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VEGF AND PODOCYTES IN DIABETIC NEPHROPATHY

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Summary

Vascular endothelial growth factor-a (VEGF-A) is a protein secreted by podocytes that is necessary for survival of endothelial cells, podocytes and mesangial cells. VEGF-A regulates slit-diaphragm signaling and podocyte shape via VEGFR2-nephrin-nck-actin interactions. Chronic hyperglycemia-induced excess podocyte VEGF-A and low endothelial nitric oxide drive the development and the progression diabetic nephropathy. The abnormal cross talk between VEGF-A and NO pathways is fueled by the diabetic milieu increased oxidative stress. Recent findings on these pathogenic molecular mechanisms provide new potential targets for therapy for diabetic renal disease.

Keywords

diabetes mellitus; diabetic nephropathy/complications; VEGF-A; endothelial nitric oxide synthase (eNOS); VEGF receptor 2 (VEGFR2); reactive oxygen species (ROS)

VEGF-A is a pleiotropic protein originally described as a vascular permeability and endothelial growth factor.^{1–3} VEGF-A is critical for endothelial cell differentiation, survival, proliferation and migration, as well as for vascular assembly, maintenance and remodeling.⁴ Deletion of the VEGF-A or its receptors genes is embryonic lethal.^{5–7} Conversely, excess VEGF-A drives pathological angiogenesis in multiple diseases, including tumors and diabetes, underscoring the importance and tight regulation of this pathway.⁸

Diabetes mellitus has increased to epidemic proportions in the last decade. Chronic complications of the disease are a major cause of morbidity and mortality. Diabetic nephropathy (DN) develops in approximately 30% of diabetic patients, representing the leading cause of end-stage renal disease worldwide.⁹ Although sustained hyperglycemia drives the clinical course and strict glycemic control decreases the incidence and progression of microvascular complications, genetic, metabolic, hemodynamic and environmental factors significantly influence DN.^{10–13} However, the precise determinants of who among diabetic patients will eventually develop DN are unknown, and the molecular mechanisms involved remain not fully defined. In this review we will focus on vascular endothelial growth factor A (VEGF-A) and podocytes, two central players in the pathogenesis of diabetic nephropathy.

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VEGF-A expression and signaling in podocytes

At difference from other tissues that cease expressing VEGF-A at the completion of development, kidney podocytes and tubular cells express VEGF-A throughout life.^{14,15} Podocytes are the major source of VEGF-A in renal glomeruli. Podocytes synthesize three VEGF-A isoforms (VEGF₁₂₁₋₁₆₅₋₁₈₉) by alternative mRNA splicing.¹⁶ VEGF-A isoforms differences in size, membrane and extracellular matrix binding properties (VEGF₁₂₁ and VEGF₁₆₅ are secreted) enable gradient formation and endothelial cell chemoattraction. VEGF₁₆₅ is the most abundant isoform. Anti-angiogenic isoforms called VEGF_{xxx}b have also been described.¹⁷ VEGF-A binds VEGF receptors 1 and 2 (VEGFR1 and VEGFR2), and co-receptors neuropilin 1 and 2 (NRP1 and NRP2). VEGF-A signals through VEGFR2, NRP1 and NRP2 amplify VEGFR2 signals, while VEGFR1 functions mostly as a decoy.⁸ All VEGF-A receptors are most abundant in endothelial cells but they are expressed by multiple cells, including podocytes and tubular cells (Figure 1).^{16,18,19} Hypoxia and high glucose upregulate podocyte VEGF-A protein expression.²⁰

