The heparin-binding domain confers diverse functions of VEGF-A in development and disease: a structure–function study

Dominik Krilleke, Yin-Shan Eric Ng and David T. Shima

Ocular Biology and Therapeutics, UCL Institute of Ophthalmology, 11–43 Bath Street, London EC1V 9EL, U.K.

Abstract

The longer splice isoforms of VEGF (vascular endothelial growth factor)-A, including VEGF_{164(165)}, contain a highly basic HBD (heparin-binding domain). This domain allows these isoforms to interact with and localize to the HS (heparan sulfate)-rich extracellular matrix, and bind to the co-receptor Nrp-1 (neuropilin-1). Heparin-binding VEGF-A isoforms are critical for survival: mice engineered to express exclusively the non-heparin-binding VEGF_{120} have diminished vascular branching during embryonic development and die from postnatal angiogenesis defects shortly after birth. Although it is thought that the HBD contributes to the diverse functions of VEGF-A in both physiological and pathological processes, little is known about the molecular features within this domain that enable these functions. In the present paper, we discuss the roles of the VEGF HBD in normal and disease conditions, with a particular focus on the VEGF_{164(165)} Isoform.

Introduction

VEGF (vascular endothelial growth factor)-A is the most prominent member of the PDGF (platelet-derived growth factor)/VEGF family of secreted dimeric growth factors, a family that consists of PI GF (placental growth factor), VEGF-B, VEGF-C, VEGF-D and the virus-derived VEGF-E variants [1,2]. PDGF/VEGF growth factors are pivotal in regulating vascular development in the embryo, as well as the formation of new blood vessels and vessel homeostasis in the adult [3]. VEGF-A is a potent chemoattractant for monocytes, a cell type implicated in pathological angiogenesis, and can activate and induce monocYTE chemotaxis across endothelial cell monolayers [4]. Cellular responses to VEGF are primarily mediated by binding to VEGFR (VEGF receptor)-1 [Flt-1 (Fms-like tyrosine kinase 1)] and VEGFR-2 [KDR (kinase insert domain receptor)/Flk-1 (fetal liver kinase 1)] [5], two structurally similar receptor tyrosine kinases predominantly expressed on vascular endothelial cells [6,7]. There is abundant evidence for unique receptor functions, and co-receptors such as Nrps (neuropilins) and HSPGs (heparan sulfate proteoglycans) can also modulate VEGF-A functions.

VEGF exists as multiple biochemically distinct protein isoforms which differ mainly by their ability to interact with Nrps and HSPGs. The isoforms are produced as a result of alternative mRNA splicing of a common transcript from a single VEGF gene [8]. In humans, although multiple spliced isoforms have been identified, the most common and well-studied isoforms are composed of 121, 165 and 189 amino acids, and the murine homologues lack one amino acid per isoform. All VEGF isoforms share the same N-terminal amino acid sequence, which contains the binding sites for VEGFR-1 and VEGFR-2, but they may or may not contain sequences encoded by exons 6 and 7 in the C-terminus (Figure 1). This C-terminal region encodes two HBDs (heparin-binding domains), each of which confers the ability to bind HS (heparan sulfate) and HSPGs on cell surfaces and basement membranes, thus determining the localization of the VEGF-A isoforms in the extracellular space [9,10]. VEGF_{121}, the shortest isoform, lacks an HBD and is freely diffusible upon secretion. In contrast, the longer splice form VEGF_{189} has two HBDS encoded by exon 6 and exon 7, and is almost completely sequestered in the extracellular matrix and on the cell surface [9,11]. VEGF_{165}, the prototypic and most abundant VEGF isoform, differs from VEGF_{121} by the inclusion of a basic peptide encompassing 44 residues encoded by exon 7. The moderate affinity for heparin enables VEGF_{165} to act as both a soluble and a cell-bound factor. This region also contains binding determinants for Nrp-1, a VEGF_{165} isoform-specific co-receptor that has been shown to be expressed on endothelial cells and certain tumour cells [12]. Nrp-1 is thought to associate with VEGFR-2 upon VEGF_{165} binding, enhancing VEGF-2 activity and signalling [13]. The exact roles of Nrp-1 in VEGF-A function are poorly understood, but there is a growing body of evidence that Nrp-1 may participate in VEGF-A signalling independent of VEGFR-2 [14,15].

Modulation of VEGF-A function by HSPG

The mechanisms by which HSPGs and certain GAGs (glycosaminoglycans) regulate the activity of heparin-binding VEGF-A isoforms have proven to be complex, as these matrix...
Figure 1 | Human VEGF-A isoforms
Schematic representation of the three major VEGF isoforms depicted as monomers. The eight exons of the human VEGF-A are alternatively spliced to produce several distinct isoforms that differ in the presence or absence of exon 6 and 7, the HBDs. Binding sites for heparin and the different receptors are indicated. Arrowhead, plasmin cleavage site. Exons are not drawn to scale.

