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The Alternatively Spliced Anti-Angiogenic Family of VEGF Isoforms VEGF_{xxx}b in Human Kidney Development

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Abstract

Background/Aim—Vascular endothelial growth factor (VEGF), required for renal development, is generated by alternative splicing of 8 exons to produce two families, pro-angiogenic VEGF_{xxx}, formed by proximal splicing in exon 8 (exon 8a), and anti-angiogenic VEGF_{xxx}b, generated by distal splicing in exon 8 (exon 8b). VEGF₁₆₅b, the first described exon 8b-containing isoform, antagonises VEGF₁₆₅ and is anti-angiogenic in vivo.

Methods—Using VEGF_{xxx}b-specific antibodies, we investigated its expression quantitatively and qualitatively in developing kidney, and measured the effect of VEGF₁₆₅b on renal endothelial and epithelial cells.

Results—VEGF_{xxx}b formed 45% of total VEGF protein in adult renal cortex, and VEGF₁₆₅b does not increase glomerular endothelial cell permeability, it inhibits migration, and is cytoprotective for podocytes. During renal development, VEGF_{xxx}b was expressed in the condensed vesicles of the metanephros, epithelial cells of the comma-shaped bodies, invading endothelial cells and epithelial cells of the S-shaped body, and in the immature podocytes. Expression reduced as the glomerulus matured.

Conclusion—These results show that the anti-angiogenic VEGF_{xxx}b isoforms are highly expressed in adult and developing renal cortex, and suggest that the VEGF_{xxx}b family plays a role in glomerular maturation and podocyte protection by regulating the pro-angiogenic pro-permeability properties of VEGF_{xxx} isoforms.

Keywords

Kidney development; Vascular endothelial growth factor ₁₆₅b; Angiogenesis

Introduction

Vascular endothelial growth factor A (VEGF-A) is a potent angiogenic factor that induces endothelial cell migration, proliferation, differentiation and regeneration [1]. In kidneys of embryos to adults, VEGF-A is expressed in presumptive and mature glomerular epithelial cells (podocytes) and tubular epithelial cells [2-7]. Normal glomerulogenesis requires the

coordinated induction of epithelial differentiation, endothelial invasion, and growth of tubular and vascular tissues. In mice, specific overexpression or deletion of the *VEGF-A* gene in podocytes results in glomerular dysfunction [8, 9]. A podocyte-specific cre-recombinase knockout of even a single gene copy leads to nephrotic syndrome, uraemia and death 9 weeks post-partum, whilst complete knockouts died a few hours post-partum [8]. In mice, glomerular overexpression of the most widely studied isoform of VEGF-A, VEGF₁₆₅ results in death a few days post-partum with renal haemorrhages [8]. In VEGF inhibition studies, murine pups treated at postnatal day 0 with VEGF-blocking antibodies exhibit marked glomerular abnormalities, with many glomeruli lacking capillary tufts [4]. Similarly, treatment of murine pups with mFlt (1-3)-IgG (a soluble VEGF receptor-1 chimeric protein) postnatally on day 1 and day 8, results in marked glomerular defects, including loss of endothelial cells, mesangial matrix accumulation and hypocellularity [10]. These results suggest that tight control of VEGF-A expression is required for normal glomerular development and well-being.

The close temporal and spatial association of VEGF-A expression (by podocytes) and its receptors (on glomerular endothelial cells, GEnc) suggests that VEGF-A plays a pivotal role in the maintenance of glomerular integrity through the existence of a paracrine loop [11], and dysregulation of glomerular VEGF-A expression has been implicated in a wide range of renal diseases in humans [11]. Moreover, VEGF-A acts as an autocrine growth factor on both proliferating and differentiating glomerular visceral epithelial cells (podocytes) [9], and this results in prolonged survival and resistance to apoptosis, associated with changes in intracellular calcium concentration [12].