During kidney organogenesis VEGF-A expressed in the metanephric mesenchyme acts as a chemoattractant for endothelial cells and directs their migration towards developing nephrons.²¹ VEGF-A is required for normal development of glomerular capillaries²² and to acquire their fenestrated phenotype.^{23, 24} In addition, tightly regulated VEGF-A is necessary to establish and maintain normal podocyte phenotype, i.e. foot processes linked by slit-diaphragms, as revealed by both loss and gain of function mouse models.^{25–27} Moderate podocyte VEGF₁₆₄ overexpression during organogenesis disrupts podocyte differentiation, and impairs slit-diaphragm development, resulting in congenital nephrotic syndrome in newborn mice.²⁷ Massive VEGF₁₆₄ overexpression in podocytes resulted in collapsing glomerulopathy in newborn mice.²² After birth podocyte VEGF-A gain of function induces reversible podocyte effacement associated with nephrin down-regulation (a minimal change-like disease) in mice.²⁷ Notably, postnatal moderate podocyte VEGF-A gain of function did not induce glomerular endothelial damage, consistent with VEGF-A trophic effect on endothelial cells, while it caused extensive foot process effacement and proteinuria.^{26,27} These changes were reversible upon removal of the transgene expression, providing evidence of a cause-effect relationship between VEGF-A and foot process effacement.^{26,27} Surprisingly, in adult mice podocyte VEGF-A gain of function caused thickening of the glomerular basement membrane, glomerulomegaly and mesangial proliferation in addition to foot process effacement²⁶, a phenotype strikingly similar to early diabetic nephropathy or Class IIa DN.²⁸ Thus, podocyte VEGF-A gain-of-function induces diverse glomerular phenotypes at specific developmental stages.

VEGF-A is a survival factor for podocytes in culture and in vivo

Podocyte VEGF-A deletion in developing mice resulted in podocyte apoptosis, leading to glomerulosclerosis²², whereas ablation of podocyte VEGF-A in mature mice resulted in thrombotic microangiopathy, associated with podocyte and endothelial cell damage.²⁹ We found that acute podocyte VEGF-A knockdown in mature mice induces down-regulation of fibronectin and α v β 3 integrin, resulting in decreased α v β 3 integrin activity, endothelial cell damage, glomerular basement membrane lamination, foot process effacement and acute kidney injury.³⁰ Upon exposure to VEGF-A murine and human podocytes significantly reduce apoptosis rate by activating Akt signaling.^{16,31,32} VEGF-A-induced protection from apoptosis and activation of the Akt survival pathway were abrogated by anti-VEGFR2 neutralizing antibody, a VEGFR2 kinase inhibitor and bevacizumab (Avastin, Genentech), demonstrating that VEGFR2 transduces VEGF-A autocrine survival signals in podocytes.^{16,32} VEGF-A also activates the PI3K-Akt survival pathway via VEGFR2 reducing apoptosis in endothelial and tubular epithelial cells.^{18,33} VEGF-A autocrine

signaling is required for endothelial cell survival in vivo, a function that is not rescued by paracrine VEGF-A.³⁴ Murine and human podocytes express VEGFR2 in culture and in vivo, as shown by our group and others.^{16, 26, 32, 35} VEGF₁₆₅ induces nephrin and podocyte VEGFR2 tyrosine phosphorylation in vivo, causing foot process effacement without evidence of podocyte loss.²⁶ These VEGF-A gain of function findings are consistent with a report demonstrating that phosphorylated nephrin interacts with the p85 subunit of PI3K and triggers Akt activation, Bad phosphorylation and inhibition, thereby promoting inhibition of detachment-induced podocyte apoptosis.³⁶

