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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.

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

medium for PAE-KDR cells or 0.05% FBS in MCDB131 medium (Sigma, St. Louis, MO) for HUVECs prior to addition of growth factors. HEK293T cells were purchased from ATCC and maintained in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich), supplemented with 10% FBS and 1% penicillin/streptomycin (Sigma).

2.5. Western blotting

After treatments, cells were washed with ice-cold phosphate-buffered saline and lysed using ice-cold lysis buffer [50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10% glycerol, PhosSTOP and protease inhibitor tablets (Roche Applied Science)]. The lysates were clarified by centrifugation (10,000 ×g at 4 °C for 15 min), and the total protein concentrations were determined with BCA Protein Assay kit (Thermo Scientific). The lysates were adjusted to the same total protein concentration, supplemented with SDS-PAGE sample buffer, and heated at 95 °C for 5 min. Proteins were separated using SDS-PAGE, followed by transfer to nitrocellulose membranes (Pure Nitrocellulose Membrane, Bio-Rad). Membranes were blocked in 5% w/v BSA in 1× TBS and 0.1% Tween 20 and incubated with primary antibodies overnight at 4 °C. HRP-conjugated secondary antibodies (Pierce), a chemiluminescent-based detection system (SuperSignal West Dura Extended Duration Substrate, Pierce), and CL-XPosure films (Thermo Scientific) were used.

2.6. Immunoprecipitations

The phosphorylation of VEGFR2 Tyr-951, Tyr-996, Tyr-1054/1059 and Tyr-1212 was analyzed from PAE-KDR cells and co-precipitation of Nrp1 and VEGFR2 was done from HUVECs. Whole cell extracts were prepared in lysis buffer and pre-cleared with 50 μl of Protein G Agarose (GE Healthcare) for 1 h at 4 °C. The lysates were incubated with 5 μl of antibody and 50 μl of Protein G Agarose overnight at 4 °C with rotation. Precipitated complexes were washed three times in lysis buffer and eluted in SDS-PAGE sample buffer at 95 °C for 5 min.

2.7. Binding assays

Relative VEGFR binding affinities were measured with solid phase competition assays. For VEGFR1 and VEGFR2 binding assays sVEGFR1-Fc and sVEGFR2-Fc proteins were pre-incubated with a dilution series of VEGF proteins and the mixture was transferred to the VEGF-A_{165} coated plate. For VEGFR3 binding assay sVEGFR3-Fc protein was pre-incubated with a dilution series of VEGF proteins and the mixture was transferred to a VEGF-C coated plate. Nrp1 and Nrp2 binding was analyzed by coating 96-well plates with the analyzed VEGF proteins. A dilution series of sNrp1-Fc and sNrp2-Fc proteins was incubated on the plates. Nrp antagonist EG00229 (Ark Therapeutics Group Plc) was used to inhibit sNrp1-Fc binding to VEGF-A_{165} and Vammin coated plates. Bound soluble receptors were detected by incubating the plates with an anti-human IgG-HRP (Sigma) and detected using TMB reagent (Sigma) by measuring absorbance at 450 nm and 650 nm and subtracting 650 nm readings from 450 nm readings. IC_{50} values were determined by fitting sigmoidal dose response curves to the data by non-linear regression in Prism 5 (GraphPad Software Inc.).

2.8. Cell proliferation, in vitro angiogenesis and immunofluorescence assays

HUVECs were seeded onto 96-well plates at 10,000 cells/well. After 24 h, MCDB131 medium (Sigma, St. Louis, MO) supplemented with 0.1% BSA (Sigma) was changed to the wells. Cells were allowed to synchronize for 16 h. Cellular proliferation was stimulated by the addition of indicated concentrations of recombinant VEGFs to the wells for 48 h. Relative amount of living cells in the wells was measured with MTS-reagent (Promega, Madison, WI) according to the manufacturer's instructions. In vitro angiogenesis assay for evaluating tube-like structures was performed using V2a kit (TCS Cellworks). For the quantification of the tubules, 12 pictures were taken from the same sites in each well using a guiding grid. The area of the tube-like structures was quantified from the pictures using Angiosys (TCS Cellworks) software. For the immunofluorescence stainings, HUVECs were stimulated with the recombinant proteins, fixed with 4% PFA/PBS and immunostained with anti-VEGFR2 and anti-Nrp1 antibodies. Statistical analysis was done with One-way ANOVA and Bonferroni's corrected *t*-test. $P < 0.05$ was considered statistically significant.

2.9. Miles assay

A Miles assay for analysis of vascular permeability in the rabbit skin was performed as described earlier [45].

