Coexpression of neuropilin-1, Flk1, and VEGF₁₆₄ in developing and mature mouse kidney glomeruli

BARRY ROBERT,¹ XUEMEI ZHAO,² AND DALE R. ABRAHAMSON¹
Departments of ¹Anatomy and Cell Biology and ²Molecular and Integrative Physiology,
University of Kansas Medical Center, Kansas City, Kansas 66160-7400
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Robert, Barry, Xuemei Zhao, and Dale R. Abrahamson. Coexpression of neuropilin-1, Flk1, and VEGF₁₆₄ in developing and mature mouse kidney glomeruli. Am J Physiol Renal Physiol 279: F275–F282, 2000.—Neuropilin-1, a neuronal cell surface semaphorin III receptor protein important for axonal guidance in developing peripheral nervous system efferents, has also been identified as a vascular endothelial growth factor (VEGF) receptor on endothelial cells. To evaluate its expression in kidney, we carried out RT-PCR on newborn and adult total renal RNAs. A 403-bp product, which was predicted to be that from neuropilin-1 mRNA, was found in both samples. Nucleotide sequencing confirmed that these products encoded neuropilin-1. Northern analysis of newborn and adult kidney RNA showed specific hybridization to appropriately sized bands of ~6 kb. In situ hybridization with a mouse-specific antisense neuropilin-1 ³⁵S-cRNA probe showed distinct glomerular localization on sections from both newborns and adults. Similar patterns of hybridization were seen in sections treated with antisense cRNA probes against another VEGF receptor, Flk1, and with VEGF probes. However, the VEGF hybridization signal was markedly less in adult glomeruli than those for neuropilin-1 and Flk1. Because neuropilin-1 specifically binds VEGF₁₆₄ in humans, we carried out RT-PCR on mouse kidney RNA with primers that amplified the three alternatively spliced isoforms of VEGF mRNA. Our analysis showed that for both newborn and adult kidneys, the relative abundance of VEGF mRNA was VEGF₁₆₄ > VEGF₁₂₆ > VEGF₁₈₈. We conclude that the expression of neuropilin-1, in conjunction with Flk1 and VEGF₁₆₄, jointly contributes to the development and maintenance of glomerular capillaries.

Embryonic blood vessels develop through a number of processes, including cell migration, aggregation, differentiation, and assembly, into recognizable vascular structures (2). The molecular regulation of these events includes activation of certain transcription factors, cell-cell and cell-matrix adherence, and interactions between growth factors and their receptors. Vascular endothelial growth factor (VEGF) and two of its receptor tyrosine kinases, termed Flk1 and Flt1 in the mouse, are crucial during the earliest phases of embryonic blood vessel development (26). Homozygous Flk1 (32)- and Flt1 (15)-null mutants die between embryonic (E) day 8.5 and 9.5 due to abnormal vascular development. In Flk1-null mutants, there is a complete failure of endothelial and hematopoietic cell differentiation. Although some limited endothelial cell development occurs in Flt1 mutants, these animals are unable to assemble organized vascular networks required for sustaining embryogenesis. Remarkably, both homozygous and heterozygous null VEGF mutants die between E8.5 and 9.5 and between E11 and 12, respectively, because of vascular aberrations (4, 14). This signifies not only that VEGF is critical for blood vessel formation but also that expression from just one allele is insufficient for vascular development.

Recently, neuropilin-1 has been shown to be a third receptor for VEGF (34 and reviewed in 16). This molecule was originally described as a neuronal transmembrane receptor that, on binding the ligand semaphorin III, evokes neurite repulsion and growth cone collapse (17, 25). Both neuropilin-1 and a related molecule (neuropilin-2) contain three unique motifs in their extracellular regions: two domains bearing homologies to the complement components C1r and C1s and bone morphogenetic protein 1 (BMP-1); two domains that resemble coagulation factors V and VIII; and a single MAM domain (typical of the protein tyrosine phosphatase μ and endopeptidase meprin) (16, 26). Neuropilin-1 and -2 have relatively short intracellular segments that lack cytoplasmic signal transduction domains, which indicates that they probably do not function as independent receptors (16, 26). Nevertheless, in mice with a targeted disruption of the neuropilin-1 gene, the normal pattern of peripheral nervous system efferent fiber growth is imprecise, and fibers of major peripheral nerves arborize abnormally and sometimes overshoot their intended targets (23). Similar to neuropilin-1 mutants, semaphorin III-null mutants also exhibit abnormalities in peripheral nerve projections (36).