VEGF-A regulates slit-diaphragm signaling and podocyte shape via VEGFR2

VEGF-A induces dose-dependent podocin upregulation, increases podocin-CD2AP interaction, downregulates and stimulates nephrin phosphorylation in cultured podocytes.^{16,31,37} Podocyte VEGF-A gain of function in mice causes reversible nephrin downregulation and phosphorylation, associated with foot process effacement and proteinuria.^{26,27} In this model we detected VEGFR2-nephrin interaction and VEGF-induced VEGFR2 phosphorylation, demonstrating a crosstalk between VEGF-A and nephrin signaling pathways in vivo.²⁶ VEGFR2-nephrin interaction was confirmed in podocytes and COS-7 cells transfected with the corresponding plasmids by co-immunoprecipitation and mass spectrometry.³⁷ A direct interaction between VEGFR2 and nephrin cytoplasmic domains was detected by GST-binding assay and overlay assay. VEGFR2-nephrin interaction is modulated by phosphorylation.³⁷ Since nephrin expression in the kidney is limited to podocytes, these findings strongly implicate VEGFR2 signaling in this cell type. In one report VEGFR2 floxed mice carrying nephrin-Cre showed no glomerular phenotype.³⁸ The absence of phenotype might be due to partial VEGFR2 knockdown, given that VEGFR2 heterozygotes are normal,⁶ or Cre-mediated recombination not resulting in protein loss.³⁹ This cannot be ascertained because quantitation of Cre expression in podocytes was not performed to assess whether VEGFR2 ablation was complete.^{38,39} Our laboratory and others demonstrated podocyte VEGFR2 expression in vivo by immunoelectron microscopy and co-immunoprecipitation.^{26,35} We found that podocyte VEGFR2-nephrin binding partners form a multi-protein complex that includes nck and actin.³⁷ Upon VEGF-A stimulation this complex induces significant cell shape change and decreases cell size³⁷, in agreement with the foot process effacement phenotype reported in mice.^{27, 40} Taken together, these findings identify VEGFR2 as a novel component of the slit-diaphragm signaling complex, and support the concept that extracellular VEGF-A signals are transduced to the slit-diaphragm and the podocyte cytoskeleton cell autonomously through the VEGFR2-nephrin-nck-actin complex, thereby regulating cell shape and function. Podocytes generate contractile forces via non-muscle myosins interaction with α -actinin-4 and actin, which are thought to enable dynamic shape adjustments in response to glomerular capillary pressure changes.⁴¹⁻⁴³ In agreement with our findings in podocytes, VEGF-A signaling induces changes in endothelial cell morphology in vitro and in vivo.^{44,45}

The precise mechanism of VEGF-induced nephrin downregulation is not fully defined downstream from VEGFR2. VEGFR2 Y1175 auto-phosphorylation mediates PLC γ binding and leads to PKC activation.⁴⁶⁻⁴⁸ Notably, the effect of hyperglycemia on nephrin- β -arrestin2 interaction, increasing nephrin endocytosis in podocytes, is mediated by PKC α .^{49,50} In endothelial cells PKC α is a negative regulator of VEGF signaling and eNOS phosphorylation.⁵¹ Collectively, these data raise the possibility that VEGFR2 signaling may play an important role in the regulation of nephrin endocytosis through PKC α .

VEGF in diabetic nephropathy

Chronic hyperglycemia stimulates VEGF-A synthesis and secretion, and triggers a series of interconnected metabolic and hemodynamic effects that contribute to VEGF-A increase, and lead to DM microvascular complications (Figure 2). Circulating VEGF-A levels are elevated in adult diabetic nephropathy patients⁵² and in T1D children and adolescents.⁵³ *VEGFA* polymorphisms have been associated with diabetic nephropathy and retinopathy.¹⁰ Since VEGF-A is considered a short-range morphogen, having mostly paracrine, autocrine and intracrine functions⁵⁴, tissue and cell-specific VEGF levels are thought to be important for DM complications. Human kidney biopsies showed high VEGF-A mRNA at early stages of DN, and lower VEGF-A expression in advanced DN, specifically in sclerotic glomeruli and mesangial nodules.^{55, 56} The decline in VEGF-A expression as overt DN progresses is thought to be due to podocyte dropout.^{57, 58} Urinary VEGF-A was elevated in type 2 diabetic patients.⁵⁹ Similarly, in multiple rodent models of DN kidney VEGF-A was found increased at early and late stage of DN.^{57, 60} Urine VEGF-A was elevated in diabetic mice too, bearing no correlation with their albuminuria.⁴⁰

