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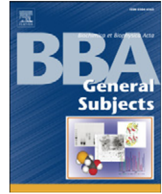

The impact of the receptor binding profiles of the vascular endothelial growth factors on their angiogenic features

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ABSTRACT

Background: Vascular endothelial growth factors (VEGFs) are potential therapeutic agents for treatment of ischemic diseases. Their angiogenic effects are mainly mediated through VEGF receptor 2 (VEGFR2).
Methods: Receptor binding, signaling, and biological efficacy of several VEGFR2 ligands were compared to determine their characteristics regarding angiogenic activity and vascular permeability.
Results: Tested VEGFR2 ligands induced receptor tyrosine phosphorylation with different efficacy depending on their binding affinities. However, the tyrosine phosphorylation pattern and the activation of the major downstream signaling pathways were comparable. The maximal angiogenic effect stimulated by different VEGFR2 ligands was dependent on their ability to bind to co-receptor Neuropilin (Nrp), which was shown to form complexes with VEGFR2. The ability of these VEGFR2 ligands to induce vascular permeability was dependent on their concentration and VEGFR2 affinity, but not on Nrp binding.
Conclusions: VEGFR2 activation alone is sufficient for inducing endothelial cell proliferation, formation of tube-like structures and vascular permeability. The level of VEGFR2 activation is dependent on the binding properties of the ligand used. However, closely similar activation pattern of the receptor kinase domain is seen with all VEGFR2 ligands. Nrp binding strengthens the angiogenic potency without increasing vascular permeability.
General significance: This study sheds light on how different structurally closely related VEGFR2 ligands bind to and signal via VEGFR2/Nrp complex to induce angiogenesis and vascular permeability. The knowledge of this study could be used for designing VEGFR2/Nrp ligands with improved therapeutic properties.
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1. Introduction

VEGF-A was originally isolated as the factor causing vascular permeability in tumors [1] and it was later found to be one of the most important mediators of vasculogenesis and angiogenesis [2]. Since then, several related family members have been identified from humans [VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PlGF)] [3], viruses (VEGF-E proteins) [4] and snake venoms (VEGF-F proteins) [5]. Anti-angiogenic drugs targeting VEGF system are already on the market and pro-angiogenic therapies to treat ischemic diseases are under development [6,7].

VEGF receptor 2 (VEGFR2) is considered the main receptor mediating VEGF family-induced angiogenesis [8,9]. VEGFR2 is a receptor tyrosine kinase, which upon activation auto/trans-phosphorylates its tyrosine residues. This triggers the activation of several downstream signaling cascades leading to different cellular functions including survival, proliferation, and migration, all required for angiogenesis [10]. VEGFR2 phosphorylation sites have been mapped to Tyr-951, Tyr-996, Tyr-1054, Tyr-1059, Tyr-1175, and Tyr-1212 [11,12]. The main downstream pathways activated after VEGFR2 stimulation by VEGF-A include PI3K pathway leading to endothelial cell survival via Akt [13] and increased NO production via eNOS [14,15], and phospholipase Cγ (PLCγ)–MAPK pathway leading to cell proliferation via p44/p42 MAPK (Erk1/2) [16]. Src activation has been suggested leading to vascular permeability via disruption of vascular endothelial (VE)-cadherin-mediated intercellular junctions [17] in a VEGF/T-cell-specific adapter

Abbreviations: VEGF, vascular endothelial growth factor; VEGFR2, VEGF receptor 2; Nrp, Neuropilin; PlGF, placenta growth factor; PLCγ, phospholipase Cγ; VE-cadherin, vascular endothelial cadherin; TSAd, T-cell-specific adapter; HSPGs, heparan sulfate proteoglycans; Hsp27, Heat shock protein 27; His-tag, hexahistidine tag; sNrp1-Fc, soluble Nrp1 IgG Fc fragment fusion protein; HEK293T cells, human embryonic kidney 293T cells; PAE cells, porcine aortic endothelial cells; HUVECs, human umbilical vein endothelial cells

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(TSAd)-dependent manner [18]. VEGFR1 is known to act as a regulator of vasculature formation during development as an inactive decoy receptor [19], but its role in adult organisms is still controversial. VEGFR3 mediates mainly lymphangiogenesis, but it has recently been suggested also having a role in angiogenesis in tip to stalk cell conversion [20].