Isoforms of VEGF-A, termed according to their amino acid number, are generated by the differential splicing of eight exons of the full-length pre-mRNA from a single *VEGF-A* gene. The differential splicing of exons 6 and 7 generates isoforms with differing heparin-binding affinities [13], whilst the differential splicing of exon 8 (the terminal exon) generates two families of isoforms, pro-angiogenic and anti-angiogenic, which differ by only six amino acids at their C-terminus [14]. The pro-angiogenic VEGF-A isoforms, i.e. VEGF₁₂₁, VEGF₁₆₅ and VEGF₁₈₉ (collectively termed VEGF_{xxx}, where xxx is the number of amino acids encoded), are formed by the selection of a proximal splice site in exon 8, termed exon 8a, which results in an open reading frame of 6 amino acids being translated. The anti-angiogenic VEGF-A isoforms are generated by the use of a more distal splice site in exon 8, termed exon 8b, resulting in an open reading frame of the same number of nucleotides as proximal (or pro-angiogenic) splice variants, but encoding a different amino acid sequence. Thus, the resulting proteins are of the same amino acid length as the conventional isoforms and are collectively termed VEGF_{xxx}b [15]. The first anti-angiogenic isoform to be identified from human renal cortex was VEGF₁₆₅b [14]. VEGF₁₆₅b inhibits VEGF₁₆₅ and hypoxia-driven angiogenesis in vivo in rat, rabbit and mouse models of physiological and pathological angiogenesis [16, 17]. VEGF₁₆₅b does result in weak and tardy signalling through MAPK in microvascular endothelial cells in vitro [18] and induces a rapid but transient puff of fluid extravasation upon first exposure in intact microvessels in vivo but does not stimulate a sustained change in water permeability of microvessels [19]. VEGF₁₆₅b therefore does appear to have a stimulatory physiological role. VEGF_{xxx}b at the protein level appears to be the dominant isoform in many adult tissues, such as ocular tissues, colon and pancreatic islets [15 and Bates, unpubl. data]. VEGF_{xxx}b may therefore play a role in defining the physiological phenotype of the normal mature glomerulus (high permeability to water, low to protein in the absence of angiogenesis).

In most studies of VEGF-A in developing or mature glomerulus, a role of VEGF₁₆₅ or of other pro-angiogenic splice variants has been investigated or assumed. Previous studies have used antibodies that detect both families of VEGF-A isoforms (pan-VEGF antibodies) as

there were no antibodies or probes that distinguished between the VEGF_{xxx} and VEGF_{xxx}b families of isoforms. The only published report of VEGF₁₆₅b expression in developing kidney does show a significant alteration in terminal exon splicing in Denys-Drash syndrome, underlying a potential role for VEGF₁₆₅b in human glomerular developmental diseases [20]. Therefore, VEGF_{xxx}b isoform expression during normal renal development and the roles it might play are unknown. In this study, we examined expression of VEGF_{xxx}b in metanephric kidneys from human fetuses, and performed parallel in vitro experiments to understand the role of VEGF_{xxx}b on cell types involved in glomerular function.

Materials and Methods

Tissue Source

Human adult renal cortex was collected from the normal pole of unilateral, unipolar renal carcinoma nephrectomy specimens with local ethics committee approval (Bristol). Three human female fetuses of 10 and 12 weeks' pregnancy were obtained with local ethics committee approval (Leiden).

Immunohistochemistry and ELISA

Sections were microwave-heated in 0.01 M citric buffer saturated sodium citrate pH buffer (pH 6.0) for either 12 min at 95°C (VEGF_{xxx}b), or for 7 min at 800 W followed by 9 min at 120 W (pan-VEGF staining). Sections were washed twice with PBS, incubated with 3% hydrogen peroxide solution for 20 min, washed again, blocked with 10% BSA (Sigma; A4378) in 0.05% Tween-PBS (TBS), and then with 1.5% normal horse serum (NHS, Vector Lab; S-2000) in TBS (1 h). Sections were incubated with 8 µg/ml primary antibody (MAB3045, R&D Systems; Sigma, I8765, or Santa Cruz, 7269) in TBS (pH 7.4) with 1% BSA, washed twice with TBS, blocked again, then incubated with secondary antibody (Vector Lab; BA2000, 1:200 dilution in NHS) in TBS for 1 h, washed twice, then incubated with Vectastain ABC solution (Vector Lab; PK4000) for 45 min.

