We speculate that endogenous renal VEGF may serve to counter the tubular injury resulting from chronic ipsilateral UUO and to stimulate adaptation by nephrons of the contralateral kidney.

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