The Importance of Cellular VEGF Bioactivity in the Development of Glomerular Disease

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Key Words

Vascular endothelial growth factor · Glomerular endothelial cells · Glomerulopathy · Diabetic nephropathy · Pre-eclampsia · Hypertension

Abstract

The bioactivity of glomerular VEGF (or activity of available VEGF) is critical to the physiological maintenance of the glomerular filtration barrier. Disturbances in glomerular VEGF expression have been linked to numerous glomerulopathies, highlighting its importance in disease progression within the kidney. However, the changes in expression are not consistent between conditions; enhanced expression sometimes appears to have a renoprotective effect, yet at other times it appears destructive. Also, the level of expression can change with the progression of disease. This review focuses on how other cellular factors, such as TGF- β and nitric oxide, work in concert to affect the bioactivity, which is not necessarily the same as the expression of VEGF, in different glomerulopathies and attempts to explain some of the paradoxes between glomerulopathies. In conclusion, the bioactivity of glomerular VEGF is regulated by many factors that are themselves moderated by changes in the local glomerular environment, such as mechanical strain and hyperglycaemia. Thus, to understand VEGF signalling in glomerular disease progression, we must examine it in the context of other appropriate cellular factors.

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The Glomerular Filtration Barrier

The glomerulus forms the filtering component of each of the million functional units, or nephrons, within the kidney. The filtering properties of the glomerulus are controlled by the glomerular filtration tri-layer barrier, which consists of fenestrated glomerular endothelial cells (GEnC) that line the capillary lumen, a glomerular basement membrane (GBM) and highly differentiated glomerular epithelial cells, or podocytes. Long cytoplasmic podocyte foot processes form tight interdigitations to fully encircle each capillary, and the slit diaphragm (a modified cell junction unique to podocytes) forms a zipper-like structure at the point where the interdigitations meet. Each layer of this filtration barrier plays an important role and, as a result, damage to one or more of these layers can lead to the development of proteinuria and eventually to progressive renal disease. Hence the glomerular microenvironment is tightly regulated.

VEGF within the Glomerulus

The sensitivity of the glomerulus to local changes in mediators has been brought to light in recent years by the use of anti-cancer therapies that surprisingly resulted in proteinuria, a sign of disturbance of the glomerular filtration barrier. The target of these therapies was VEGF-

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A. Eremina et al. [1] recently described the development of proteinuria and hypertension in cancer patients taking Bevacizumab, a monoclonal antibody to VEGF-A, leading to a reduced dosage of glomerular VEGF-A. Renal biopsies revealed a phenotype of glomerular thrombotic microangiopathy, which was reversed when treatment stopped.

VEGF-A promotes angiogenesis, and belongs to the VEGF family of proteins, which also includes VEGF-B, VEGF-C, VEGF-D and placental-derived growth factor [2]. There are many isoforms of VEGF-A that are named according to their number of amino acids, i.e. VEGF₁₂₁, VEGF₁₆₅, VEGF₁₈₉, VEGF₂₀₆. The most biologically active VEGF-A isoform is VEGF₁₆₅, which predominantly signals through VEGF receptor 2 (VEGFR2), although VEGF₁₆₅ can also be 'retained' by VEGFR1, which has limited signalling potential [3]. VEGF-C and VEGF-D promote lymphangiogenesis and predominantly signal through VEGFR3, although they can also both signal through VEGFR-2 [as reviewed in 4]. VEGF-A biology has been complicated in recent years by the discovery of an entire family of sister isoforms that share the same number of amino acids, yet differ in their last 6 amino acids (VEGF_{xxx}b, where xxx denotes the number of amino acids). Historically, the design of antibodies and primers to conventional VEGF-A isoforms inadvertently also detected VEGF_{xxx}b isoforms. [5]. VEGF_{xxx}b is expressed in normal tissues and in circulating plasma and tends to dominate over the expression of conventional VEGF-A isoforms (VEGF_{xxx}A) in the basal mature state; however, VEGF_{xxx}A dominates in the angiogenic environment, such as in developing tissues and in tumour growth [6]. VEGF_{xxx}b cannot phosphorylate VEGFR2 effectively and consequently blocks VEGF_{xxx}A activation of VEGFR2. Because of the anti-angiogenic properties of VEGF_{xxx}b, it has been identified as a potential therapy in cancer development [7].