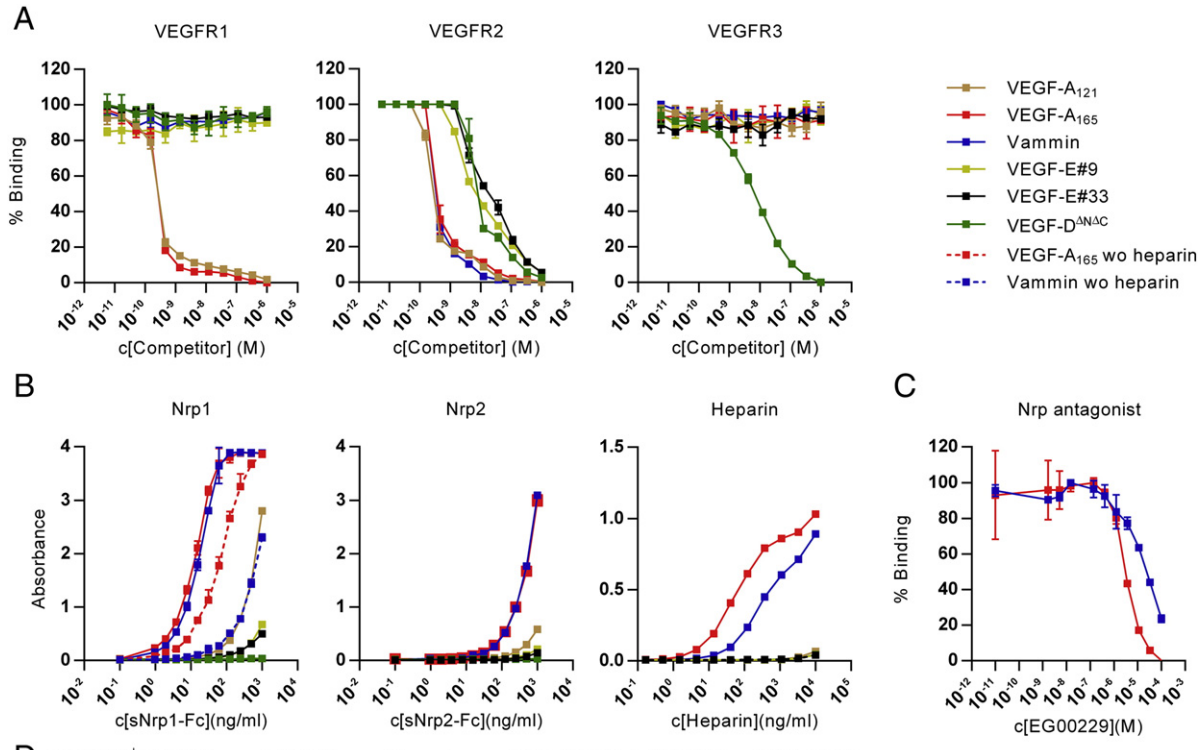
3. Results

3.1. Receptor binding profiles of VEGFs

Several VEGFR2 ligands, all potential candidates for therapeutic applications, were selected for this study to cover a wide range of different VEGF receptor binding profiles. Previously, it has been demonstrated that VEGF-A isoforms VEGF-A_{121} and VEGF-A_{165} are ligands for VEGFR1 and VEGFR2 [46,47]. They differ from Nrp binding affinity [48], and only VEGF-A_{165} binds to HSPGs [49]. VEGF-$D^{\Delta N\Delta C}$ is a ligand for VEGFR2 and VEGFR3 [50], and the engineered VEGF-E_{NZ7}/PlGF chimeras and Vammin are VEGFR2 specific [51,52]. VEGF-E_{NZ7}/PlGF chimeras have been reported being especially weak inducers of vascular permeability despite their angiogenic activity [53]. To compare these ligands side by side in the same setting, we determined the receptor binding profiles of the recombinant proteins using solid phase assays (Fig. 1). VEGFR1 bound both VEGF-A isoforms with high affinity and VEGFR2 bound to all selected VEGF ligands. Both VEGF-A isoforms (IC_{50} 2.7×10^{-10} M for VEFG-A_{121} and 3.7×10^{-10} M for VEGF-A_{165}) and Vammin (IC_{50} 3.6×10^{-10} M) had similar and markedly higher VEGFR2 binding affinity than VEGF-E_{NZ7}/PlGF chimeras (IC_{50} 6.7×10^{-9} M for chimera 9 and 1.7×10^{-8} M for chimera 33) and VEGF-$D^{\Delta N\Delta C}$ (IC_{50} 7.5×10^{-9} M) dividing the proteins into high and low VEGFR2 affinity groups (Fig. 1A and B). VEGF-A_{165} and Vammin both bound with similar affinity to Nrp1 whereas VEGF-A_{121} had an intermediate binding capacity. Both VEGF-E_{NZ7}/PlGF chimeras showed some degree of Nrp1 binding whereas VEGF-$D^{\Delta N\Delta C}$ did not bind to Nrp1. A similar but weaker ligand binding affinity profile was observed for Nrp2. In addition, VEGF-A_{165} and Vammin were able to bind biotinylated heparin. Since VEGFR binding assays were all performed in the presence of heparin (100 ng/ml), we also tested VEGF-A_{165} and Vammin binding to Nrp1 in its absence. The result showed decreased binding with both proteins, but the decrease was the most evident in the case of Vammin (Fig. 1B). The binding of both VEGF-A_{165} and Vammin to Nrp1 was prevented by a small molecule Nrp antagonist EG00229 [54] that competes for the VEGF binding pocket occupation in the B1 domain of Nrp (Fig. 1C).

3.2. VEGFR2 activation and downstream signaling

To determine the capability of the VEGFs to induce the activation of the VEGFR2 tyrosine kinase, we performed a series of phosphorylation assays (Fig. 2). The maximal tyrosine phosphorylation of Tyr-1175 was seen with the high affinity VEGFR2 ligands at 10–50 ng/ml whereas the low affinity ligands required concentrations starting from 250 ng/ml (Fig. 2A). The subsequent characterization of the ability of these ligands to induce the phosphorylation of the other VEGFR2 tyrosine residues was performed using the concentrations yielding the maximal phosphorylation of Tyr-1175. All ligands were able to induce the phosphorylation of Tyr-951, Tyr-996, Tyr-1054/1059, and Tyr-1212 (Fig. 2B). The high affinity ligands VEGF-A_{165} and Vammin showed faster receptor



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IC50 (M)	VEGF-A_{121}	VEGF-A_{165}	Vammin	VEGF-$D^{\Delta N\Delta C}$	VEGF-E#9	VEGF-E#33
VEGFR1	2.7×10^{-10}	2.8×10^{-10}				
VEGFR2	2.7×10^{-10}	3.7×10^{-10}	3.6×10^{-10}	7.5×10^{-09}	6.7×10^{-09}	1.7×10^{-08}
VEGFR3				8.9×10^{-09}		

