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Research Article

A VEGF-A splice variant defective for heparan sulfate and neuropilin-1 binding shows attenuated signaling through VEGFR-2

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Abstract. The development of functional blood and lymphatic vessels requires spatio-temporal coordination of the production and release of growth factors such as vascular endothelial growth factors (VEGFs). VEGF family proteins are produced in multiple isoforms with distinct biological properties and bind to three types of VEGF receptors. A VEGF-A splice variant, VEGF-A_{165}b, has recently been isolated from kidney epithelial cells. This variant is identical to VEGF-A_{165} except for the last six amino acids encoded by an alternative exon. VEGF-A_{165}b and VEGF-A_{165} bind VEGF receptors 1 and 2 with similar affinity. VEGF-A_{165}b elicits drastically reduced activity in angiogenesis assays and even counteracts signaling by VEGF-A_{165}. VEGF-A_{165}b weakly binds to heparan sulfate and does not interact with neuropilin-1, a coreceptor for VEGF receptor 2. To determine the molecular basis for altered signaling by VEGF-A_{165}b we measured VEGF receptor 2 and ERK kinase activity in endothelial cells in culture. VEGF-A_{165} induced strong and sustained activation of VEGF receptor 2 and ERK-1 and -2, while activation by VEGF-A_{165}b was only weak and transient. Taken together these data show that VEGF-A_{165}b has attenuated signaling potential through VEGF receptor 2 defining this new member of the VEGF family as a partial receptor agonist.

Keywords. VEGF, splicing, heparin, neuropilin, tyrosine kinase, angiogenesis, signal transduction, MAP kinase.

Introduction

Angiogenesis, the formation of new blood vessels from preexisting ones, is a complex and tightly regulated process. The development of fully functional vessels requires spatio-temporal coordination of the production, release and deposition in the extracellular matrix (ECM) of several growth factors such as vascular endothelial growth factors (VEGFs), angiopoietins, transforming growth factor-β, fibroblast growth factor and platelet-derived growth factor (PDGF) by many cell types (reviewed in [1–3]). This process is counterbalanced by angiogenesis inhibitors such as ECM-derived polypeptides or specific inhibitory molecules like PEDF [4, 5]. Impaired vessel function is associated with many pathologies, for instance in atherosclerosis [6], diabetic retinopathy [7, 8], arthritis [9], malignant cell growth [10–12], neurodegenerative disease such as amyotrophic lateral sclerosis (ALS) [10, 13] and in preeclampsia, a placental insufficiency observed in some patients in the late stage of pregnancy

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[14]. These disorders arise from mutation or impaired expression of various signaling molecules regulating angiogenesis, in particular of angiogenic growth factors such as VEGFs and their receptors that directly operate on endothelial cells.

VEGFs regulate physiological and pathological angiogenesis and belong to a gene family that includes VEGF-A, -B, -C, -D [3], placenta growth factor (PlGF) [15], *orf* virus-encoded VEGF-like proteins called VEGF-E [16–18] and a series of snake venoms collectively called VEGF-F [19–22]. VEGF family growth factors form cysteine cross-linked dimers folded into a cysteine knot structure [23]. All mammalian VEGFs are produced in multiple isoforms upon alternative splicing and proteolytic processing giving rise to a plethora of functionally divergent proteins [24, 25]. Although structurally very similar, the different VEGFs display distinct properties and bind to specific subtypes of VEGF receptors. VEGFs are produced by normal and transformed cells upon exposure to hypoxia [26, 27] and promote endothelial cell growth, migration and survival, but also regulate blood vessel permeability and vasodilation (reviewed in [10]). The importance of VEGF in vascular development has been documented in knockout mice where disruption of a single allele of the VEGF-A gene leads to embryonic lethality [28, 29]. Even subtle changes in VEGF expression during fetal development [28, 30], a block in the expression of a particular VEGF-A splice variant [31] or disruption of one allele of the gene encoding VEGFR-2 result in abnormal blood vessel formation and are embryonic lethal [33]. VEGF is also important for tumor growth; blocking VEGF with antibodies, soluble receptor or inhibiting VEGF receptor kinase activity have shown promising clinical results in tumor therapy [10].