Cytotoxicity, ELISA Flow Cytometry and Migration Assays

VEGF ELISA [21], cytotoxicity [12], apoptosis [22], and migration [23] were determined as previously described.

Culture of GEnC

GEnC derived from decapsulated glomeruli isolated from normal human kidney (according to the supplier's data sheet) were obtained at passage 2 from the Applied Cell Biology Research Institute (ACBRI, Kirkland, Wash., USA). Cells were cultured in endothelial growth medium 2 - microvascular (EGM2-MV; Cambrex, Wokingham, UK), made up from endothelial basal medium 2 (EBM2; Cambrex) and fetal calf serum (5%), antimicrobial agents and growth factors as supplied. Cells being prepared for, or being used in, experiments were cultured in EGM2-MV without VEGF.

Measurement of Trans-Endothelial Electrical Resistance

Trans-endothelial electrical resistance (TEER) is a measure of ion flux and is inversely related to the fractional area of pathways open to water and small molecules across a cell monolayer. Tissue culture inserts containing polycarbonate supports (0.4 µm pore size, Nalge Nunc International, Rochester, N.Y., USA) were seeded with GEnC at 100,000 cells/cm². Measurement of TEER of GEnC monolayers was performed using an Endohm 12 electrode chamber and EVOMx voltmeter (World Precision Instruments, Sarasota, Fla., USA) as previously described [24]. Medium was replaced with serum-free medium (EBM2).

Baseline TEER was measured after 1 h and the culture medium was again replaced, this time with SFM alone (control) or containing 1 nM VEGF₁₆₅ (R&D Systems) or 1 nM VEGF_{165b}. TEER was remeasured at 15, 30 and 60 min. Previous work has demonstrated a peak response to VEGF between 30 and 60 min in this assay.

Results

VEGF_{xxx}b Expression in Adult Renal Cortex

To determine quantitatively the contribution of VEGF_{xxx}b isoforms to the total VEGF expression in normal adult kidneys, VEGF_{xxx}b and total VEGF were measured in protein extracted from freshly frozen renal cortex. Total protein was measured using the commercially available ELISA, and VEGF_{xxx}b levels measured by a comparable ELISA but using a biotinylated detection antibody specific to the C terminus of VEGF_{xxx}b. Total VEGF concentrations in normal renal cortex averaged of 54.2 ± 14 ng/mg protein. VEGF_{xxx}b concentrations averaged 25.8 ± 9.6 ng/mg ($n = 3$; fig. 1), or $45 \pm 5\%$ of the total VEGF. This was similar to the relative proportion of total VEGF that was VEGF_{165b} measured in protein extracted from normal isolated glomeruli collected from human nephrectomy specimens ($46.6 \pm 18\%$, $n = 3$).

VEGF_{xxx}b Staining in Adult Kidney

The antibody to VEGF_{xxx}b used for immunohistochemistry is an affinity-purified mouse monoclonal IgG₁ antibody, Cat MAB3045, commercially available through R&D Systems, which has been characterised previously [15, 16, 25]. It binds recombinant VEGF_{165b}, and shows expression of VEGF_{165b}, VEGF_{180b}, VEGF_{121b}, VEGF_{183b} and VEGF_{145b}, collectively termed VEGF_{xxx}b, but not VEGF₁₆₅. Western blotting has previously shown that all the proteins recognised by this antibody are also recognised by commercial antibodies raised against VEGF-A. This antibody does not recognise the VEGF_{xxx} isoforms, but does recognise recombinant VEGF_{165b} and VEGF_{121b}, conclusively demonstrating that this antibody is specific for VEGF_{xxx}b [16]. VEGF_{xxx}b staining was limited to a significant proportion of podocytes (fig. 2a), but present in parietal epithelial cells, macula densa and proximal and distal tubules of the renal cortex (fig. 2b). VEGF_{xxx}b staining was also observed in the vasa recta, collecting ducts and ascending thin and thick loop of Henle (fig. 2b). In the epithelial cells of the ascending thick loop of Henle, strong intracellular staining was observed, whereas in the epithelial cells of the collecting ducts, staining was highly localised to the tips of the apical surface (short arrow) and to the basolateral cytoplasm (long arrow, fig. 2c). A similar trend was observed for pan-VEGF staining (fig. 2d-f). Staining was never seen when an isotype-matched IgG antibody was used as a control (fig. 2g-i) under the same conditions.