Fig. 1. The receptor binding profile of the recombinant VEGF proteins. (A) Competitive binding curves of soluble VEGFR-Fc fusion proteins to VEGF-A (VEGFR1 and VEGFR2) or VEGF-C (VEGFR3) coated plates. The values are expressed as mean ± SD. (B) Binding curves of soluble Nrp-Fc proteins and Heparin–biotin to recombinant VEGF protein coated plates. The values are expressed as mean ± SD. (C) Inhibition of the binding of sNrp1-Fc to VEGF-A_{165} and Vammin coated plates by a small molecule Nrp antagonist. The values are expressed as mean ± SD. (D) Determined IC_{50} values of the recombinant VEGF proteins for VEGFR binding.

activation kinetics than the low affinity VEGF-$D^{\Delta N\Delta C}$. The high affinity ligand signal peaked already at 5 min whereas the signal of VEGF-$D^{\Delta N\Delta C}$ peaked at 15 to 30 min (Fig. 2C). The downstream signaling was studied by the determination of the phosphorylation status of Erk1/2, eNOS and Hsp27 (Fig. 3). Similar activation of these pathways was obtained using all VEGFR2 ligands when they were used at concentrations determined earlier to lead to equal VEGFR2 activation.

3.3. Biological activity of VEGFR2 ligands

To determine the biological activity of VEGFs on endothelial cells, serum starved HUVECs were grown in the presence of VEGF proteins (Fig. 4). All VEGFs induced HUVEC proliferation, but Nrp binding forms VEGF-A_{165} and Vammin were the strongest ligands with statistically significant differences to weakly Nrp binding VEGF-A_{121} and Nrp

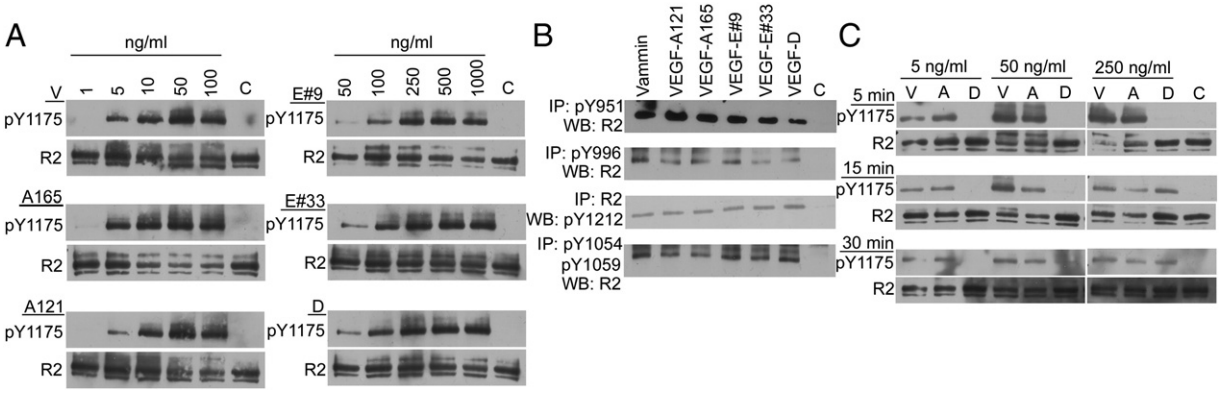

Fig. 2. VEGFR2 activation. (A) HUVECs were stimulated with Vammin (V), VEGF-A_{165} (A_{165}), VEGF-A $_{121}$ (A_{121}), VEGF-E $_{NZ7}$/PlGF chimeras 9 and 33 (E#9 and E#33), VEGF-$D^{\Delta N\Delta C}$ (D), or with medium (C) for 15 min. Whole cell lysates were prepared and immunoblotted with antibodies against VEGFR2 phosphorylated at Tyr-1175 or total VEGFR2. (B) PAE-KDR cells were stimulated with 50 ng/ml Vammin, VEGF-A_{121}, and VEGF-A $_{165}$, or with 500 ng/ml VEGF-E/PlGF chimeras 9 and 33 (VEGF-E#9 and VEGF-E#33) and VEGF-$D^{\Delta N\Delta C}$, or with medium (C) for 15 min. Whole cell lysates were immunoprecipitated (IP) and immunoblotted (WB) with the indicated antibodies. (C) HUVECs were stimulated with Vammin (V), VEGF-A_{165} (A), VEGF-$D^{\Delta N\Delta C}$ (D), or with medium (C). Whole cell lysates were prepared and immunoblotted with antibodies against VEGFR2 phosphorylated at Tyr-1175 or total VEGFR2.

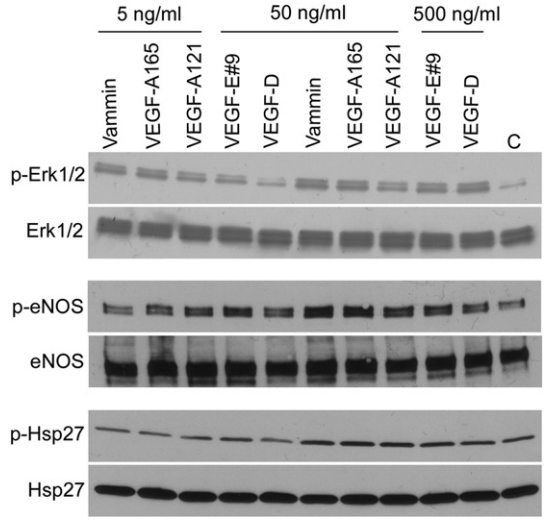

Fig. 3. VEGFR2 downstream signaling. HUVECs were incubated with different VEGFs or with medium (C) for 15 min. Whole cell lysates were prepared and immunoblotted with antibodies against Erk1/2 phosphorylated at Thr-202/Tyr-204, eNOS phosphorylated at Ser-1177, or Hsp27 phosphorylated at Ser-82. Equal loading was confirmed using antibodies against total Erk1/2, eNOS, or Hsp27.