VEGF and renin-angiotensin system

The renin-angiotensin system plays a pivotal role in DN.⁶¹ In addition to Ang II -AT1-mediated glomerular hypertension and albuminuria, Ang II increases VEGF-A, TGF β , and oxidative stress.⁶² Diabetes-induced increase in ACE enhances bradykinin degradation, which does not alter BP but significantly decreases NO availability.⁶³ Transactivation of bradykinin receptor 2 and VEGFR2 induces eNOS activation.^{64, 65} Impairment of this mechanism likely contributes to the severity of DN in mice with bradykinin receptor deletion, and in humans carrying the *ACE*D allele.^{63, 66} By contrast ACE2, which cleaves Ang II into Ang 1–7, is decreased in the diabetic kidney.⁶⁷ *ACE2* deletion enhances albuminuria and hypertension.⁶⁸ In line with these findings, mice overexpressing *ACE2* developed milder DN, had higher Ang 1–7, lower Ang II, decreased VEGF-A, TGF β , collagen IV deposition, oxidative stress and albuminuria.⁶⁹

VEGF, reactive oxygen species and nitric oxide

Oxidative stress in diabetes mellitus results from excess reactive oxygen and nitrogen species (ROS/RNS) derived from the polyol pathway, glucose oxidation, advanced glycation, and mitochondrial electron transfer chain, which are not cleared by antioxidants (SOD, catalase, glutathione peroxidase).^{70, 71} ROS/RNS increase VEGF-A by stabilizing HIF-1 α .⁷² and by activating notch signaling.⁷³ VEGF-A activates eNOS via PI3K/Akt, and thereby stimulates nitric oxide (NO) generation.^{74–76} However, in the presence of superoxide (O₂^{•−}) NO[•] rapidly forms peroxynitrite (ONOO[−]), effectively increasing ROS rather than NO and stimulating guanylate cyclase.⁷⁰ Accumulating evidence suggest that the crosstalk and positive feedback between VEGF-A and NO pathways plays a central role in the pathogenesis of diabetic complications.^{40, 77–79} Indeed, *eNOS* null mice express higher VEGF-A than wild type mice and develop advanced DN, whether diabetes is induced with STZ or by genetic mutations (*Db/Db* or Akita).^{80, 81, 82} A modest decrease in eNOS (~30%), similar to that associated with human *NOS3* polymorphisms linked to severe DN,⁸³ is sufficient to worsen DN in mice.⁸² Surprisingly, oxidative stress decreased in diabetic *eNOS*^{+/-} and ^{-/-} leading to exclude it as a factor in the exacerbation of DN.⁸² In the absence of diabetes, *eNOS* null mutants develop hypertension, micro and macrovascular disease, owing to endothelial damage caused by about 40% decrease in NO availability.^{82, 84}

VEGF and advanced glycation end-products

Advanced glycation end products (AGEs), covalently bound glycosylated proteins and lipoproteins, increase in diabetic humans and animals and contribute to DN pathogenesis.⁸⁵

AGEs induce increased VEGF-A in vitro and in vivo.^{86,87} AGEs bind to several receptors (RAGE and AGE-R1-3) located in multiple renal cell types, including podocytes.^{87,88} AGE-RAGE interaction activates NADPH oxidase, thereby increasing cytosolic ROS, and activates PKC and NF κ B pathways leading to release of VEGF, TGF β and CTGF.^{89,90} Inhibition of NADPH oxidase or PKC α in diabetic rats decreased VEGF-A, superoxide, collagen IV and fibronectin accumulation, albuminuria and glomerulosclerosis.⁹⁰ Consistent with this, RAGE and aldose reductase null mice had lower VEGF and developed milder DN.^{91,92}