VEGFR2 ligands induce angiogenesis in various in vitro and in vivo models [21–23]. Increased vascular permeability and the following edema are the severe dose limiting side-effects of therapies aiming for therapeutic vascular growth using VEGF gene transfer [21,24,25]. Therefore, it would be highly beneficial for therapeutic purposes if the vascular permeability effect and angiogenesis could be separated. There are several reports implying that vascular permeability caused by VEGFs is mainly mediated by VEGFR2: 1) VEGF-A induces a rapid increase in vascular permeability at low concentrations, whereas VEGFR1 specific ligands PlGF and VEGF-B do not increase permeability [26–28]. 2) A mutant of VEGF-A that is specific for VEGFR2 retains the vascular permeability increasing activity, whereas VEGFR1 specific mutant does not increase permeability [29]. 3) VEGFR3 specific VEGF-C mutant does not induce vascular permeability [30]. However, there are also conflicting reports showing that actually VEGFR1 would be either solely responsible [31] or have a synergic effect [32] with VEGFR2 for the induction of vascular permeability.

VEGF/VEGFR system is further regulated by Neuropilin (Nrp) co-receptors (Nrp1 and Nrp2) and heparan sulfate proteoglycans (HSPGs). Alternative splicing of mRNA and proteolytic modifications are able to regulate the binding of VEGFs to these co-receptors. Nrp1 is required for angiogenesis as Nrp1 deficient mice are embryonically lethal due to cardiovascular defects [33]. In endothelial cells, Nrp1 functions likely by aiding the formation or stabilizing the VEGF/VEGFR2 complexes [34]. It has also been suggested that Nrp1 may have its own signal transduction mechanisms not mediated by VEGFR2 [35]. The role of HSPGs is to function as a reservoir of growth factors in tissues and to participate in the formation of growth factor concentration gradients [36]. Despite intensive research, the exact mechanism on how Nrp:s contribute to VEGF signaling and function has not been established.

The three-dimensional structures of VEGF family members have been extensively studied [37]. Despite a relatively low conservation of the primary structures, the three-dimensional structures of VEGFs share a highly conserved VEGF homology domain. This domain mediates the binding to VEGFR2 Ig like domains 2 and 3 by similar mechanisms shared by all VEGFR2 ligands [38]. Binding to Nrp:s is not dependent on such a defined homologous domain, as protein domains with no apparent homology can bind to Nrp:s. Most of the VEGF proteins binding to Nrp:s, however, have a rather short C-terminal sequence that fits into a binding pocket in Nrp b1 domain [39].

The key question is how the minor differences in the structure and co-receptor binding profiles of the different natural and modified VEGF family members affect the biological outcomes of the ligand–receptor interactions and if this knowledge could be used to design therapeutics for either anti- or pro-angiogenic applications. To answer these questions we compared a panel of VEGFR2 ligands to evaluate their properties regarding: 1) receptor binding and intracellular trafficking, 2) the activation of signaling pathways, 3) the stimulation of cellular proliferation, 4) the stimulation of tubulogenesis, and 5) the stimulation of vascular permeability. It was found that the ligands differed from their VEGFR2 binding affinities and VEGFR2 activation kinetics, but the induced phosphorylation pattern was similar. The VEGFR2 ligands that also bind to Nrp had the strongest angiogenic power measured as cell proliferation and formation or tube-like structures, whereas either Nrp or VEGFR1 binding did not have additive effect on acute vascular permeability.

2. Materials and methods

2.1. Antibodies and proteins

Antibodies against VEGFR2, phospho-VEGFR2 (Tyr-1175), phospho-VEGFR2 (Tyr-951), phospho-VEGFR2 (Tyr-996), phospho-VEGFR2 (Tyr-1212), Heat shock protein (Hsp) 27, phospho-Hsp27 (Ser82), p44/48 MAPK (Erk1/2), phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), eNOS, and phospho-eNOS (Ser1177) were all from Cell Signaling Technologies. Phospho-VEGFR2 (Tyr1054/Tyr1059) was purchased from Abcam and Nrp (C-19) antibody from Santa Cruz Biotechnology. Alexa Fluor 488 donkey anti-goat antibody came from Invitrogen and Donkey anti-rabbit rhodamine from Jackson ImmunoResearch. VEGF-A_{111} and sVEGFR1-Fc proteins were obtained from R&D Systems. Nrp antagonist EG00229 was produced by Ark Therapeutics.