non-binding ligands at 10 ng/ml and 100 ng/ml concentrations. Increasing the concentration of the ligands not binding to Nrp was not able to induce as high response, but, instead, the effect saturated to a lower level. The high efficacy of VEGF-A_{165} and Vammin was repeated with statistical significances in the in vitro angiogenesis assay at 1 ng/ml concentration, and a clear trend was also seen with the higher concentrations. VEGF-A_{121} showed an intermediate activity whereas VEGF-$D^{\Delta N\Delta C}$ and VEGF-E_{NZ7}/PlGF chimeras had a minor effect. Also, in this assay, the weaker activity of the low affinity VEGFR2 ligands was not compensated by increasing their concentrations (Fig. 4A). To asses if this was due to the lack of Nrp binding, we generated the Nrp binding deficient mutants of VEGF-A_{121} and VEGF-A_{165} by blocking their free C-terminus, required for Nrp binding [39], with a His-tag. Mutant proteins had unchanged VEGFR2 binding capability, but were devoid of Nrp1, Nrp2, and heparin binding (Fig. 4D). When compared with the wild type proteins in the in vitro angiogenesis assay, VEGF-A_{165}-His and VEGF-A_{111}, totally lacking the Nrp binding domain, were found to have a statistically significantly reduced activity compared to VEGF-A_{165} at 1 ng/ml and 10 ng/ml concentrations. Also, there was a clear trend in the reduced ability of VEGF-A_{121}-His to form tube-like structures compared to VEGF-A_{121}. The reduced activities of the mutant proteins were not compensated by an increasing concentration of the growth factor except in the case of VEGF-A_{165}-His (Fig. 4B). Based on visual inspection, the VEGF-A forms capable of binding to Nrp were able to induce more sprouting (Fig. 4C).

3.4. Acute vascular permeability

The capability of the VEGF proteins to induce acute vascular permeability was evaluated using Miles assay in rabbit skin (Fig. 5). For the assay, the recombinant VEGF proteins were divided into a high affinity group (VEGF-A isoforms and Vammin) used with doses from 1 ng to 500 ng and a low affinity group (VEGF-$D^{\Delta N\Delta C}$ and VEGF-E_{NZ7}/PlGF chimeras) used with doses from 100 ng to 10000 ng. All proteins induced acute vascular permeability when sufficient doses were used