In mammals VEGF-A is expressed in many variants upon alternative splicing of a single precursor mRNA. Four major splice variants, VEGF-A_{121}, VEGF-A_{165}, VEGF-A_{189} and VEGF-A_{206} have been described [34, 35]. The longer isoforms show binding affinity to the ECM, particularly to heparan sulfate glycosaminoglycans (HSPG), while VEGF-A_{121} is a soluble, acidic polypeptide that does not interact with HSPG [35]. The longer versions of VEGF-A bind to additional receptors such as neuropilin-1 and -2, which play a role in cell adhesion and in nerve and vessel guidance [36–38]. Increased activation of VEGFR-2 in the presence of heparan sulfate (HS) and neuropilin-1 or -2 has been observed for VEGF-A_{165}, the isoform most prominently expressed in mammals [37–39]. The most basic VEGF variant, VEGF-A_{189}, is sequestered in the ECM and shows no biological activity unless mobilized by heparinases, while VEGF-A_{206} is proteolytically cleaved and released from the ECM by metalloproteinases. Recently, new VEGF-A splice variants have been described which carry an alternative C terminus encoded by exon 9 instead of exon 8 [40, 41]. The best characterized member of these proteins is $VEGF_{165}$b which was isolated from kidney epithelial cells. This protein showed anti-angiogenic properties counteracting VEGF-A_{165} *in vitro* and *in vivo*.

VEGFs signal through cell surface receptor tyrosine kinases related to the PDGF receptor family. VEGF-A binds to both VEGF receptor 1 (VEGFR-1) and -2 (VEGFR-2) [3, 10], while PlGF and VEGF-B exclusively bind to VEGFR-1 [42–44].VEGF-C and VEGF-D are specific ligands for VEGFR-2 and VEGFR-3, which regulate both blood and lymphatic vessel development [45].Viral VEGF-Es and some snake venom VEGF-F variants exclusively activate VEGFR-2 [18–20].

Here we study the biological activity of VEGF-A_{165}b in which the C-terminal domain encoded by exon 8 was replaced by an exon 9-encoded sequence. This protein binds VEGF receptors with the same affinity as VEGF-A_{165}, shows reduced binding to HS, does not bind neuropilin-1 and displays altered signaling properties through VEGFR-2 and ERK kinases.

Materials and methods

Materials. VEGF-A_{165}, VEGF-A_{165}b, VEGF-A_{121}, VEGF-E and PlGF were produced in *Pichia pastoris* using the pPICZαA expression system (Invitrogen, San Diego, CA, USA) [46]. Myelin basic protein (MBP) and heparin (H-1027) were from Sigma (St. Louis, MO, USA). Bovine serum albumin (BSA) was obtained from Promega (Madison, WI, USA). ERK antibodies were K-23 for ERK-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) pan-ERK (E17120, Transduction Laboratories, BD Biosciences, Franklin Lakes, NJ, USA), phospho-ERK (Cell Signaling, Cambridge, MA, USA), and rabbit polyclonal anti-ERK-2 (F135, a generous gift from Dr. D. Fabbro, Novartis, Basel, Switzerland). Rabbit anti-mouse antibody and alkaline phosphatase conjugated species-specific antibodies were from Southern Biotech (Birmingham, AL, USA). Monoclonal anti-VEGF antibody MAB293 and MAB3045 (specific for the exon 9 variant), polyclonal rabbit anti-VEGF AB293 and polyclonal anti-Tie-2 antibodies were from R&D Systems Inc. (Minneapolis, MN, USA). Protein A Sepharose CL-4B was from GE Healthcare and [γ-^{32}P]ATP was from ICN (Costa Mesa, CA, USA). Antibodies against VEGFR-2 and pY1175 were from Santa Cruz (A3) and Cell Signaling, respectively.