The first evidence that neuropilin-1 might play a role in blood vessel development emerged when it was constitutively expressed in transgenic mice (24). Mice overexpressing neuropil-1 exhibit several cardiovas-
cular abnormalities, including hypervascularization, dilated blood vessels, and heart malformations (24). Additionally, neuropilin-1-null mutants die in utero between E10.5 and 12.5 (23) and exhibit a wide variety of cardiovascular defects, including deficiencies in yolk sac vessels, deranged neural vascularization, agenesis and transposition of the great vessels, and persistent truncus arteriosus (21). Furthermore, when neuropilin-1 is coexpressed in cells with KDR (human homolog of murine Flk1), neuropilin-1 behaves as a VEGF co-receptor with KDR, and binds specifically to the human VEGF<sub>165</sub> isoform (34).

At least three VEGF isoforms are synthesized in the mouse by alternative splicing: VEGF<sub>120</sub>, VEGF<sub>164</sub>, and VEGF<sub>188</sub> (3, 26). Whether these different isoforms play distinct roles is not entirely clear, but VEGF<sub>120</sub> lacks the heparin-binding domains encoded within exons 6 and 7, does not bind to matrix- or cell-associated heparan sulfate proteoglycans, and hence is more soluble in tissue than the larger VEGF<sub>164</sub> and VEGF<sub>188</sub> variants (26). Additionally, some experiments suggest that the VEGF<sub>164</sub> and VEGF<sub>120</sub> isoforms elicit the most angiogenic responses (26). On the other hand, mice expressing only VEGF<sub>120</sub> have impaired postnatal cardiac angiogenesis and die of cardiac failure before postnatal day 14 (5), which indicates that VEGF<sub>120</sub> by itself cannot supplant the functions of the other isoforms. Animals deficient in VEGF<sub>164</sub> and VEGF<sub>188</sub> also display postnatal vascular defects in other sites, including glomeruli, but these lesions have not yet been described in detail (5).

Kidney development in the mouse begins at E10 as the ureteric bud impinges on a cord of metanephric blastemal mesenchyme (31). Nephrogenesis results from reciprocal signals between epithelial cells of the ureteric bud and mesenchymal cells of the blastema (19, 27). Concurrently, development of renal vasculature begins at ~E11 when cells expressing Flk1 are first observed within the metanephric blastema alongside the ureteric bud (28). Thereafter, as each emerging glomerulus reaches the comma-shaped stage, endothelial precursors invade its vascular cleft and the glomerular capillary tuft begins to form (1). Although the general morphological features of renal vascular development have been described, molecular mechanisms directing this process are still poorly defined. In this study we examined developing and mature mouse kidneys for expression of neuropilin-1. Using in situ hybridization we compared the distribution patterns of neuropilin-1, Flk1, and VEGF in newborn and adult kidneys. Because neuropilin-1 is an isoform-specific VEGF receptor, we also evaluated the relative expressions of the three VEGF isoforms in immature and mature mouse kidney.

MATERIALS AND METHODS

Animals. All mice used in these experiments were taken from colonies of outbred CD-1 mice founded in stocks obtained from Charles River (Wilmington, MA).