VEGF_{xxx}b Staining in Developing Glomerulus

To investigate VEGF_{165b} expression in the developing glomerulus, immunohistochemistry was carried out on sections of human fetal renal tissue. Immunohistochemical staining for VEGF_{xxx}b of 10- and 12-week-old fetuses showed clear expression in the developing nephron that was noticeably stronger than the surrounding mesenchyme (fig. 3a, 10 weeks; fig. 3b, 12 weeks). Staining was very intense in all stages of nephrogenesis from the condensed vesicle stage onwards (fig. 4a-d). Staining with an antibody to all isoforms of VEGF confirmed that VEGF was located throughout the developing kidney (fig. 3c, 10 weeks; fig. 3d, 12 weeks). Interestingly, there were no areas of the kidney that stained for VEGF_{xxx}b but did not stain for pan-VEGF. In contrast, there were a number of areas, including in the mesenchyme, where VEGF_{xxx}b antibodies did not detect expression, but the pan-VEGF antibody did (fig. 4a vs. fig. 4e). No staining was seen using any isotype-matched affinity-purified mouse IgG (fig. 3e, 10 weeks; fig. 3f, 12 weeks).

In the condensed vesicle (fig. 4b), VEGF_{xxx}b staining was greater than the surrounding mesenchyme (fig. 4a, b). The greatest intensity of staining could be seen during epithelialisation, and staining was highest in both the apical and basolateral parts of the primitive epithelial cell and weakest in the nuclear regions, indicating a cytoplasmic subcellular localisation. Pan-VEGF staining was also apparent in these regions in the condensed vesicle (fig. 4e, f). As development proceeded to comma- (fig. 4c, g) and S-shaped bodies (fig. 4d, h), VEGF_{xxx}b staining (fig. 4c, d) became even more restricted to the apical and basolateral parts of the primitive columnar epithelial cells of the developing nephron, as did pan-VEGF staining (fig. 4g, h). Of note was the more diffuse VEGF_{xxx}b staining in the glomerular cleft (fig. 4d, h), the site at which endothelial cells will invade. This pattern of staining, observed in the primitive epithelial cells and glomerular cleft appeared to be more diffuse in the capillary loop stage of glomerulogenesis (fig. 5a), in contrast to pan-VEGF which appeared stronger (fig. 5a in comparison with 5b contrasts with 4d in comparison with 4h). As the glomerulus was formed (fig. 5c), VEGF_{xxx}b staining appeared to diminish in the developing glomerular visceral epithelial (podocytes) and endothelial cells (fig. 5e), but there was still marked staining in the parietal epithelial cells lining Bowman's capsule, and cells of the maculae densa (fig. 5e). Pan-VEGF expression appeared to be maintained through glomerular maturation, and stained up more glomerular epithelial cells than VEGF_{xxx}b (fig. 5d, f). In comparison, in the mature glomeruli, VEGF_{xxx}b staining was limited to a subpopulation of podocytes (fig. 2a). This appeared to be true for pan-VEGF staining too, but the pan-VEGF antibody identified more podocytes than the VEGF_{xxx}b antibody (fig. 2d).

VEGF_{xxx}b Staining in Developing Tubules of the Primitive Renal Cortex

Throughout the developmental stages, examined VEGF_{xxx}b staining was clearly seen in both the proximal and distal portions of the convoluted tubules (fig. 6a). More specifically, staining was seen both in the apical and basolateral parts of the primitive epithelial cells. In addition, staining was seen in all areas of tubule development in the renal cortex. Comparable pan-VEGF staining was seen in the tubules of the developing renal cortex (fig. 6b).