VEGF-A is highly expressed by podocytes and plays an important role in the formation of the glomeruli during development [8], but curiously is also highly expressed within the adult glomerulus despite little or no angiogenesis occurring beyond development. We and many other groups have investigated this paradox in recent years and demonstrated a paracrine role for VEGF-A in GEnC signalling and fenestration formation [9]. We also discovered a surprising autocrine role for VEGF in human cultured podocytes, despite the lack of VEGFR2 expression (although other researchers have demonstrated VEGFR2 expression in murine podocytes) [10]. VEGF promoted podocyte survival in culture and was shown to be associ-

ated with podocytes in human glomeruli sections using immunogold staining and electron microscopy. Furthermore, we demonstrated that blockade of endogenous VEGF reduced nephrin (a podocyte-specific cell adhesion molecule which is one of the main components of the slit diaphragm) phosphorylation in cultured human podocytes and demonstrated the dependence on nephrin for VEGF survival signalling [11]. We have also demonstrated that VEGF_{xxx}b sister isoforms are normally expressed in human glomeruli alongside conventional VEGF isoforms throughout development and at maturity [12]. Functionally, in contrast to VEGF₁₆₅, VEGF₁₆₅b increases resistance in human GEnC monolayer integrity and inhibits GEnC migration, although it also promotes survival in podocytes [12]. This suggests that the anti-angiogenic VEGF_{xxx}b isoforms may regulate the local dosage of conventional VEGF_{xxx} isoforms and they are already emerging as players in glomerular disease. Schumacher et al. [13] describe the downregulation of VEGF₁₆₅b mRNA expression in laser-microdissected glomeruli from patients with Denys-Drash disease, whilst high levels of conventional VEGF₁₆₅ remain and, as a result, the glomeruli are not capable of developing passed the s-shaped body stage. Another potential player in glomerular disease from the VEGF family of proteins is VEGF-C. We have demonstrated the expression of VEGF-C by podocytes, which has functional effects on both cultured GEnC and podocytes [14, 15]. Interestingly, VEGF-C activates VEGFR2 not VEGFR3 in cultured GEnC resulting in a reduction in macromolecular passage, which is in sharp contrast to the increased macromolecular passage by VEGF-A [14]. Therefore, VEGF-C has the potential to affect the local dosage of glomerular VEGF-A by competing with signalling effects through the same receptor.

The literature on VEGF in glomerular disease grows monthly. This minireview will focus on the most recent original publications that have progressed our understanding of some regulators that affect the bioactivity of cellular VEGF in the progression of glomerular diseases, namely pre-eclampsia, diabetic nephropathy and hypertension, three common renal related conditions associated with changes in VEGF expression. VEGF-A bioactivity describes both expression levels and, importantly, the availability of expressed VEGF-A. For instance, highly expressed VEGF-A may not be very biologically active due to confounding factors within its local environment, such as naturally occurring competing ligands and alternative receptors/binding proteins. Conversely, its activity may be enhanced by proteins that act synergistically with it.

Evidence for Involvement of VEGF in Glomerulopathologies

More than a decade of research has implicated VEGF-A in various glomerulopathies revealing dramatic but often apparently contradictory changes in its expression, suggesting that a balance of glomerular VEGF-A may be important in maintaining the normal physiology of the filtration barrier, but that other players are also involved. For example, glomerular VEGF-A expression is increased in many glomerulopathies, such as human crescentic glomerulonephritis [16] and minimal-change nephropathy [17], but also decreased in glomerulopathies such as diabetic nephropathy [17] (although this is after a transient increase in VEGF [18]). Exogenous VEGF treatment accelerated glomerular recovery in experimental thrombotic microangiopathy [19], yet podocyte-specific overexpression of VEGF-A led to collapsing glomerulopathy [20]. Blockade of endogenous VEGF with a VEGF aptamer in rats had no effect on the course of proteinuria in puromycin aminonucleoside nephropathy and passive Heymann nephritis experimental models [21] and did not induce proteinuria in normal rats after 21 days' treatment [22]. Yet, neutralisation of VEGF by sFlt-1 induced proteinuria [23], suppression of endogenous VEGF in adult mice caused swelling and vacuolation of endothelial cells [22], and podocyte-specific deletion of VEGF-A induced proteinuria and endotheliosis [20].

The importance of exquisite control of dosage of glomerular VEGF-A has been demonstrated in elegant work by Eremina et al. [20] using podocyte-specific mouse models of heterozygous VEGF-A deletion or overexpression. Heterozygous *VEGF-loxP^{+/−},Neph-Cre^{+/−}* mice developed nephrotic syndrome and end-stage renal failure at 9 weeks, whereas podocyte-specific knock-out of VEGF-A in the adult kidney resulted in thrombotic microangiopathy, mimicking the phenotype of that seen when patients took bevacizumab [1]. In contrast, podocyte-specific overexpression of VEGF-A resulted in a phenotype of collapsing nephropathy, evident at 2.5 weeks [20]. It seems that the importance of glomerular VEGF is not the amount per se but the active amount in relation to what is required by the glomerulus.