VEGF downstream signals in DN

Irrespective of the mechanism driving VEGF-A in DM, its increase disregulates multiple signaling pathways and induces abnormalities that characterize diabetic glomerulopathy (Figure 3). Elevated VEGF-A associates with glomerulomegaly and excessive vessels in mice and humans with DN.^{40,52,93} Although high glucose and TGF β are known to induce cell growth,⁹⁴ several lines of evidence indicate that high local VEGF-A mediates the glomerulomegaly typically observed in DN. First, VEGF neutralizing antibodies block glomerular hypertrophy in diabetic rodents.^{95,96} Second, podocyte VEGF-A gain-of-function induces glomerulomegaly in the absence of diabetes and exacerbates the glomerular enlargement induced by diabetes.^{26,40} Third, VEGF-induced glomerulomegaly is reversible upon removal of the transgene induction.²⁶ Fourth, podocyte VEGF knockdown decreases glomerular volume in non-diabetic mice, and prevents the development of glomerulomegaly in diabetic mice.⁹⁷

Podocyte VEGF-A gain-of function in diabetic mice increases albuminuria ten-fold above control diabetics, by disrupting the glomerular filtration barrier via foot process effacement (Figure 4), nephrin downregulation,⁴⁰ and probably by increasing endothelial fenestration and disrupting cell-cell interactions.⁹⁸ VEGF-A-induced nephrin disregulation is mediated by VEGFR2 signaling³⁷, likely via PKC α activation,^{49,50} even in the absence of podocyte loss.²⁶ However, additional angiogenic factors, such as semaphorin3a, PDGF B, and angiopoietin 2, which are also increased in DN, may contribute to VEGF-A-induced proteinuria in diabetic mice.^{40, 99,100} In the context of high VEGF, angiopoietin2 destabilizes vessels and induces angiogenesis^{78,100}, while excess sema3a causes podocyte effacement and endothelial damage *per se*.^{101,102} Consistent with this possibility, Ang1 deletion worsened DN,¹⁰³ while PDGFR β deletion⁹⁹ and anti-angiogenic factors, such as angiostatin, tumstatin, endostatin and VEGF blockers (neutralizing antibody, kinase inhibitor or sVEGFR1) decreased albuminuria in rodent DN models.^{35,78,95,96,104–108}

VEGF-A induces mesangial proliferation and stimulates TGF β activation, TGF β RII and α 3 collagen IV synthesis by podocytes and mesangial cells.^{109–112} Accordingly, podocyte VEGF-A gain-of-function in diabetic mice resulted in Kimmelsteil-Wilson-like nodular glomerulosclerosis.⁴⁰ High glucose and VEGF-A induce rapid activation of the TGF β latent complex into active TGF β , mediated by MMP2 and MMP9, triggering cell growth and proliferation.⁹⁹ TGF β 1 signaling is thought to be responsible for mesangial cell proliferation and extracellular matrix expansion observed in human and rodent DN.^{28,58,113} VEGF-A and TGF β induce connective tissue growth factor (CTGF), a secreted protein of the CCN family and downstream mediator of TGF β , critically important in fibrosis.¹¹⁴ CTGF is increased in human DN, in rodent models of DN and in mesangial cells exposed to high glucose, TGF β or VEGF-A.¹¹⁵ CTGF is mitogenic for mesangial and endothelial cells, induces synthesis of collagen IV and fibronectin and associates with the extracellular matrix. Interestingly, CTGF binds VEGF₁₆₅ and inhibits VEGF signaling, an interaction cleaved by MMP2 and MMP9.^{116,117} Nitric oxide is a strong repressor of CTGF in mesangial cells, and thereby inhibits mesangial cell proliferation, their adhesion to extracellular matrix, collagen IV and fibronectin synthesis.¹¹⁸ In the context of DN, hyperglycemia, high VEGF, MMP activation

and low NO availability establish a positive feedback loop between TGF β and CTGF, likely to drive glomerular basement membrane thickening and mesangial matrix deposition (Figure 3).