Total RNA isolation. Kidneys from newborn and adult mice were collected and immediately frozen in liquid nitrogen. Tissues were then transferred to glass homogenizers on ice, and 1 ml Trizol Reagent ( Gibco-BRL, Gaithersburg, MD) was added per 75 mg tissue. Kidneys were homogenized quickly on ice followed by a 5-min incubation at room temperature, and homogenates were centrifuged at 12,000 g for 10 min at 4°C. Supernatants were transferred to fresh tubes and extracted with 200 µl of chloroform/ml Trizol. After centrifugation at 14,000 g for 10 min at 4°C, the upper aqueous phase, containing total RNA, was pipetted to a new tube, and RNA was precipitated by adding 0.5 ml isopropanol/ml Trizol. The precipitated RNA was collected by centrifugation at 12,000 g at 4°C for 10 min. The RNA pellet was washed with 75% ethanol, centrifuged for 5 min at 7,500 g at 4°C, and briefly air-dried. Pellets were redissolved in diethylpyrocarbonate-treated water, and total RNA was quantified spectrophotometrically by reading at A<sub>260</sub>.

RT-PCR. RT-PCR was carried out on total RNA by using the Access RT-PCR System (Promega, Madison, WI) following the manufacturer’s protocol. To detect the three major mouse VEGF isoform transcripts (VEGF<sub>120</sub>, VEGF<sub>164</sub>, and VEGF<sub>188</sub>), the following mouse-specific primers were designed: sense primer, 5’-ATAGCGGATGGAAAACCCTGC-3’ (nucleotides 334–353, GenBank accession no. M95200) and antisense primer, 5’-TATAGGCCTACTTGGGGAGC-3’ (nucleotides 639–658). For detection of neuropilin-1 transcripts the following sense primer, 5’-GGCTGGCCTGTTGTCGCGCCA-3’ (nucleotides 358–378) and antisense sequence, 5’-ATAGCGGATGGAAAACCCTGC-3’ (nucleotides 741–761, GenBank accession no. D50086) were prepared. For RT-PCR reactions, 1 µg total RNA/reaction was used. Reverse transcription was carried out at 48°C for 45 min followed by an avian myeloblastosis virus-RT/denaturation step for 2 min at 94°C. PCR amplification was carried out over 40 cycles of incubations at 94°C for 30 s, 60°C for 1 min, and 68°C for 2 min with a final extension at 68°C for 7 min. The RT-PCR products were then resolved by agarose gel electrophoresis. Ethidium bromide-stained gels were then analyzed on a GlycoDoc Gel Imaging System by using Analytical Software version 2.47 (Bio-Rad Laboratories, Hercules, CA).

Hybridization probes. To detect neuropilin-1, a cDNA corresponding to bases 358–761 (20) was obtained by RT-PCR and subcloned into the pCRII-TOPO vector (Invitrogen, Carlsbad, CA). For Flk1, a partial cDNA clone encoding nucleotides 3333–4310 was subcloned into a pSPT2 vector (6). For VEGF, a cDNA fragment consisting of 117 bp of exon 7 (bases 506–622) (7), which is specific for mouse VEGF<sub>164</sub> and VEGF<sub>188</sub>, was derived by RT-PCR and subcloned into a pCRII-TOPO vector. Antisense <sup>32</sup>P-labeled cRNA probes were synthesized by using appropriate RNA polymerases and used for Northern blot hybridizations. To provide in situ hybridization probes, <sup>35</sup>S-labeled sense and antisense cRNAs were prepared by using SP6, T7, or T3 polymerases. All probes used for in situ hybridizations had a specific activity of 0.2 × 10<sup>6</sup> disintegrations/min (dpm)/µg.

Northern blot hybridization. Equal quantities (12 µg) of total RNA from newborn and adult kidneys were electrophoresed in a formaldehyde agarose gel and passively transferred to Hybond-N+ nylon membrane (Amersham, Arlington Heights, IL) in 20 × SSC overnight. The membrane was then ultraviolet crosslinked (UV Stratalinker 1800, Stratagene, La Jolla, CA) and prehybridized, hybridized, and washed as previously described (10). Briefly, hybridization took place at 68°C for 2 h in 3 × SET (1 × SET: 150 mM NaCl, 5 mM EDTA, and 10 mM Tris - HCl, pH 8.0), 20 mM phosphate buffer (pH 7.2) containing 250 µg denatured tRNA/ml, and 10% dextran sulfate. Blots were hybridized...
incubated with 35S-labeled antisense cRNA probes for neuropilin-1, Flk1, or VEGF 164/188 in 50% formamide buffer at 45°C for 4 h. After hybridization and washing, sections were then treated with RNase A (20 μg/ml) at 37°C for 20 min, and RNAse A-resistant hybrids were detected by autoradiography by using Kodak NTB-2 liquid emulsion. Sections were then counterstained with hematoxylin and eosin. Sections similarly hybridized with 35S-labeled sense probes served as negative controls.