Tissue culture. Human umbilical vein endothelial cells (HUVE cells, Promocell, Heidelberg, Germany) were used up to passage 8. Cells were grown in endothelial cell growth medium (ECGM) containing the supplement C-39210 (Promocell). They were starved overnight in endothelial cell basal medium (ECBM) supplemented with

0.1% fetal bovine serum (FBS, Invitrogen, Gibco-BRL). Porcine aortic endothelial cells (PAE cells) overexpressing VEGFR-1 or VEGFR-2 or VEGFR-2 together with neuropilin-1 [47] were maintained in Ham's F12 medium containing 10% FBS. BAEC cells were grown in DMEM with 10% donor calf serum.

Cloning and expression of VEGFs. The methylotrophic yeast *Pichia pastoris* was used to express all VEGF isoforms. Our constructs were derived from the canine isoform of VEGF-A, which is shorter by one amino acid than the human isoforms [48]. The exon 8 encoded sequence (Cys Asp Lys Pro Arg Arg) was replaced by the sequence Ser Leu Thr Arg Lys Asp encoded by human exon 9. All expression vectors were constructed by the PCR subcloning method [49] with the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) using pPICZαAVEGF-A_{165}, a vector for expression of VEGF-A_{165} in *Pichia pastoris* [48], as template. The following primers were used in the first PCR reaction: VEGF-A_{165}b primer 1: 5′-GCGACTGGTTCCAATTGACAAG-3′, VEGF-A_{165}b primer 2: 5′-GATGATGATGATGTCGACTCATCAGTCTTTCCTGGTGAGAGATCTGCAAGTAGTTCGTTTAACTCAAG-3′, VEGF-A_{159} primer 1: 5′-GCGACTGGTTCCAATTGACAAG-3′ and VEGF-A_{159} primer 2: 5′-GATGATGATGATGGTCGAATCATCTGCAAGTACGTTCGTTTAACTC-3′. The constructs were linearized with *Sac*I and transferred into *Pichia pastoris* strain X-33 by electroporation (Easyselect™ Pichia Expression Kit, Invitrogen). Clones were selected for protein expression (Easyselect™ Pichia Expression Kit, Invitrogen). The proteins were produced in a 3.7 l fermenter (Bioengineering, Wald, Switzerland) as described [46].

Purification of VEGF-A_{165}b and VEGF-A_{159}. Proteins were affinity purified with an ÄKTAprime™ system (GE Healthcare). Cell-free medium was collected from yeast cultures, sterile filtered through 0.45-µm MF™ membrane filters (Millipore, Billerica, MA, USA) and concentrated to 200 ml by ultrafiltration (Schleicher & Schuell GmbH, Dassel, Germany). Buffer was exchanged with binding buffer (20 mM sodium phosphate, pH 7.4, 0.5 M NaCl and 10 mM imidazole) and loaded onto a 50-ml Ni^{2+}-NTA column (GE Healthcare) equilibrated with binding buffer. The material was eluted by gradually increasing the imidazole concentration to 500 mM. The peak fractions were analyzed by SDS-PAGE, pooled, concentrated using a YM 10 ultrafiltration membrane (Millipore) and dialyzed overnight at 4 °C against PBS.

Radioiodination and cell binding assays. ^{125}Iodine labeling of recombinant VEGF-A_{165} was performed with the IODO-GEN method according to the Pierce protocol (Pierce, Rockford, IL, USA). Briefly, 0.5 mCi $Na^{125}I$ (IMS-30, GE Healthcare) was activated for 6 min at room temperature (RT) by incubation in a tube coated with 50 µg iodogen and mixed with a solution containing 10 µg VEGF-A_{165} for 9 min at RT. The reaction was stopped with 1 mM DTT and the iodinated protein was separated from free iodine on a PD10 gel filtration column (GE Healthcare) equilibrated with 0.5% BSA, 1 mM citric acid in PBS. The specific radioactivity of VEGF-A_{165} was 62 000 cpm/ng. PAE cells expressing VEGFR-1 or VEGFR-2 or VEGFR-2 together with neuropilin-1 were grown overnight to subconfluency in 24-well tissue culture plates and incubated for 4 h at 4 °C with 0.1 nM ^{125}I-labeled VEGF-A_{165} with or without the indicated amount of cold competitor. Each data point was determined in triplicate. Unbound radioactive protein was washed off with MEM-HBS, 25 mM HEPES, with or without L-glutamine (Amimed, Allschwil, Switzerland) containing 0.25% BSA. Cells were then lysed in 200 µl 1 M NaOH and radioactivity determined in a Beckman γ counter.