VEGF_{xxx}b Staining in the Primitive Renal Medulla

VEGF_{xxx}b staining appeared to be more specifically localised in the primitive epithelial cells of the developing nephron (fig. 6c), whereas pan-VEGF staining was intense and widespread throughout the renal medulla (fig. 6d), VEGF_{xxx}b staining was seen in both the apical and basolateral sides, but not as intense in the central, perinuclear regions in the epithelial cells of the distal tubules extending into the medulla and of the collecting ducts (fig. 6c). Furthermore, it appeared that where the more distal portions of the convoluted tubules differentiate into their specialised transporting segments (the thin and thick loop of Henle) VEGF_{xxx}b staining was less intense (fig. 6c). Weak VEGF_{xxx}b staining was also observed in the endothelial cells of the vasa recta (fig. 6c).

The Effect of VEGF₁₆₅b on Human Glomerular and Endothelial Cells in vitro

Alterations in expression may reflect changes in function in the embryo, in the adult and in disease. The role of VEGF_{xxx}b in the developing human kidney is not known. Although VEGF₁₆₅b has been shown to inhibit endothelial cell migration in response to VEGF₁₆₅, it is not known whether this inhibition can be balanced by controlling the expression level of VEGF isoforms. To determine whether VEGF₁₆₅b could dose-dependently affect endothelial cells in vitro, the effect of VEGF₁₆₅b on human endothelial cell migration was estimated. Figure 7a shows that VEGF₁₆₅b dose-dependently inhibited HUVEC migration responses to VEGF₁₆₅, with an IC₅₀ of 0.29 ± 0.03 -fold excess (i.e. 40 ng/ml VEGF₁₆₅ was 50% inhibited by 11.4 ± 1.4 ng/ml VEGF₁₆₅b, n = 3). This is consistent with

downregulation of VEGF_{165b} during the endothelial invasion phase of glomerular development. To determine whether VEGF_{165b} might have positive benefits during glomerular development, we measured the effect of VEGF_{165b} on podocyte cytotoxicity. VEGF_{165b} dose-dependently decreased cytotoxicity of primary cultured podocytes (fig. 7b) with an EC₅₀ of 107 ± 1.2 pM, showing that VEGF_{165b} had a cytoprotective effect (n = 8). The LDH assay measures only the number of cells releasing a cytoplasmic protein and hence does not distinguish between apoptosis and necrosis. Interestingly, VEGF_{165b} did not affect podocyte cell proliferation ($16.5 \pm 1.1 \times 10^3$ cpm/cell compared with $15.8 \pm 1.0 \times 10^3$ cpm/cell, n = 6), thus suggesting an anti-apoptotic effect on human podocytes. This was confirmed by flow cytometry using annexin V and propidium iodide staining (fig. 7c). Whereas serum starvation induced a significant proportion of the cells to undergo apoptosis (region A in fig. 7c₁), this was inhibited by treatment with 0.3 nM VEGF_{165b} (fig. 7c₂). To determine whether VEGF_{165b} could affect glomerular endothelial barrier function a trans-electrical endothelial resistance (TEER) in vitro assay of glomerular endothelial permeability was used. Although VEGF₁₆₅ significantly reduced glomerular endothelial TEER (indicating an increase in monolayer permeability), VEGF_{165b} resulted in a significant increase in TEER, and VEGF_{165b} inhibited the increase induced by VEGF₁₆₅ (fig. 7d, n = 4). Thus in contrast to VEGF₁₆₅, VEGF_{165b} prevents endothelial cell migration and reduces monolayer permeability, while maintaining podocyte cell survival in vitro.

Discussion

The role of VEGF in renal function and development has been the subject of intense scrutiny since VEGF expression was demonstrated in the renal cortex and medulla by antibody staining, RT-PCR, in situ hybridisation and Northern blotting, in both normal and disease states. VEGF is highly expressed in the kidney - more so than nearly any other tissue, but very few studies have accounted for the VEGF_{xxx}b variants that are anti-angiogenic [16, 20, 25]. mRNA encoding the VEGF_{xxx}b splice variants were first described in normal renal cortex, and VEGF_{165b} protein was first identified in human podocytes by isoform-specific siRNA [26]. The experiments described here, however, are the first to quantitate the contribution of VEGF_{xxx}b to the total VEGF expressed. The finding that, in normal renal cortex, almost half of the VEGF found is VEGF_{xxx} has significant implications for our interpretation of the many studies that have investigated VEGF expression in normal renal tissues and disease states [15]. The finding that VEGF_{xxx}b isoforms are a highly significant component of the total VEGF in renal tissues implies an as yet unknown physiological relevance.