Figure 1

Role of heparin-binding VEGF isoforms in normal development: lessons from mouse studies
Bioavailability and spatial distribution of VEGF-A can be controlled via production of both soluble and matrix-bound isoforms. A finely balanced VEGF-A concentration gradient consisting of isoforms with differential heparin-binding affinities is necessary for proper blood vessel branching morphogenesis in mice [24]. In the Vegf120/120 mice, which were generated by targeted deletion of exons 6 and 7 of the vegf gene and consequently are devoid of heparin-binding VEGF-A variants [25], the naturally occurring chemotactic VEGF-A gradient and matrix-derived chemoattractive signals of VEGF-A are lacking. As a consequence, migration of sprouting vessel tips is defective, branching complexity is decreased and capillaries are enlarged compared with wild-type animals [24]. VEGF120, despite showing similar potency to VEGF164 in promoting endothelial cell proliferation in vitro, is not sufficient to drive extension of endothelial tip cell filopodia and proper vascular branching. Normal vessel architecture is fully restored, however, in mice engineered to solely express VEGF164 (Vegf164/164) or a combination of both VEGF120 and VEGF189 (Vegf120/189) [26,27]. Thus VEGF164 appears to be sufficient for normal vascular development, but VEGF120, which lacks certain functional properties provided by the HBD in the longer isoforms, is not. The Vegf120/120 mice also exhibit specific defects in developmental bone vascularization [28] and in postnatal angiogenesis of the myocardium [25], embryonic retina [27] and kidney glomerulus [29]. VEGF164 has previously been shown to participate in neuronal patterning in vivo. Using mouse genetics and explant cultures, Schwarz et al. [30] showed that VEGF164 is both necessary and sufficient for the correct migration of facial motor neuron somata by acting as a guidance cue for Nrp-1-expressing motor neurons. This study confirms the importance of the HBD in VEGF-A-mediated vascular and neuronal development.

Role of heparin-binding VEGF-A isoforms in cancer
Deregulated VEGF-A expression contributes to the development of solid tumours by promoting tumour angiogenesis [31]. The pathophysiological roles of the different VEGF-A isoforms appear to be affected significantly by the microenvironment, such as expression of VEGF-A receptors and extracellular matrix components at different anatomical sites of tumours [32]. Various studies have shown that soluble VEGF121 is less effective than heparin-binding VEGF-A isoforms in supporting the neovascularization needed for tumour survival. For example, human glioma cells overexpressing VEGF121 fail to induce tumour progression when implanted in the subcutaneous space in mice, whereas overexpression of VEGF165 and VEGF189 strongly augments neovascularization and tumour growth relative to parental cells [32]. VEGF120 is also incapable of supporting maximal
growth of tumour cells in mice receiving allografts of VEGF-A-null embryonic fibroblasts stably expressing individual VEGF isoforms [33]. In this study, the diffusible VEGF120 recruited peripheral vessels, but was incapable of vascularizing the tumour itself. In contrast, VEGF164 induced both internal and external tumour vascularization, fully rescuing growth of the angiogenesis-deficient parental tumour cells. Furthermore, heparin-binding isoforms may confer a growth advantage in certain human tumours, as altered VEGF-A expression patterns in favour of the longer isoforms have been associated with tumorigenesis and poorer outcomes in certain cancers [34,35]. These data suggest that heparin-binding VEGF-A isoforms may be particularly important in tumour angiogenesis, and that isoform-specific antagonists may specifically target tumour angiogenesis [33,36].

Intraocular disease and the predominant role of the VEGF165(164) isoform

Many studies suggest a causal role of VEGF-A activity in proliferative neovascular pathologies of the eye [37]. In DR (diabetic retinopathy), ROP (retinopathy of prematurity) and the wet form of AMD (age-related macular degeneration), abnormal VEGF-A expression causes uncontrolled neovascular growth and promotes vascular haemorrhages and leakage, conditions that eventually lead to irreversible retinal damage and blindness [37]. During pathological proliferative retinopathy, hypoxia-induced VEGF-A production by ischaemic retinal cells results in aberrant vessel growth that can extend beyond the retinal surface into the vitreous cavity. Among the various isoforms, VEGF164 appears to be preferentially involved in pathological retinal neovascularization. This isoform selectively accumulates in the ischaemic retina in a commonly used animal model of ROP [26,38]. Indeed, VEGF164 isoform-specific inhibition in this model has been shown to be as effective as pan-VEGF-A blockade in preventing neovascularization [26,38]. Although VEGF120 and VEGF164 are equally potent at rescuing retinal neuronal cell apoptosis when injected intraocularly after ischaemic injury, VEGF164 causes oedema and haemorrhage that is not detected in retinas treated with VEGF120 [39]. Taken together, these studies strongly suggest a pathological role for heparin-binding VEGF164 in retinal disorders.