RESULTS

Neuropilin-1 expression in mouse kidney. To screen for possible renal expression of neuropilin-1, we first performed RT-PCR on total RNA isolated from newborn and adult mouse kidneys. Oligonucleotide primers were designed based on the published cDNA sequence of neuropilin-1 (20), which amplified a 403-bp segment of neuropilin-1 mRNA corresponding to nucleotides 358–761. An appropriately sized PCR product was generated from both newborn and adult kidney total RNA (Fig. 1). Nucleotide sequencing of PCR products from both samples showed that they were 99 and 98% identical, respectively, to the previously reported sequence of murine neuropilin-1 (GenBank accession no. D50086). To further analyze neuropilin-1 expression during kidney development, we utilized our RT-PCR-generated neuropilin-1 probe for Northern blot analysis. A transcript of ~6 kb was observed in total RNA harvested from newborn and adult mouse kidneys (Fig. 2), which is in close agreement with that observed previously in human tissues (34).

Although RT-PCR and Northern blot hybridization clearly indicated the expression of neuropilin-1 mRNA in kidney, these experiments provided no information regarding the cellular localization of the receptor. We therefore carried out in situ hybridization experiments. In the newborn mouse kidney, neuropilin-1 transcripts were most abundant in glomeruli (Fig. 3). A more diffuse hybridization signal was also observed in the outer nephrogenic zone of the newborn kidneys (Fig. 3), a site that continues to undergo nephrogenesis for ~7 days after birth. This region contains undifferentiated mesenchyme, ureteric bud tips, early nephric figures, and nascent capillaries. In adults, neuropilin-1 was also expressed chiefly in glomeruli, but the level of expression was greatly reduced from what was seen in newborns (Fig. 3). Only background levels of signal were observed over tubular epithelial cells. In sections incubated with sense neuropilin-1 probes as controls, no positive signals were detected.

In situ hybridization for Flk1 and VEGF. As indicated earlier, neuropilin-1 has recently been shown to be a receptor specific for the VEGF165 isoform. Additionally, cells expressing both Flk1 and neuropilin-1 show enhanced VEGF165 binding and VEGF165-mediated chemotaxis compared with cells expressing only Flk1 (34). Because mRNAs and proteins for both Flk1 (12, 18, 33) and VEGF (3, 12, 18, 33) have been reported in developing and mature kidneys, we sought to compare the pattern of Flk1 and VEGF mRNA expression using the same techniques we had used to examine neuropilin-1 synthesis.

Hybridization of the Flk1 probe in newborn kidney tissue was most evident over glomeruli and in the subcapsular nephrogenic zone (Fig. 4). In the adult, Flk1 expression was predominately seen in glomeruli (Fig. 4) and, as has been observed before in Flk1/lacZ heterozygous mice (28), Flk1 expression was not evident in arterioles and arteries or in tubular epithelial cells (Fig. 4). The glomerular expression patterns for Flk1 mRNA in both newborns and adults were therefore very similar to that seen for neuropilin-1 (cf. Figs. 3 and 4) and are strongly suggestive of glomerular capillary endothelial localization.

The strongest hybridization signals for VEGF in newborn kidney were evident in glomeruli and medullary collecting duct epithelium (Fig. 5). Additionally,
the hybridization over early capillary loop-stage glomeruli of newborns showed intense labeling in ringlike patterns, consistent with high levels of expression by developing podocytes (Fig. 5). In adult kidney, VEGF continued to be expressed, but the hybridization patterns were weaker over glomeruli and more diffuse in the remainder of the adult cortex (Fig. 5). In sections incubated with sense Flk1 or VEGF probes, no specific hybridization was detected.