VEGF Regulation in Glomerulopathologies

The delicate balance of glomerular VEGF-A is reflected by the diversity of glomerular diseases linked to changes in its expression such as pre-eclampsia, diabetic

nephropathy, hypertension, mesangioproliferative glomerulonephritis, thrombotic microangiopathy and crescentic glomerulonephritis. To dissect the complexity of VEGF dosage and function in glomerular disease, it is necessary to look at individual conditions in more detail.

Pre-Eclampsia

Pre-eclampsia affects 5% of pregnancies worldwide and is characterised by hypertension and proteinuria. These symptoms are associated with increased production of circulating soluble VEGFR1 (sFlt-1) and soluble endoglin (TGF- β receptor) by the placenta, which reduce the local bioactivity of both VEGF-A and transforming growth factor- β (TGF- β). Surprisingly, in normotensive pregnancies the proportion of plasma VEGF-A that is VEGF_{165b} increases from 18% at 12 weeks gestation to 49% at pre-delivery, suggesting that the control of action of conventional VEGF-A isoforms is necessary in later pregnancy [24]. However, in contrast to the anti-angiogenic factors sFlt-1 and sEndoglin, there is a failure to upregulate VEGF_{165b} early on in pregnancies that later become pre-eclamptic. The authors speculate that this may be due to a maternal vasculature response to correct the defective implantation process, or indeed the lack of VEGF_{165b} may be the cause of it. These results suggest that the anti-angiogenic effects of sFlt-1 and sEndoglin on the placental environment may differ from those of VEGF_{165b}, potentially due to the alternative signalling effects of VEGF_{165b} through VEGFR1, but this warrants further investigation.

In rats, the reduction in VEGF-A dosage in pre-eclampsia is thought in turn to reduce the activation of endothelial nitric oxide synthase (eNOS) and thus block the vasodilatation normally seen in microvessels [25], contributing to the development of systemic endotheliosis. VEGF-A, and possibly TGF- β [as reviewed in 26], are also thought to be involved in GEnC fenestration maintenance, which may explain the susceptibility of GEnC to damage [26]. TGF- β is known to regulate VEGF-A expression in pericytes and podocytes, and to alter splice site decision between the pro- and anti-angiogenic isoforms [27], thus sEndoglin-1 may also indirectly regulate VEGF-A dosage in pre-eclampsia. These studies suggest that a reduced dosage of local VEGF-A, as well as TGF- β , lead to the progression of pre-eclampsia through reduced vasodilatation and reduced fenestration formation and that drug strategies targeting both sFlt-1 and sEndoglin may be beneficial. Importantly, the progression of pre-eclampsia is not solely dependent on reduced availability

of one growth factor, but on the sum of the reduced activities of these growth factors.

A recent study on kidney biopsies from pre-eclamptic and normal pregnant women identified a reduction in both nephrin and synaptopodin (a maturity marker of podocytes) expression in pre-eclamptic glomeruli, with no effect on podocin (a podocyte-specific hairpin protein) expression [28]. These findings were confirmed as a direct effect of reduced VEGF-A dosage in mice which received either anti-VEGF antibodies or sFlt-1 [28]. This work was supported by *in vitro* work, which also demonstrated nephrin shedding by podocytes in pre-eclampsia sera [29]. Interestingly, this was not a direct effect of the sera on podocytes, but occurred when cultured podocytes were incubated with conditioned media from GEnC that had been incubated with the pre-eclampsia sera. The authors showed that this was due to the release of endothelin-1 by the pre-eclamptic sera from GEnC and furthermore demonstrated that reduced VEGF-A availability allowed endothelin-1 release from endothelial cells. This work demonstrates that reduced VEGF-A bioactivity in pre-eclampsia affects both the endothelial and epithelial layers of the filtration barrier, both directly and indirectly. In summary, in pre-eclampsia VEGF bioactivity is too low, thus therapies to increase VEGF bioactivity would be beneficial.