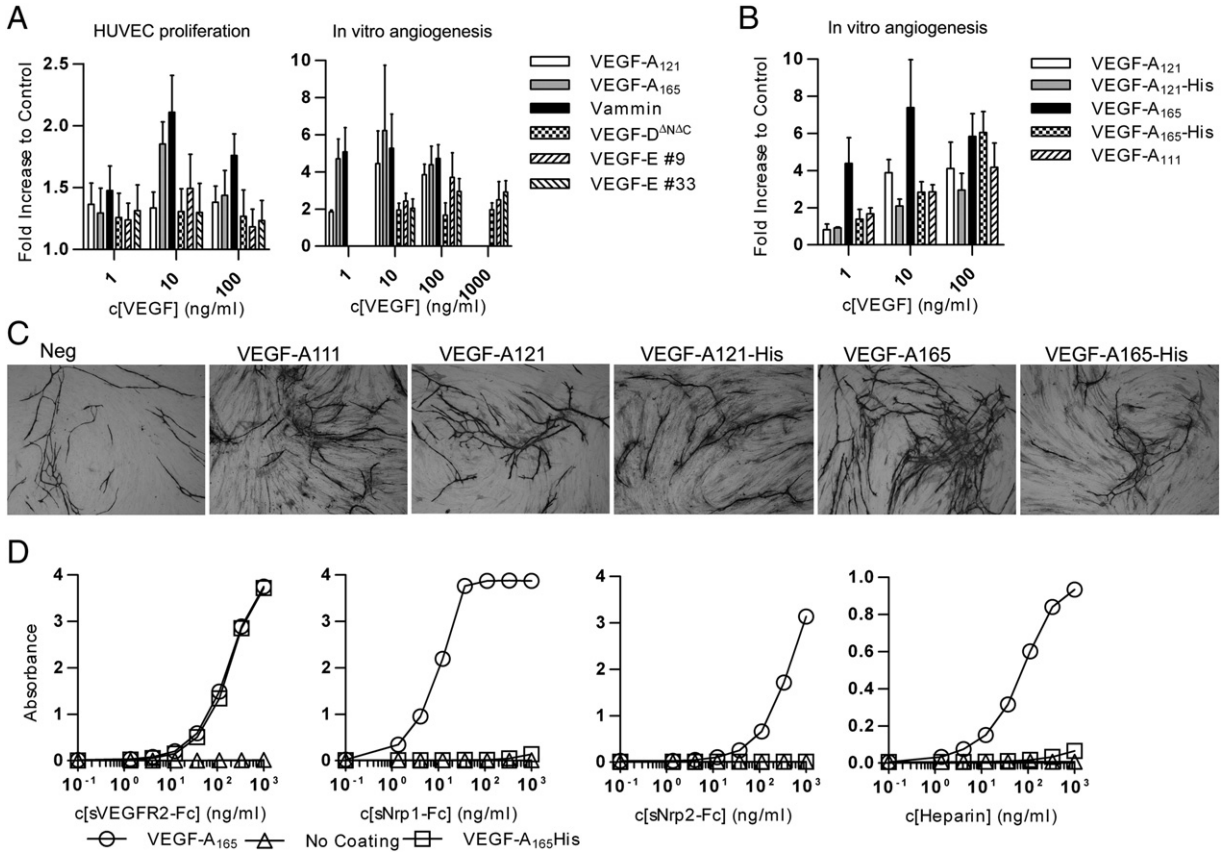

Fig. 4. The biological activity of the recombinant VEGF proteins. (A) HUVEC proliferation and in vitro angiogenesis assay for evaluating tube-like structures with recombinant VEGF proteins. Data represents mean fold changes ± SD. (B) In vitro angiogenesis assay for evaluating tube-like structures with recombinant VEGF-A mutant proteins. Data represents mean fold changes ± SD. (C) Illustrative pictures of the angiogenic sprouts induced by 10 ng/ml recombinant VEGFs in in vitro angiogenesis assay. (D) Comparison of the receptor binding profiles of VEGF-A_{165} and VEGF-A_{165}-His. The values are shown as mean ± SD.

(Fig. 5A). The determined relative VEGFR2 binding affinities and VEGFR2 activation profiles correlated with the ability of these proteins to induce acute vascular permeability (Fig. 5B). Notably, the two VEGF-A isoforms with different Nrp binding properties had practically identical responses.

3.5. VEGFR2/Nrp complex formation

As the interaction of Nrp with VEGFs seemed to be essential to obtain the maximal angiogenic effect, its mechanism was further studied by confocal microscopy (Fig. 6). In unstimulated cells, VEGFR2 was typically found mainly on the plasma membrane and intracellularly in the Golgi region and Nrp1 was found both on the plasma membrane and in intracellular vesicles. The plasma membrane-derived VEGFR2 was internalized after treatment with VEGFs. The high affinity ligands (VEGF-A isoforms and Vammin) induced efficient internalization already at 15 min time point and at 30 min time point the receptor was found mainly in intracellular vesicles (Fig. 6A), which was verified by the quantification of VEGFR2 intensity in internalized vesicles (Fig. 6B). Nrp1 was internalized after treatments with VEGF-A_{165} and Vammin but only very weakly with other ligands (Fig. 6A). The internalized Nrp1 co-localized with VEGFR2 in the intracellular vesicles, which was verified by the quantification of Nrp1 intensity inside VEGFR2 positive vesicles (Fig. 6B). The formation of VEGFR2/Nrp1 complexes by VEGF-A_{165} and Vammin was verified by a co-precipitation assay

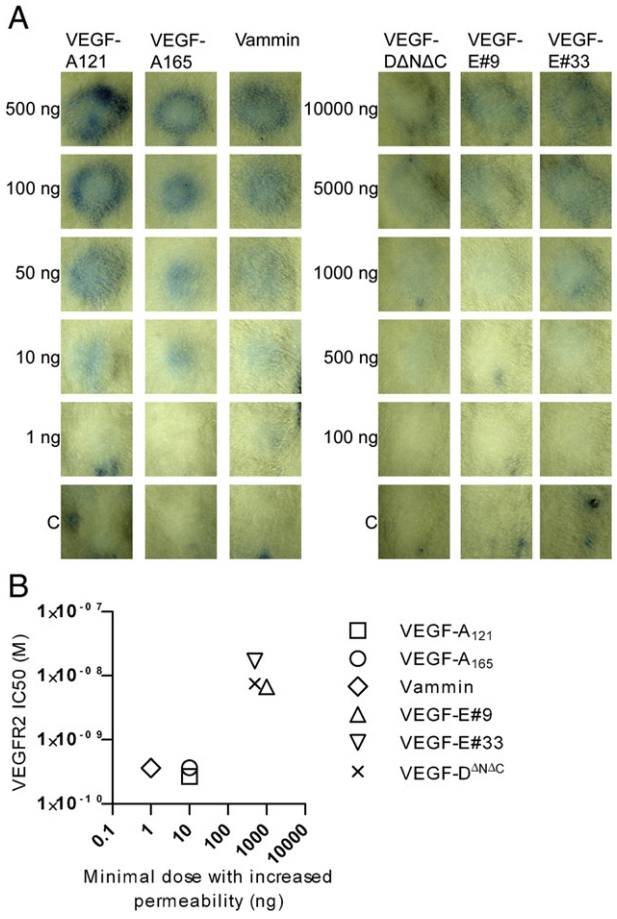

Fig. 5. Vascular permeability and its correlation with VEGFR2 binding affinity. (A) Miles assay on rabbit skin with the recombinant VEGF proteins. The proteins were divided into high affinity (VEGF-A forms and Vammin) and low affinity (VEGF-$D^{\Delta N\Delta C}$ and VEGF-E_{NZ7}/PlGF chimeras) groups and used at concentrations able to stimulate VEGFR2 activation. (B) The correlation of the determined minimal doses causing vascular permeability with the VEGFR2 binding affinities.