Expression of VEGF isoforms. Three isoforms of VEGF have been identified in the mouse: VEGF$_{120}$, VEGF$_{164}$, and VEGF$_{188}$ (3). Because neuropilin-1 has been found to be a receptor specific for VEGF$_{165}$, we were interested in examining the relative expression levels of the different VEGF isoforms in newborn and adult kidney. Using RT-PCR and a single set of primers designed to amplify all three of the major VEGF isoforms, we detected VEGF$_{120}$ (a 193-bp product),
VEGF164 (a 325-bp product), and VEGF188 (a 397-bp product) in developing and mature kidney (Fig. 6). Under the conditions used in this semiquantitative experiment, VEGF164 mRNA was by far the most abundant product on ethidium bromide-stained agarose gels (Fig. 6A), followed by VEGF120, and then VEGF188, and this was confirmed by densitometry (Fig. 6B).

DISCUSSION

In the present study we sought to analyze the expression of neuropilin-1 mRNA in developing and mature mouse kidneys and compare its expression pattern to those for Flk1 and VEGF. First, we detected neuropilin-1 mRNA in newborn and adult kidneys by RT-PCR and Northern hybridization. Second, on in situ hybridizations in newborn kidneys, neuropilin-1 mRNA was most prominently exhibited in glomeruli and diffusely over the nephrogenic outer cortex. In adult kidney, neuropilin-1 mRNA was again observed in glomeruli, but the overall expression levels were less than what was seen in newborns. Third, the neuropilin-1 expression pattern coincided exactly with that for Flk1 mRNA in both newborns and adults. Fourth, VEGF mRNA was also highly expressed in glomeruli of newborns, specifically in podocytes, but in adult kidneys was much less abundant. Finally, RT-PCR analysis illustrated that, although all three major VEGF isoforms were expressed in newborn and adult mouse kidney, VEGF164 was by far the most prevalent.

The evolution of glomerular epithelial and endothelial cells during nephrogenesis occurs in a temporally and spatially coordinated manner, but the molecular...
regulators of these cell differentiation events are poorly understood. Nevertheless, the appropriately timed expression of Flk1/VEGF during glomerulogenesis has implicated this receptor-ligand pair as early mediators of glomerular capillary development. Flk1 is expressed by endothelial precursors in the metanephros before their assembly into recognizable blood vessels and is highly expressed in the developing glomerular endothelium (28, 29). VEGF mRNA, on the other hand, has been specifically localized to tubular and glomerular epithelial cells of developing kidneys (3, 12, 18, 33). Heretofore, the expression of another recently identified VEGF receptor, neuropilin-1, has only been shown to be present in adult human kidney by Northern blot analysis (34). In the study presented here, we illustrate that neuropilin-1 mRNA was also expressed in newborn mouse kidney, and furthermore, was primarily localized to glomeruli in both newborns and adults. Because the level of resolution of the in situ hybridization technique used here makes precise cellular localization difficult, we are presently engaged in using other approaches to image neuropilin-1 expression more precisely. Nevertheless, the pattern of glomerular binding observed with 35S-labeled antisense probes strongly suggests that neuropilin-1 is expressed by glomerular endothelial cells.

By comparison, in situ hybridizations on newborn and adult kidneys demonstrated that the expression of Flk1 closely paralleled that for neuropilin-1. The localization of Flk1 mRNA to glomeruli also agrees with our previously reported findings using anti-Flk1 antibodies (29) and mice containing lacZ in place of one Flk1 allele (28). In those studies we demonstrated that Flk1 is highly expressed by endothelia of glomeruli, capillaries, and arterioles in newborn mouse kidneys and becomes limited to glomeruli and peritubular capillaries in adults. Taken together with the present findings, these results are intriguing in view of recent data demonstrating that cells coexpressing neuropilin-1 and Flk1 exhibit fourfold greater VEGF binding and enhanced VEGF165-mediated chemotaxis in vitro (34). This therefore raises the possibility that, during glomerular capillary development, responses to VEGF may be potentiated in cells expressing both neuropilin-1 and Flk1. As mentioned earlier, the use of other localization techniques may define whether the same endothelial cell populations express both receptors, and we are presently pursuing this question.