***In vivo* chicken embryo chorioallantoic membrane (CAM) assay and histological analysis of CAMs.** Protein (1 µg) was applied to a 0.5% methylcellulose carrier placed onto the CAM of shell-free grown chicken embryos (Lohman LSL strain, Animalco, Staufen, Switzerland) at embryonic day 8. Angiogenesis was monitored for up to 4 days after sample application under a stereomicroscope and pictures were taken. A solution of 20% Lipovenös (Fresenius Kabi, Stans, Switzerland) was injected into the CAM immediately before photographing to improve the contrast. For histological analysis, CAMs were fixed in 3.5% formaldehyde at 4 °C. Areas to be analyzed were cut out and washed in PBS for 30 min at 4 °C. Samples were incubated for 30 min at RT in 0.85% NaCl, rinsed twice in 50% EtOH in 0.85% NaCl, then in 70% EtOH in PBS for 20 min at RT and embedded in paraffin. Sections were deparaffinized in xylene and stained with hematoxylin (Medite AG, Nunningen, Switzerland) and eosin (Chroma-Gesellschaft, Münster, Germany). Average pixel intensities of digitized pictures were quantified with ImageJ software (NIH, Bethesda, MD, USA).

***In vivo* chamber implant angiogenesis assay.** Sterile tissue chambers made of perfluoro-alkoxy-Teflon® were filled with 500 µl molten 0.8% w/v agar containing 20 U/ml heparin (Novo Nordisk A/S, Bagsvaerd, Denmark) with or without growth factor (46 or 92 nM) and implanted aseptically into female mice (FVB) (Charles River Laboratories, les Oncins, France). Four days after implantation, chambers were recovered from the animals and the vascularized tissue that had formed around each implant was removed and weighed. Tissue samples were homogenized in RIPA buffer (50 mM Tris-HCl, 121 mM NaCl, 1 mM EDTA, 6 mM EGTA, 1% NP-40, 20 mM NaF, 1 mM Pefabloc SC, 1 mM Na_3VO_4). Samples were then centrifuged for 1 h at 7000 rpm. The supernatant

was filtered using a 0.45-µm syringe filter (Acrodisc® GF, Gelman Sciences, Ann Arbor, MI, USA) to avoid fat contamination. The amount of hemoglobin present in the filtrate was determined spectrophotometrically at 540 nm using a Drabkin reagent kit (Sigma). For Tie-2 determination Nunc maxisorb 96-well plates were coated overnight at 4 °C with the capture anti-Tie-2 antibody (Upstate Inc., Charlottesville, VA, USA) at a concentration of 2 µg/ml. Wells were washed three times with Tris phosphate-buffered saline (TPBS) and blocked by incubating with 3% Top-Block (Juro, Lucerne, Switzerland) for 2 h. Wells were then washed three times with TPBS and protein lysates were added for 2 h. After washing, a complex of anti-Tie-2 antibody (R&D Systems) and alkaline phosphatase conjugated anti-goat antibody (Pierce Biotechnology) was applied for 1 h at RT. After washing three times with TPBS, Tie-2 antibody complexes were detected by incubating with p-nitrophenyl phosphate (4-NPP) (Sigma) and the absorbance was determined in an ELISA reader at 405 nm. Recombinant human extracellular domain of Tie-2 fused to the constant region of human IgG1 dissolved in RIPA buffer was used as standard.