2.2. Cloning of the expression vectors

The sequence coding for IL-3 signal peptide and a hexahistidine tag (His-tag) was cloned into pDonr201 vector (Invitrogen). The open reading frames of snake venom Vammin, human VEGF-A_{165}, human VEGF-A_{121} and chimeras 9 and 33of Orf virus-derived VEGF-E_{NZ7} and human PlGF (chimeric VEGF-E $_{NZ7}$/PlGF plasmids were received as generous gifts from Dr. Masabumi Shibuya) were cloned into this vector to generate cDNA:s encoding VEGF proteins with an N-terminal His-tag under IL-3 signal peptide. VEGF-A_{165}-His protein was generated by cloning tPA signal sequence into pDonr201 vector (Invitrogen) and subsequent ligation of a cDNA encoding human VEGF-A_{165} with His-tag in the protein C-terminus. Clones were completely sequenced to verify the sequences before cloning into pBVboostFG system expression vector [40], and recombinant baculoviruses were generated as described earlier [41]. The inserts encoding human soluble Neuropilin-1-IgG Fc fragment fusion protein (sNrp1-Fc) and human sNrp2-Fc were amplified from pIgPlus-hNRP1 and pIgPlus-hNRP2 plasmids and subcloned into a pAdCMV expression vector. Vectors encoding human VEGF-$D^{\Delta N \Delta C}$, human sVEGFR2-Fc, and human VEGF-A_{121}-His were generated as described previously [42]. Human VEGF-C and human sVEGFR3-Fc were prepared as described previously [43].

2.3. Protein expression and purification

Recombinant proteins were produced using recombinant baculoviruses in High Five™ cells (Invitrogen) or in transfected human embryonic kidney 293T cells (HEK293T). Purification was done from clarified culture media using BD Talon Metal Affinity Resin (Clontech). The resin was agitated in clarified medium for 2 h and packed into chromatography columns for washing (20 mM $NaPO_4$ and 500 mM NaCl, pH 7.4) and elution (20 mM $NaPO_4$, 500 mM NaCl, 200 mM imidazole, pH 7.4). Polishing was done with HisTrap™ HP column (GE Healthcare) in 20 mM $NaPO_4$ and 500 mM NaCl. Prior to washing, the buffer was supplied with 60 mM imidazole and the proteins were eluted using a stepwise imidazole gradient from 40 mM to 500 mM. The Fc-fusion proteins were purified with Protein A Sepharose™ Fast Flow (GE Healthcare) using the washing buffer containing 20 mM $NaPO_4$ and 100 mM NaCl, pH 7.0, and elution buffer 100 mM Na-citrate and 100 mM NaCl, pH 3.5. 1 M Tris–HCl, pH 9, was used to neutralize the pH. Buffer was changed to 20 mM $NaPO_4$ and 100 mM NaCl, pH 7.4, with HiTrap Desalting column (GE Healthcare). Protein purity was verified using SDS-PAGE and Coomassie staining.

2.4. Cell culture

Porcine aortic endothelial (PAE)-KDR cells [44] were cultured in F12 nutrient mixture (Invitrogen) supplemented with 10% FBS, 1% penicillin/streptomycin (Sigma), and 400 μg/ml of G418 (InvivoGen). Human umbilical vein endothelial cells (HUVECs) isolated from umbilical cords obtained from the maternity ward of Kuopio University Hospital with the approval of local Ethics Committee were used at early (I–V) passages and grown on plastic surface coated with 0.05% gelatin/10 μg/ml fibronectin (Sigma) in EBM Endothelial Cell Basal Medium supplied with EGM SingleQuots (Lonza). For experimental purposes, confluent cells were pre-incubated overnight with serum-free F12

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