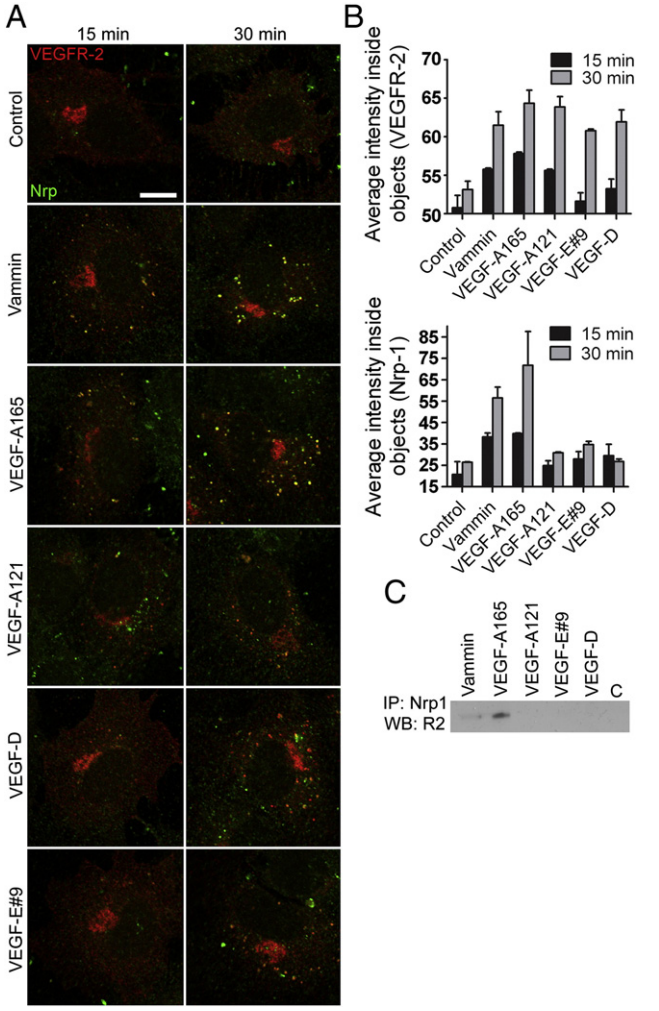

Fig. 6. VEGF-induced VEGFR2 and Nrp1 internalization and complex formation. (A) HUVECs were stimulated with 50 ng/ml Vammin, VEGF-A_{165}, and VEGF-A_{121}, or with 500 ng/ml VEGF-E_{NZ7}/PlGF chimera 9 (VEGF-E#9) and VEGF-$D^{\Delta N\Delta C}$, or with medium (Control), fixed, permeabilized and stained for VEGFR2 (red) and Nrp1 (green). (B) The quantification of the average intensities of VEGFR2 internalized inside vesicles and Nrp1 internalized inside VEGFR2 positive vesicles. Data represents mean numbers of average density ± SEM. (C) To verify complex formation, similarly treated samples at 30 min time point were immunoprecipitated (IP) with antibody against Nrp1 and immunoblotted (WB) with VEGFR2 antibody.

(Fig. 6C). Other ligands did not induce complex formation at levels detectable by this assay.

4. Discussion

The recently determined structures of VEGF-A, VEGF-E [38], and VEGF-C [55] as a complex with the ligand binding domains (Ig like domains 2 and 3) of VEGFR2 show a highly conserved binding mechanism, even though the individual amino acid residues buried in the contact interface are quite different. The resolved three-dimensional structures of VEGF-$D^{\Delta N\Delta C}$ [56] and Vammin [57] also show very similar tertiary structures compared with other VEGFR2 ligands (Fig. 7A), and these growth factors can directly compete with each other for receptor binding. Therefore, it is likely that all VEGFR2 ligands bind to the same site in the receptor using highly similar mechanisms. To try to answer the question if the small variations seen in the receptor binding, as recently seen with VEGFR1 ligands VEGF-B and PlGF [58], and the recruitment of Nrp co-receptors, can lead to distinct activation of VEGFR2 we performed a series of experiments characterizing VEGFR2 activation mechanisms and angiogenic effects induced by these ligands.

In addition to different binding profiles to VEGFR1 and VEGFR3, competitive binding assays showed a clear difference with binding to VEGFR2 between high affinity VEGF-A isoforms and Vammin and lower affinity VEGF-$D^{\Delta N\Delta C}$ and VEGF-E_{NZ7}/PlGF chimera forms. Structurally highly conserved VEGF homology domain of the VEGF family members is responsible for the binding to all VEGFRs. On the contrary, quite varied structures can bind to Nrp:s. VEGF-A_{165}, PlGF-2, and VEGF-B_{167} have a somewhat homologous separate Nrp/heparin binding domains, but it has been shown that also VEGF-A_{121} [48], the propeptides of VEGF-C and VEGF-D [59], and some forms of VEGF-E [60] bind to Nrp:s. In this study, we show that also recombinant Vammin is capable of binding to Nrp:s. Most of these Nrp binding forms possess a positively charged C-terminal tail ending at arginine (Fig. 7B), and in the case of VEGF-A_{165}, this tail has been shown to fit into the binding pocket of the Nrp B1 domain. The high affinity interaction of VEGF-A_{165} is further strengthened by another minor binding site missing in VEGF-A_{121} [39]. As predicted, all our proteins possessing the positively charged C-terminal tail showed some binding to Nrp:s. VEGF-E_{NZ7}/PlGF chimeras with low binding capability have a C-terminal tail of PlGF-1 resembling the tail of VEGF-A_{121} which had moderate binding affinity. The high binding affinity of Vammin to Nrp:s was surprising since it had previously been determined not to be able to bind to Nrp [52], and based on its primary structure, it contains only a minimal Nrp binding site resembling sequence in its C-terminus. The presence of heparin has been previously shown to increase the affinity of $VEGF_{165}$ for Nrp1 [61], and as expected, the binding capability of both VEGF-A_{165} and Vammin to Nrp1 dropped in the absence of heparin. However, the decrease was much greater in the case of Vammin, indicating that the interaction between Vammin and Nrp is highly dependent on heparin, whereas VEGF-A_{165} with the large Nrp binding domain with two defined Nrp binding sites is less dependent on heparin.