Heparin affinity chromatography. VEGF was applied to a 1 ml HiTrap heparin HP column (GE Healthcare) equilibrated with varying salt concentrations in 20 mM Tris (pH 7). VEGF was eluted with a linear salt gradient reaching a final concentration of 1.2 M and column fractions were monitored on an ÄKTA prime system (GE Healthcare) operated at a flow rate of 1 ml/min.

Neuropilin-1 binding in ELISA. Nunc microtiter plates were coated overnight at 4 °C with 2.8 µg/ml recombinant neuropilin-1 (R&D Systems). Plates were washed twice with PBS and blocked in PBS containing 2% BSA for 1 h at 37 °C. Unspecific binding was determined on duplicate plates treated with 2% BSA in PBS. After washing, the different VEGF isoforms were added to the wells at the indicated concentrations, followed by a 1 h incubation at 37 °C. After washing with PBS, bound VEGF was detected using a mixture of monoclonal anti-VEGF (R&D Systems) and alkaline phosphatase conjugated anti-mouse antibody. Plates were rinsed three times with Tris-buffered saline (20 mM Tris/HCl, pH 7.5, 150 mM NaCl, TBS) and alkaline phosphatase activity was determined colorimetrically with 4-NPP, absorbance at 410 nm was quantified in an ELISA reader.

Immunoprecipitation and *in vitro* immune complex kinase assays. VEGFR-2 activity was determined in Western blots using a phospho-specific antibody directed against Y1175 of VEGFR-2 (Cell Signaling). ERK-1 and -2 activity was determined in immune complex kinase assays with MBP as substrate. Cells were plated in 6 cm plates and grown in Ham's F12 or ECGM for 24 h and starved overnight in medium plus 0.1% FBS. Cells were stimulated at 37 °C, washed once with cold PBS containing 100 µM Na_3VO_4, and lysed in 500 µl lysis buffer. Lysates were cleared by centrifugation at 13 000 rpm and incubated for 2 h with anti-ERK-1 and anti-ERK-2 polyclonal antibody. Protein A Sepharose (60 µl) were added to each sample for an additional hour at 4 °C and the beads were washed once with TNET (50 mM Tris, pH 7.5, 140 mM NaCl, 5 mM EDTA, 1% Triton X-100), TNE (50 mM Tris, pH 7.5, 140 mM NaCl, 5 mM EDTA), and H_2O and equilibrated in kinase buffer (20 mM HEPES pH 8, 20 mM $MgCl_2$, 2 mM $MnCl_2$ and 1 mM DTT). Immunoprecipitated ERK-1 and -2 were incubated in kinase buffer containing 5 µCi [γ-^{32}P]ATP and 10 µg MBP for 15 min at 30 °C. Kinase reactions were stopped by the addition of sample buffer and the samples were separated by SDS-PAGE, transferred to PVDF membranes and exposed to X-Ray film. PVDF membranes were further analyzed by Western blotting for quantification of ERK-1 and -2. Unspecific binding was blocked with 3% gelatin in TBS. Mouse anti-pan-ERK was used as first antibody, alkaline phosphatase conjugated anti-mouse as secondary antibody. Blots were developed with a mixture of NBT (nitro-blue tetrazolium chloride, Sigma) and BCIP (5-bromo-4-chloro-3′-indolylphosphate *p*-toluidine, Sigma) diluted in development buffer (100 mM Tris, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$). Quantification of the data was performed with ImageQuant software (Molecular Dynamics, GE Healthcare). The films were scanned and the ratio of the intensities of the bands corresponding to the pY1175 and the MBP signal, respectively, were corrected for the intensity of the bands reflecting the total amount of VEGFR-2 and ERK-1 and -2, respectively.

Results

VEGF production and purification. All VEGFs were produced in the yeast *Pichia pastoris* [48] as described before and purified via an N-terminal His_6 tag on Ni^{2+}-NTA chelating Sepharose. The identity of the proteins was confirmed by Western blot analysis and by Edman sequencing (data not shown). To investigate whether the sequence encoded by exon 8 of VEGF-A_{165} has a specific function in receptor activation, we also produced a recombinant variant, VEGF-A_{159}, that lacks the last six amino acids encoded by either exon 8 or 9.