Pan-VEGF and VEGF_{xxx}b Staining Patterns Compared

VEGF-A, both mRNA and/or protein, of unknown isoform family has been detected in the presumptive and mature podocytes and primitive columnar epithelial cells of the developing nephron, in both rodent and human tissues [2, 4-7, 27, 28]. In this study, we sought to determine the presence and localisation of VEGF_{xxx}b proteins in human metanephric kidneys and compare its spatiotemporal staining pattern to that detected by pan-VEGF antibodies. We detected VEGF_{xxx}b in metanephric kidneys from 10- and 12-week fetuses using immunohistochemical staining. Our pan-VEGF staining of the metanephric kidney is in close agreement with previous studies; VEGF was detected in presumptive and mature podocytes and in the primitive columnar epithelial cells of the nephron [2, 4-7, 29]. VEGF_{xxx}b isoforms appear to be present in a subset of cells that express VEGF, as there were no areas in the metanephric kidney that stained for VEGF_{xxx}b isoforms but not for pan-VEGF but some areas that stained positively for pan-VEGF but not for VEGF_{xxx}b. In the adult kidney, the presence of VEGF in the convoluted tubules is in contrast to in situ hybridisation studies, which show in adult tissues the primary source of renal VEGF

synthesis to be the podocyte [30], suggesting the possibility of glomerular-derived VEGF protein uptake by tubular cells.

Glomerulogenesis and VEGF_{xxx}b

Glomerulogenesis is dependent on reciprocally inductive interactions between renal endothelial cells and nephron epithelial cells, but although various genes [31-34] and growth factors [9, 11, 35-37] have been implicated at specific stages, the molecular regulators of the cell differentiation events are poorly understood. A dosage sensitivity to VEGF exists within the developing glomerulus [8], similar to that seen when VEGF expression was manipulated throughout the embryo [38, 39]. As VEGF₁₆₅b has been shown to counteract some of the effects of VEGF₁₆₅s, and has a dose-dependent effect on podocyte survival, it is likely that dosage sensitivity of glomerulogenesis to VEGF_{xxx}b may also be a critical component of normal renal cortex formation, and a recent study showing that transgenic mice over-expressing VEGF₁₆₅b in mouse podocytes have reduced glomerular permeability characteristics supports this suggestion [40].

Previous Studies on VEGF

Apart from the original isolation of VEGF₁₆₅b mRNA from renal cortex [14] and protein in the glomeruli [16], and the identification of VEGF₁₆₅b mRNA and protein in differentiated, but not proliferating conditionally immortalised podocyte cell lines [26], previously used methodologies either did not detect VEGF_{xxx}b isoforms (RT-PCR using primers in the proximal part of exon 8), or did not distinguish VEGF_{xxx}b isoforms from VEGF_{xxx} isoforms. The only study that has addressed this examined microdissected mRNA from fetal, child and adult glomeruli, and found that expression of VEGF₁₆₅b mRNA was lower in the S- and C-shaped bodies than in adult or child glomeruli. The decreasing protein expression we see here from condensed vesicle through S- and comma-shaped bodies to immature glomeruli may therefore be a result of this endogenous downregulation at the mRNA level, temporally shifted slightly, as the VEGF protein is turned over more slowly than the mRNA. Schumacher et al. [20] also noted higher VEGF₁₆₅b expression in the adult glomeruli compared with VEGF₁₆₅. Unfortunately, antibodies that specifically detect VEGF_{xxx} isoforms are not yet available, but it appears likely that most of the VEGF staining in adult glomeruli is VEGF₁₆₅b. Interestingly, in that study, Schumacher et al. [20] demonstrate a complete loss of VEGF₁₆₅b in Denys-Drash glomeruli, indicating a link to WT1, a finding recently confirmed by over-expression studies in vitro [41]. Podocyte-specific knockout of VEGF during development resulted in a lack of formation of glomeruli and renal failure immediately after birth followed by death within 6 h [8], presumably because endothelial cells fail to migrate into the glomerulus (as is evidenced by a lack of phenotypically discernable endothelial cells in the glomerulus), and thus aberrant microvessel formation and glomerular filtration. VEGF knockouts, however, also are VEGF_{xxx}b knockouts, so it is not clear which part of the phenotype is dependent on VEGF_{xxx}b knockout. Inhibitors of VEGF such as VEGF-TRAP [42], sFlt-1 [43], bevacizumab [44] and other monoclonal antibodies to VEGF, shown to affect renal function, are also likely to affect the VEGF_{xxx}b isoforms. Therefore, it is not clear whether the results in studies previously carried out on the inhibitory role of VEGF in glomerular function were due to the pro-angiogenic isoforms, or the anti-angiogenic isoforms, or both.