We used the level of Tyr-1175 phosphorylation as the main indicator of VEGFR2 activation due to its crucial importance for mediating the effects of VEGF-A [8]. We were able to measure its phosphorylation over a wide range of concentrations of different VEGFs in cells expressing only VEGFR2 or both VEGFR2 and Nrp1. There was a clear correlation between the VEGFR2 binding affinity and Tyr-1175 phosphorylation of the ligands. All tested VEGFs also induced the phosphorylation of the other known VEGFR2 tyrosine phosphorylation sites and the known major downstream signaling pathways. This supports a simple model for VEGF induced receptor activation, where a dimeric VEGF ligand links the receptor monomers together with efficacy determined by the ligand binding affinity to form the active receptor dimer. The binding of the ligands that share a highly homologous tertiary structure leads to a highly similar response on the level of the receptor kinase domain activation. However, the receptor activation kinetics clearly differed between low and high affinity ligands. Maximal response to the high affinity ligands was seen in 5 min time point but the response to VEGF-$D^{\Delta N\Delta C}$ peaked instead at 15–30 min time points. The same effect was seen in receptor internalization kinetics. After 15 min stimulation with the high affinity ligands, VEGFR2 was clearly internalized to cytoplasmic vesicles. VEGF-$D^{\Delta N\Delta C}$ and VEGF-E_{NZ7}/PlGF chimera-induced VEGFR2 internalization was slower. There could also be differences to which cytoplasmic vesicle VEGFR2 goes dependent of the ligand used to stimulate the receptor [62]. These results imply that the low affinity ligands need longer time to cross-link and activate the receptor, whereas the high affinity ligands act more quickly. However, this strong response of the high affinity ligands triggers the subsequent downregulation mechanisms that lead to VEGFR2 internalization by endocytosis and either recycling back to the plasma membrane or degradation [63]. VEFG-A expression is regulated by the oxygen availability in tissues [64], whereas VEGF-D expression is rather constitutive [65]. These growth factors have likely evolved for different purposes: VEGF-A is a highly potent initiator of angiogenic responses whereas the function of VEGF-D may be to maintain a steady low level VEGFR2 signaling. VEGF-$D^{\Delta N\Delta C}$ recombinant protein has previously been shown to be more unstable compared to other VEGF family members, due to an unpaired cysteine residue in the dimer interface, leading to the formation of inactive monomeric forms [42,56,66]. Whereas this does not likely reduce the activity of the protein in binding, signaling and permeability experiments with short duration, the longer term assays for cellular proliferation and tube-like structure formation are prone to be affected by the instability.

The complex formation between Nrp1 and VEGFR2 was verified with confocal microscopy and co-precipitation when Nrp binding ligands were used. The complex formation induced by Vammin was not as efficient as induced by VEGF-A_{165} in the co-precipitation assay, but this is expected, since Vammin lacks another Nrp binding site

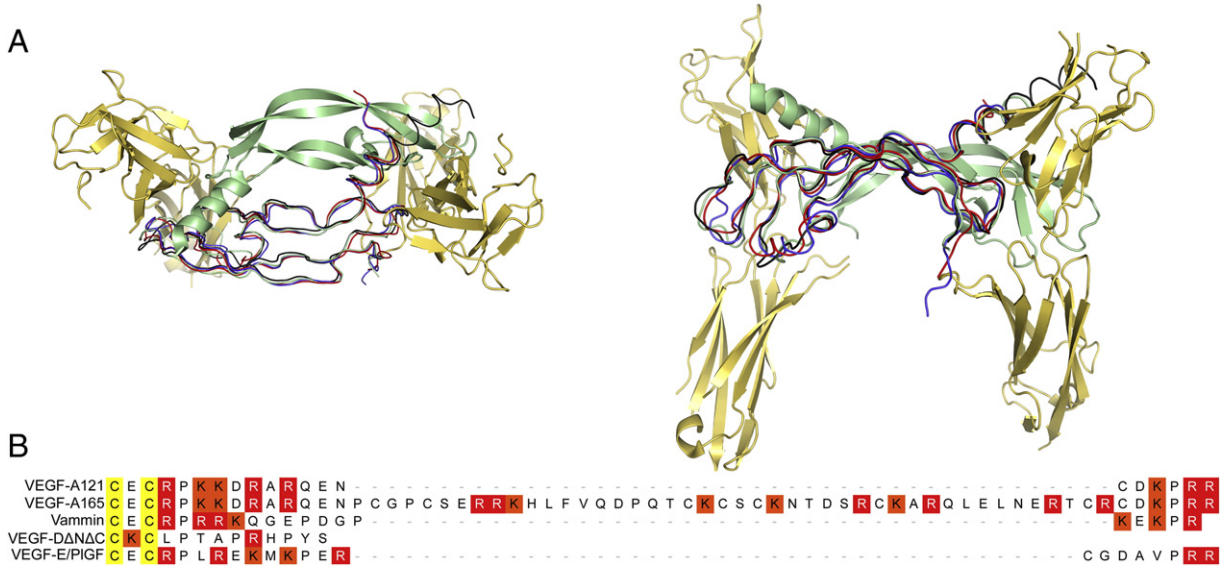

Fig. 7. Three dimensional structure and sequence comparisons of the VEGF proteins. (A) Three dimensional structures of the VEGF proteins show a highly similar fold. The determined crystal structures of VEGFR2 ligand chains represented as ribbon [VEGF-A in red (PDB ID: 2VPF) [75]; VEGF-D in black (PDB ID: 2XV7); Vammin in blue (PDB ID: 1WQ8)] superimposed with one monomer of the crystal structure (PDB ID: 2X1W) of VEGF-C dimer (green) as complex with two monomers of VEGFR2 (yellow). (B) The comparison of the C-terminal Nrp/Heparin binding domains of VEGF proteins. The two cysteine residues marking the end of the highly homologous VEGF homology domain are highlighted with yellow. The basic amino acids important for heparan sulfate interaction are highlighted with red (Arginine) and orange (Lysine).