Diabetic Nephropathy

Diabetic nephropathy, one of the most common causes of end-stage renal disease (ESRD), is a microvascular disease characterised by thickening of the GBM, mesangial expansion and glomerular hypertrophy in its early stages followed by mesangiolysis and nodular sclerosis. VEGF expression is upregulated in the early stages of both human [30] and experimental [31] diabetic nephropathy, falling with the progression of sclerosis probably as a consequence of reduced function or indeed loss of VEGF-producing podocytes [32]. This is due to hyperglycaemic (high glucose) and hypertensive (mechanical stretch, increased angiotensin II, Ang II, availability) environments, and in this instance increased VEGF bioactivity is considered to be detrimental to glomeruli.

Unfortunately, animal models of diabetic nephropathy do not resemble human disease very closely because they only tend to develop the early stages of diabetic nephropathy and do not develop histological features of advanced disease or ESRD. In diabetes, NO levels remain low due to a number of factors [see 35]. A breakthrough in an experimental diabetic nephropathy model was achieved by Nakagawa et al. [33], who developed eNOS

knock-out mice in which they induced diabetes. The diabetic eNOS knockout animals developed mesangial expansion, mesangiolysis, glomerular microaneurysms and nodular glomerulosclerosis, which were largely prevented by insulin. This predisposition to diabetic nephropathy was thought to be due to the development of systemic hypertension and heterogeneous afferent arteriolar lesions in the absence of eNOS. The knockout animals were later shown to express more VEGF than wild type, which further increased with the induction of diabetes [34]. Unusually, enhanced VEGF expression was maintained even in severely sclerotic glomeruli. VEGF was shown to act as a chemotactic agent for VEGFR1-positive macrophages and induce their activation and hypertrophy resulting in GEnC injury [34]. VEGF expression and macrophage infiltration was reduced by insulin treatment. The addition of exogenous NO blocked macrophage infiltration at least in part due to negative regulation of macrophage VEGFR1 expression, suggesting that NO can modify the local bioactivity of VEGF through increased availability for VEGFR2. Nakagawa [35] hypothesised that in human disease increased VEGF expression is detrimental because NO bioavailability is reduced in diabetes, leading to the uncoupling of the otherwise protective VEGF-NO signalling pathways. In diabetes, therefore, VEGF bioactivity is too high in the glomerulus and strategies to reduce its activity would be beneficial.

Hypertension

Hypertension is the second most common cause of ESRD, and overactivation of the renin-angiotensin system (RAS), resulting in increased production of Ang II, is important in its pathogenesis. Ang II induces vasoconstriction throughout the body, including the afferent and efferent glomerular arterioles. VEGF upregulation has been demonstrated in two models of hypertension: (1) spontaneously hypertensive rats (SHRs), which have decreased plasma and intrarenal Ang II, and (2) transgenic rats [(mRen2)-27] with increased activity of the RAS system resulting in increased plasma and intrarenal Ang II. VEGF expression was increased further in (mRen-2)-27 rats compared to SHRs, suggesting an additive effect of Ang II to mechanical strain [31]. Interestingly, the blockade of VEGF bioactivity by Zactima® (Vandetanib ZD6474, a potent and selective inhibitor of VEGFR2), resulted in GEnC loss in control animals and led to marked progression of the hypertensive disease. This suggests that glomerular VEGFR2 signalling is essential for the capacity of the glomerulus to respond to stress and that,

Fig. 1. Summary of VEGF availability in different glomerular conditions, some of its cellular regulators and putative interactions between conditions. In pre-eclampsia, there is reduced VEGF and TGF- β bioactivity, which blocks VEGF and TGF- β -mediated vasodilation and endothelial maintenance, but promotes endothelin-1 secretion from GEnC. In diabetic nephropathy, hyperglycaemia increases both VEGF and TGF- β expression, but increased ROS reduces NO availability. This leads to reduced

VEGF-mediated VEGFR2 signalling. In hypertensive environments, stretch and increased Ang II availability increase VEGF expression, leading to increased downstream VEGFR2 signalling. In pre-eclampsia, the hypertensive effects on VEGF are overridden by sFlt-1 which reduces the local bioactivity of VEGF. In diabetic nephropathy, the hypertensive environment is overridden by the combination of the hyperglycaemic environment and oxidative stress.

in contrast to diabetic nephropathy, an increased intrarenal VEGF bioactivity appears to be beneficial. In addition, it was shown that Vandetanib reduced the expression of perlecan mRNA in (mRen2)-27 rats, thus VEGF blockade also had a detrimental effect on GBM components. The increased expression of VEGF by both stretch and Ang II in hypertension is potentially an adaptive response of the VEGF-NO axis (that remains coupled under these circumstances) to increase NO release and counteract vasoconstriction.