The mechanism by which VEGF164 exerts its pathological function in ischaemic retinal disease may be related to the pro-inflammatory actions of VEGF-A in the presence of the HBD [26]. VEGF164 is likely to be the most pro-inflammatory isoform in the eye, capable of inducing leucocyte recruitment (leukostasis) to the limbal and retinal vascular endothelium [26,40]. On an equimolar basis, VEGF164 more potently induces retinal leukostasis and corneal inflammation than does VEGF120 [40,41]. A comparison of VEGF120 with VEGF164 demonstrated that VEGF164 more potently induces both expression of ICAM-1 (intercellular adhesion molecule-1) and translocation of P-selectin to the surface of HUVECs (human umbilical vein endothelial cells) [40]. Both ICAM-1 and P-selectin are important mediators of vascular inflammation. In the retina, increased levels of VEGF-A and ICAM-1 have been shown to coincide with elevated leucocyte counts during experimental diabetes, with the VEGF164 isoform accounting for 80% of total VEGF-A detected [42,43]. VEGF-A may also act directly on certain cells of the immune system, including monocytes, lymphocytes and dendritic cells, as
these cells have been shown to express active VEGF-A receptors [44,45]. Retinal inflammation and vessel leakage in both early and established diabetes can be significantly reduced when VEGF164 is inhibited by an isoform-specific aptamer antagonist [41]. This is not surprising, given that VEGF165(164) expression predominates over other isoforms in patients with DR and in an animal model of this disease [43,46]. Taken together, these observations suggest that VEGF165(164) is pro-inflammatory and the major pathological isoform in models of neovascular eye disease and in human DR, although the exact mechanistic roles of the HBD remain to be determined.

Molecular characterization of the VEGF164 HBD

The differential functions of the VEGF isoforms suggest that the HBD plays a critical role in modulating VEGF-A isoform activity. Neither the sequence nor the structure of this domain (residues 111–165 of VEGF165) bears any similarity to known heparin-binding proteins outside the VEGF family of growth factors [47]. The HBD comprises 55 residues, with clearly defined N-terminal and C-terminal subdomains (residues 1–29 and 29–55 respectively), each containing a two-stranded, antiparallel, β-sheet and two disulfide bonds which dominate the fold. In addition, a single α-helix is located in the C-terminal subdomain, where it is packed against the β-sheet. The charge of this overall highly basic domain (pI ∼11) is distributed rather unevenly across the surface, with a surplus of positively or negatively charged amino acids on each side of the domain (Figure 2). The loop region at the interface of the two subdomains (residues 11–17) and the relative orientation of the subdomains within the HBD remain poorly defined. The increased flexibility of this domain compared with the rest of the protein complicates accurate prediction of heparin-binding sites within the HBD. Using a mutagenesis approach to identify residues that are critical for mediating heparin binding, a principal heparin-binding site consisting of Arg13, Arg14 and Arg49 (corresponding to Arg123, Arg124 and Arg159 respectively of VEGF165) has recently been proposed [48]. The residues, although non-contiguous in sequence, are located along the interface of the clearly defined N-terminal and C-terminal subdomains, where they form a continuous binding surface. This model is supported by a refined NMR solution structure showing that the heparin-binding residues are in close contact with each other [49]. Spatial proximity of heparin-binding residues in a three-dimensional context is characteristic for many heparin-binding proteins [50].

Mutant VEGF164 variants in which Arg13, Arg14 or Arg49 have been replaced with alanine are severely compromised in their ability to bind to heparin and HS/HSPGs, although they retain wild type-like potency in inducing endothelial gene expression in vitro and angiogenesis in an ex vivo aortic ring assay (Figure 3) [48]. Binding to Nrp-1 was only minimally reduced, so these mutants might provide a tool to help differentiate between Nrp-1 and HS/HSPG effects on VEGF-A signalling. The VEGF164 mutants exhibited similar affinity towards VEGFR-2 [48]. These results suggest that activation of VEGFR-2 is responsible for the proliferative activities of VEGF-A on endothelial cells, and that the HBD does not play a direct role in VEGFR-2 activation. However,
the mutants exhibited a reduced binding affinity for VEGF-R1, which is also expressed by leucocytes and mediates VEGF-induced migration [44]. The increased affinity of VEGF164 for VEGF-R1 relative to VEGF120 has previously been linked to the presence of the HBD [10], and the data from the HBD mutants confirm such a functional connection. It is possible that the HBD contributes to the binding energy of the VEGF-R1–VEGF-A interaction. VEGF-A, in particular the isoforms containing the HBD, may act as a chemoattractant for inflammatory cells by ligating and stimulating VEGF-R1. As a result, elevated local VEGF164 concentrations may provide recruitment signals for VEGF-R1-expressing inflammatory cells. It will be interesting to see whether the non-heparin-binding VEGF164 HBD mutants, which bind to VEGF-R1 with lower affinity, have reduced pro-inflammatory activities compared with wild-type VEGF164 and activities similar to VEGF120 in various disease models.

Conclusions

The studies described above highlight the importance of understanding the specific contributions and interplay of the different VEGF-A isoforms in normal physiology and in vascular pathologies. The pathobiology of the VEGF-A HBD remains a matter of considerable interest, and there is growing evidence that the heparin-binding isoforms have additional functions beyond the endothelium, influencing neural and immune cells. The HBD may represent a suitable therapeutic target for pathologies associated with VEGF165 overexpression. Future studies should address the mechanism of action of the HBD in VEGF-A–induced pathologies, and the feasibility of targeting the pathological functions of the HBD for more effective and safer anti-VEGF-A therapy.

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References


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