Possible Functions of Renal VEGF_{xxx}b Expression

VEGF₁₆₅b inhibits VEGF₁₆₅-mediated endothelial cell proliferation and migration in vitro and vasodilatation in isolated arteries ex vivo [14, 16], VEGF₁₆₅-mediated physiological angiogenesis in the mesentery and the eye [16], the chicken chorioallantoic membrane and the dorsal skin chamber in mice [18], pathological VEGF-mediated angiogenesis in tumour models [16], and hypoxia-driven retinal angiogenesis in the eye in vivo [17]. VEGF₁₆₅b has

been shown to have both dominant negative and partial agonist activity on endothelial-mediated signalling [16, 18], potentially explaining its ability to both inhibit migration and protect against cytotoxicity. In contrast, no effect of VEGF₁₆₅b was seen on glomerular endothelial monolayer integrity in vitro (fig. 7c). In the developing kidney, VEGF_{xxx} isoforms are thought to mediate endothelial cell survival and migration, microvascular permeability [9] and perhaps epithelial cell survival [12]. The results shown here are consistent with the concept that the VEGF_{xxx}b isoforms also support epithelial cell survival, without increased permeability, and are downregulated during endothelial cell migration presumably to allow invasion into the glomerular cleft.

Eremina et al. [8] have shown that unrestricted expression of VEGF₁₆₅ during development is significantly detrimental, which taken together with these results suggest a balance of pro-angiogenic/anti-angiogenic VEGF-A is required for normal development and function [8, 9, 31, 45, 46]. Expression of VEGF_{xxx}b isoforms and crucially the control of distal and proximal 3' -end splicing control during kidney development may therefore play a significant role in the modulation of VEGF_{xxx}-driven responses. VEGF_{xxx}b may play a modulatory role in the developing kidney. For example, factors must limit and halt the endothelial cell invasion into the glomerular cleft at the primitive glomerulus and subsequent stages of glomerular development. To address this hypothesis, further investigation is required including conditional transgenic knock-out and over-expressing models that are designed to take account of both sides of the VEGF-A biology - angiogenesis and permeability - and perhaps, more importantly, their inhibition.

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Fig. 1.
Expression of VEGF and its two families of isoforms in normal human renal cortex.
VEGF_{xxx}b isoforms comprise over 45% of total VEGF in adult human renal cortical tissues.

Fig. 2.

Immunohistochemical staining of VEGF_{xxx}b and pan-VEGF in the adult kidney. VEGF_{xxx}b staining in the podocytes of the glomerulus (**a**, arrows), proximal and distal tubules of the renal cortex (**b**) and in the ascending thick and thin loop of Henle in the renal medulla (**c**) was clearly seen. Sections treated with a pan-VEGF antibody (**d-f**) show a comparable staining pattern. Matched mouse IgG controls were negative (**g-i**). Scale bars = 30 μ m, except inset in **a** = 10 μ m, and **f**. TLH = Thin loop of Henle; TklH = thick loop of Henle; CD = collecting ducts; VR = vasa recta.