found in VEGF-A_{165} [39] and its binding to Nrp depends strongly on heparin. The in vitro angiogenesis assay showed the interaction between Nrp1 and VEGFR2 to be crucial, since only the ligands capable of binding to Nrp:s could reach the maximal activity measured as total tubule area. Structurally, VEGFR and Nrp binding regions are located in different separately folding protein domains and can, therefore, be modified without affecting each other [38,39,67]. Supporting this, Nrp binding of VEGF-A_{165} could efficiently and specifically be blocked by addition of 6 histidine residues to the C-terminus of the protein verifying the previous findings that the free C-terminus is the major binding determinant in VEGF-A_{165} and VEGF-A_{121} [39]. Blocking this binding site or the total deletion of the Nrp binding domain in VEGF-A_{111} led to significant reduction in the efficacy of VEGF-A. In the absence of Nrp binding, the sprouting/branching of the tube-like structures also declined. The VEGF-A_{165}-His mutant with the disrupted Nrp binding site showed surprisingly high activity at the highest concentration used. Compared to the solid phase binding assay with recombinant Nrp, VEGF-A_{165}-His may interact in the in vitro angiogenesis assay weakly with Nrp through its minor Nrp binding site [39] even though the major Nrp binding site is blocked, since in the cell membrane, VEGFR2, Neuropilin and heparan sulfate proteoglycans are all present together. Supporting this, VEGF-A forms lacking both Nrp binding sites (VEGF-A_{111} and VEGF-A_{121}-His) had only moderate activity even at the highest concentrations. Since Nrp and heparin binding sites somewhat overlap, the reduced effect in the in vitro angiogenesis assay with the VEGF-A_{165}-His mutant might also come partly from the loss of the heparin binding. However, this is not the case with VEGF-A_{121} as it is not able to bind to heparin but its activity was still reduced by blocking the Nrp binding site.

Nrp binding didn't have an influence on the main downstream signaling pathways activated by the VEGFR2 ligands, and others have only found some subtle differences on the phosphorylation levels of migration and vessel branching-related signaling molecules [68–70]. The impact of Nrp on the VEGF function was more evident in biological assays, since the lack of Nrp binding could not be compensated by increasing VEGF concentrations, even though phosphorylation saturated to the same level with each VEGF when sufficient concentrations were used. Possible mechanism how Nrp affects VEGF signaling may raise therefore from 1) the recruitment of adapter proteins by Nrp C-terminus to the signaling complex, as well as from 2) spatial or 3) temporal differences in signaling between the VEGFR2/Nrp complex versus an uncomplexed VEGFR2.

Increase in vascular permeability is one of the dose-limiting side-effects of pro-angiogenic therapies [25]. VEGFR2 binding affinity of the tested ligands correlated well with the capability to increase vascular permeability in Miles assay performed in rabbit skin. All VEGFR2 ligands were capable of inducing acute vascular permeability, but the low affinity ligands VEGF-$D^{\Delta N\Delta C}$ and VEGF-E_{NZ7}/PlGF chimeras had to be used at very high concentrations. Interestingly, VEGF-A_{121}, VEGF-A_{165}, and Vammin with similar VEGFR2 binding affinities, but very different Nrp and VEGFR1 binding properties, showed highly similar responses in the Miles assay. Therefore, based on the results of this model, either VEGF-VEGFR1 or VEGF-Nrp interactions do not seem to have a major role in acute vascular permeability response.

Although the mitogenic effects of VEGFs have been shown to go through VEGFR2 [29,44,71], there are several conflicting reports about the receptor responsible for mediating the vascular permeability. It has been suggested that VEGFR1 would be the receptor for mediating vascular permeability, since a chimeric VEGF-A mutant protein, with preferential binding to VEGFR1, was nevertheless able to induce vascular permeability as efficiently as wild type VEGF-A [31]. Also, in another study, simultaneous activation of both VEGFR1 and VEGFR2 was shown to lead to synergic effect on vascular permeability [32]. However, data published by others suggests that VEGFR2 is an important receptor mediating vascular permeability [26,29,30,72] and this is verified by our data showing that several VEGFR1 binding deficient VEGFR2 ligands dose dependently induce vascular permeability. However, VEGFR1 may have a role in vascular permeability since it has been indicated to modulate VEGFR2 activation and signaling [73]. A recent report has also showed that different VEGFR1 ligands VEGF-B and PlGF can actually activate the receptor differentially leading to distinct biological responses [58], complicating the analysis of the role of VEGFR1 in induction of vascular permeability. Additionally, in all studies, the issue of cross species reactivity should be taken into consideration. VEGF-D has previously been shown to be a VEGFR3 specific ligand in mice [74], whereas it binds also to VEGFR2 in humans. However, we have not observed nor others have published other species specific differences in interactions of VEGFs.

In conclusion, we were able to show that 1) the highly conserved structure and receptor binding mechanism of VEGFR2 ligands lead to a similar activation of the receptor kinase domain, but 2) the ligands differ greatly in their binding affinity, receptor activation potency and receptor activation and internalization kinetics, 3) the acute vascular permeability response on rabbit skin is induced by all VEGFR2 ligands and is dependent on the VEGFR2 affinity and the concentration of the ligand, and 4) Nrp binding is required for the full potency of the VEGFR2 ligands. These results suggest that it might be useful for future pro-angiogenic therapies to create new modified dual VEGFR2 and Nrp ligands with moderate VEGFR2 affinity to control edema, combined to high Nrp binding affinity to retain angiogenic activity.

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