Although ample evidence indicates that ROS, AGEs, Ang II and low NO play pathogenic roles in DN, none of these factors induces a DN phenotype *per se*, in the absence of diabetes, suggesting they act in concert with the diabetic milieu. By contrast, we showed that excess podocyte *Vegf*₁₆₄ in adult mice causes glomerular abnormalities remarkably similar to human early DN and rodent DN models, i.e the triad of glomerulomegaly, mesangial expansion and thickened GBM associated with albuminuria, in the absence of diabetes.²⁶ Reversibility of the phenotype upon removal of the transgene induction provided proof of causality,²⁶ and recapitulated human early DN.⁹³ Notably, excess circulating VEGF₁₆₄ resulting from tubular overexpression stimulates chaotic capillary growth and mesangial proliferation within the glomerular tuft, a characteristic feature of mesangial diabetic nodules.¹¹⁹ Moreover, podocyte *Vegf*₁₆₄ overexpressing mice harboring *eNOS* deletion developed an advanced DN-like phenotype with glomerulosclerosis, massive proteinuria and renal insufficiency in the absence of diabetes (Veron and Tufro, unpublished results), suggesting that excess podocyte VEGF-A signals and low NO may be sufficient to induce advanced DN-like phenotype. Interestingly, deletion of the insulin receptor in podocytes or mesangial *Glut1* overexpression induced mild DN-like phenotype and glomerulosclerosis in the absence of diabetes, suggesting that loss of insulin signaling in podocytes or increased intracellular glucose may play a role in DN pathogenesis.^{120,121} The downstream mechanisms mediating regulation of podocyte function by insulin signaling remain to be established.

For many years, our understanding of DN pathogenesis was centered on mesangial cells, their proliferation and expansion of the extracellular matrix, as a major driving force for the progression of the disease. Endothelial damage was thought to result from hypertension, and podocyte loss was considered a mere consequence of the progression of the disease. The recently recognized pivotal role of VEGF-A and nitric oxide driving the development of typical DN lesions and the progression of the disease has restored some balance to the contributions of the three glomerular cell types to DN. In this paradigm, the diabetic podocyte produces excessive VEGF in the setting of low endothelial NO, stimulates growth and proliferation of mesangial and endothelial cells, leading to increased extracellular matrix accumulation, hyperfiltration and proteinuria. As glomerular damage progresses VEGF may decrease leading to mesangiolysis and endothelial cell damage, exacerbated by dysregulated RAS-driven hypertension and by elevated reactive oxygen species. Based on the pleiotropic effects of VEGF-A and NO, this view of DN pathogenesis fits the vast majority of previous and recent findings in DN animal models, the natural course of the disease in humans, and the known polymorphisms associated with severe DN. Several potential targetable steps in DN emerge from this view, including the regulation of VEGF secretion or receptor binding, bradykinin, NO availability and critical downstream signals such as PKC α and CTGF.

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Figure 1. Expression of VEGFR2 in vivo

Transmission electron microscopy from Flk-1-LacZ⁶ kidney showing LacZ deposits en lieu of VEGFR2 localized to A) foot processes near slit-diaphragm, GBM and fenestrated endothelium; B) podocytes and endothelial cell. Scale bars= 200nm (A) and 1 μm (B). Sample processed as reported.²⁶

Figure 2. Pathways of VEGF-A increase in diabetes

Figure 3. Consequences of VEGF-A increase in diabetic nephropathy

VEGF-A increases TGF β , CTGF and established a positive feedback loop, leading to extracellular matrix (ECM) accumulation and GBM thickening (orange); VEGF-A induces nephrin downregulation and foot process effacement (FPE) (green); VEGF-A stimulates eNOS, which in the setting of high ROS leads to peroxynitrite (ONOO⁻) and further ROS generation, a positive feedback loop (blue), the dashed line represents the normal negative feedback regulation of VEGF-A by NO, not operative in DN. Low NO and high ROS damage endothelial cells and induce HTN.

Figure 4. VEGF-induced thickening and distortion of podocyte foot processes in diabetic mice, observed by scanning electron microscopy. A) Control diabetic glomerulus; B) *Vegf*₁₆₄ overexpressing glomerulus showing wider foot processes. Scale bars=2 μm.