In contrast, in a separate study on (mRen2)-27 hypertensive rats, podocyte VEGF expression was increased further by the induction of diabetes with streptozotocin, resulting in GEnC apoptosis. The increased apoptosis was shown to be due to increased TGF- β expression,

which synergistically induced apoptosis with VEGF [36], suggesting that VEGF bioactivity is too high in hypertensive conditions in the presence of a hyperglycaemic environment. The inhibition of PKC (a downstream mediator of Ang II which enhances the dose of both VEGF and TGF- β) by ruboxistaurin reduced both VEGF and TGF- β expression, ameliorating albuminuria and glomerulosclerosis [36]. Ang II not only upregulates VEGF and TGF- β expression, it also upregulates platelet-derived growth factor and tumour necrosis factor, growth factors also associated with diabetic nephropathy and proteinuria [37]. Irbesartan, an Ang II antagonist, blocked the increase in expression of all of these growth factors in glomeruli of uninephrectomised, streptozotocin-induced diabetic rats [37]. These results show that VEGF

bioactivity is critical for maintaining a balance between hypertensive drivers and the adaptive response to hypertension, but that other growth factors also have important roles.

The Paradox of VEGF Expression and Function in Different Conditions

Hypertension often develops early in patients with diabetic nephropathy, yet the reason for this has remained elusive until recently. Using isolated glomeruli and *in vitro* assays, Toma et al. [38] demonstrated that high glucose produced an accumulation of succinate, an intermediate of the TCA cycle, which was also increased in diabetic mouse glomerular tissue. High levels of succinate were shown to activate its G protein-coupled receptor (GPR) 91 on GEnCs and trigger the release of renin from the adjacent juxtaglomerular apparatus. In GPR91^{+/+} mice, microperfusion of the afferent arteriole with glucose from 5.5 to 25.5 mM significantly increased renin release compared with GPR91^{-/-} mice [38], demonstrating the dependence of GPR91 signalling to convey renin release by high glucose. This provides a direct link between glucose levels and activation of RAS, suggesting a possible pathogenic mechanism for the association between diabetic nephropathy and hypertension. Since both glucose and Ang II enhance VEGF expression, and renin stimulates the production of Ang II, this increased renin release by high glucose provides an explanation for why VEGF expression levels may accumulate in hypertensive, diabetic environments.

However, hypertension is also involved in the progression of pre-eclampsia. Therefore, there is a paradox in the contrasting role of VEGF in glomerular conditions with overlapping features; VEGF expression is increased in hypertension, causing vasodilatation, yet VEGF bioactivity is reduced in pre-eclampsia, a condition that involves hypertension, causing vasoconstriction. In addition, in diabetic nephropathy VEGF bioactivity is increased, as in hypertension, yet this causes both inflammation and endothelial cell death. This simplified summary (fig. 1) highlights the contrasting roles of VEGF bioactivity under different circumstances, but these differences can be explained.

Hypertension increases glomerular VEGF (and TGF- β) expression as reviewed recently [39]. There is a large amount of literature describing attempts to establish whether VEGF expression is altered in pre-eclampsia, including analyses of protein levels in maternal serum, um-

bilical chord, and placenta, from before manifestation and during severe pre-eclampsia, but this work remains inconclusive. Nevertheless, it is clear that overall VEGF bioactivity is vastly reduced by sFlt1, suggesting that any effects of hypertension on VEGF expression (and TGF- β) are overridden in pre-eclampsia.

Increased VEGF (and TGF- β) availability reverses the progression of experimental pre-eclampsia, yet in diabetic nephropathy it drives progression of glomerular disease. Interestingly, podocyte-specific inducible overexpression of sVEGFR1 in a streptozotocin diabetic mouse model ameliorated the development of diabetic nephropathy despite enhanced expression of VEGF, by reducing the bioactivity of VEGF and, surprisingly, reducing the expression of TGF- β [40]. In diabetic nephropathy, the situation that appears to drive VEGF-induced injury instead of VEGF-induced vasodilatation is the inactivation of nitric oxide by reactive oxygen species leading to the uncoupling of the VEGF-NO axis [26]. This, in concert with enhanced TGF- β expression, culminates in a negative effect on GEnC (fig. 1).

Conclusions

It is becoming clear that the role of VEGF in glomerular physiology is highly dependent on the bioavailability of other local factors that regulate its bioactivity in relation to what is required. The microenvironment of the glomerulus is dynamic and it is impossible to experimentally control for all variables in glomerular disease; nevertheless, in addition to affecting bioavailability, the presence of VEGF effectors, such as TGF- β , endothelin-1 and NO, may completely change the signalling platform of VEGF. Thus, they will need to be considered in future experimental glomerular VEGF research.

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