Fig. 3. Overview of VEGF_{xxx}b and pan-VEGF immunohistochemical staining of the metanephric kidney. VEGF_{xxx}b (a, b) and pan-VEGF (c, d) staining was observed in specific developmental regions of the metanephric kidney of 10-week (a, c) and 12-week (b, d) fetuses. Matched mouse IgG controls were negative (e, f). Scale bars = 600 μ m.

Fig. 4. VEGF_{xxx}b and pan-VEGF immunohistochemical staining during the early stages of nephrogenesis. Intracellular VEGF_{xxx}b staining (**a-d**) and pan-VEGF staining (**e-h**) was observed during glomerular development. VEGF_{xxx}b (**a**) and pan-VEGF (**e**) staining was observed at the various stages of nephrogenesis, with a specific staining pattern. For example, in the condensed vesicle (**b, f**) the staining was more polarised as the mesenchymal cells gained epithelial characteristics. In comma-shaped (**c, g**) and S-shaped (**d, h**) bodies, VEGF_{xxx}b (**c, d**) and pan-VEGF (**g, h**) staining was localised to the primitive epithelial cells, especially on the apical side, and to the glomerular cleft. Scale bars = 30 µm, except **e**. m = Mesenchyme; GC = glomerular cleft; CV = condensed vesicle; CSB = comma-shaped body; SSB = S-shaped body; G = glomeruli. Arrows point to diffuse staining of the glomerular cleft.

Fig. 5. VEGF_{xxx}b immunohistochemical staining during glomerular maturation. Apical and basolateral VEGF_{xxx}b (**a, c, e**) and pan-VEGF (**b, d, f**) staining. **a, b** The presumptive podocytes and the glomerular cleft show diffuse staining in the capillary loop stage. As development of the glomerulus progresses and the presumptive podocytes mature, the intensity of the VEGF_{xxx}b staining decreases (**c**), whereas pan-VEGF staining is still intense (**d**) and VEGF_{xxx}b becomes more specific to a subpopulation of mature podocytes (**e**); pan-VEGF staining is more widespread in the podocytes at this stage (**f**). Scale bars = 50 μ m. MD = Macula densa. Arrows point to parietal epithelial cells.

Fig. 6. Immunohistochemical staining of VEGF_{xxx}b and pan-VEGF in the tubules of the developing renal cortex and medulla. **a, c** VEGF_{xxx}b staining was seen in the more proximal and distal regions of the convoluted tubules. Strong periluminal staining was observed in the proximal tubules and collecting ducts. **b, d** Similar immunohistochemical staining was observed for pan-VEGF. Scale bars = 30 μ m. PT = Proximal tubule; DT = distal tubule.

Fig. 7.

VEGF₁₆₅b is cytoprotective for glomerular epithelial cells, and inhibits VEGF₁₆₅-mediated migration and increased permeability of endothelial cells. **a** VEGF₁₆₅b dose-dependently inhibits endothelial cell migration induced by VEGF₁₆₅. *** $p < 0.001$, ANOVA. **b** VEGF₁₆₅b dose-dependently prevents cell death in primary cultured podocytes. *** $p < 0.001$, ANOVA. **c** VEGF₁₆₅b inhibits apoptosis induced by serum starvation. Flow cytometry of annexin V and propidium iodide-stained human conditionally immortalised podocytes (hCIP) treated with 48-hour serum starvation (SS; **c₁**) or 1 nM VEGF₁₆₅b and SS (**c₂**). V = Viable population; N = necrotic; A = apoptotic; LA = late apoptotic. **d** VEGF₁₆₅b reduces permeability of glomerular monolayers. VEGF₁₆₅b increases glomerular transendothelial electrical resistance (TEER) in cultured monolayers, VEGF₁₆₅ reduces TEER (increases permeability), and VEGF₁₆₅b inhibits the VEGF₁₆₅-mediated reduction. ** $p < 0.01$, compared with control; ++ $p < 0.01$ compared with both VEGF₁₆₅b and VEGF₁₆₅; one-way ANOVA and Bonferroni post-hoc analysis.