Several earlier experiments have shown that VEGF is crucial for normal glomerular capillary development. When VEGF-blocking antibodies are injected into newborn mice, fewer glomeruli form than normal, and many are avascular (22). Recent reports using metanephric organ cultures indicate that hypoxia, which induces VEGF expression, also stimulates endothelial development in vitro, and similar findings are obtained when organ cultures are supplemented with exogenous VEGF (37, 38). These functional studies therefore strongly implicate VEGF as a vascular morphogen in metanephros. In adults, VEGF is greatly downregulated but, as shown here and previously (3, 33), hybridization probes continued to localize to glomeruli. Although we were unable to distinguish between the various VEGF isoforms by in situ hybridizations, we demonstrated by RT-PCR with a single primer set that VEGF164 was by far the most abundant isoform in both newborn and adults, followed by VEGF120 and, finally, VEGF188. Our findings are in close agreement with RNase protection assays showing that VEGF164 is expressed at higher levels than the other isoforms in newborn mouse kidney (5). Because the cRNA probe we used for the in situ hybridization studies would hybridize only to the VEGF164 and VEGF188 isoforms, we therefore believe that the glomerular signal we observed was contributed mainly by hybridization to VEGF164 mRNA. Additionally, our data show that VEGF mRNA was expressed at high levels in newborn kidney and, because of the ringlike in situ hybridization pattern observed in capillary loop-stage glomeruli, appears to localize predominately to podocytes. This site would therefore place VEGF164 synthesis in close proximity to its receptors, Flk1 and neuropilin-1, on glomerular endothelial cells.

Evidence is accumulating that there may be important overlap in signaling mechanisms used to guide neuronal cell connections and blood vessel development (see 8 for a review). For example, members of the Eph/ephrin receptor ligand family, first described as helping direct axonal targeting in the optic tectum, have also been observed in the developing renal vasculature (8, 9). Additionally, ephrinB1 oligomers have been shown to mediate capillary-like assembly of human renal microvascular endothelial cells by specific signaling through the EphB1 receptor (35). Perhaps the expression of neuropilin-1 by endothelial cells during glomerulogenesis also helps facilitate faithful vascularization of each nephron as it develops. However, exactly how this might occur is not known. During neuronal development, neuropilin-1 transmits a chemorepulsive signal on binding semaphorin III, but when neuropilin-1 and Flk1 are coexpressed in endothelial cells, chemotactic responses to VEGF are amplified over cells expressing Flk1 alone (34). As observed earlier (34), these results indicate that two different ligands, semaphorin III and VEGF, can evoke strikingly different responses on binding the same receptor. Whether glomerular endothelial cells would normally be exposed to physiological amounts of semaphorin III, and whether VEGF would bind to branching neurons, are also presently not known. Nevertheless, the findings that Eph receptors, and now neuropilin-1, which are both known to mediate directed axonal growth, are also expressed in the developing kidney vascular system, strongly suggest a common pathway for transmitting positional information. Future studies that examine the coordinated and local expression of Flk1, neuropilin-1, VEGF, and Eph/ephrins during metanephric development should help address this issue further.

The persistent expression of Flk1, neuropilin-1, and VEGF in adult glomeruli, albeit at lower levels than those observed in immature stages, is also intriguing.
A precise role for Flk1 expression in the adult kidney glomerular endothelium has not been elucidated. Previous experiments have shown that the addition of VEGF to cultured endothelial cells induces fenestration (12, 30). Similarly, the topical application or intradermal injection of VEGF in vivo also increases permeability and induces fenestrations in endothelia of vessels that normally are not fenestrated (30). Others have postulated that VEGF/Flk1 may play a role in maintenance of the fenestrated, differentiated phenotype of the glomerular endothelium (33). Whether the sustained expression of neuropilin-1 also contributes to a fenestrated vascular phenotype of the adult glomerular endothelium or serves additional purposes remains to be defined.

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