VEGF-A_{165}b and VEGF-A_{159} bind to VEGFR-1 and VEGFR-2 with the same affinity as VEGF-A_{165}. Binding of VEGF-A variants to VEGF receptors was determined on PAE cells expressing either VEGFR-1 or VEGFR-2. Figure 1a shows that VEGF-A_{165}b and VEGF-A_{159} displaced ^{125}I-labeled VEGF-A_{165} from cells expressing VEGFR-2 with the same efficiency as VEGF-

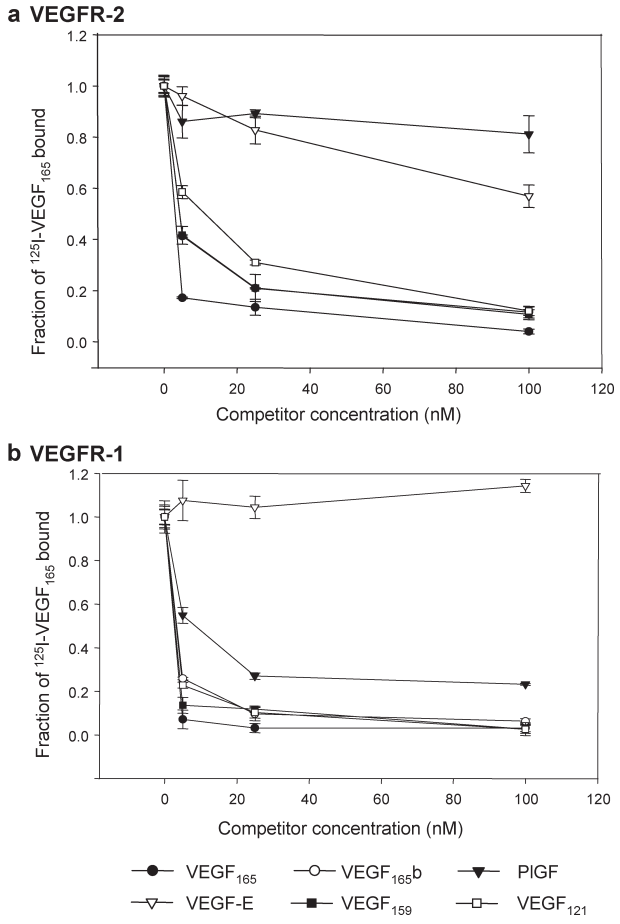

Figure 1. VEGF-A_{165}, VEGF-A_{165}b and VEGF-A_{159} bind VEGF receptors on endothelial cells with the same affinity. ^{125}I-labeled VEGF-A_{165} (0.1 nM) was competed with increasing amounts of competitor protein for 4 h at 4 °C on PAE cells expressing VEGFR-2 (*a*) or VEGFR-1 (*b*). Values are means ± SD, $n = 3$.

A_{165}. Consistent with this observation, PlGF, which does not bind VEGFR-2, did not block VEGF-A_{165} binding to VEGFR-2. Binding was also determined on PAE cells expressing VEGFR-1, where VEGF-A_{165}, VEGF-A_{165}b and VEGF-A_{159}, but not VEGF-E, blocked VEGF-A_{165} binding (Fig. 1b). These results demonstrate that the sequence encoded by exon 8 is not required for binding to VEGF receptors 1 and 2 and that the exon 9 variant showed identical binding to VEGF receptors as the exon 8 isoform.

***In vivo* angiogenesis.** A simple experimental system to qualitatively evaluate the angiogenic potential of growth factors *in vivo* is the chicken CAM. Samples (1 µg) of each VEGF variant were applied to the CAM of an 8-day-old chicken embryo and vascularization in the area of application evaluated 48 h later. Compared with PBS controls (average pixel intensity ± SD, 138 ± 13), where no alteration of the vascular network was seen, VEGF-A_{165} induced a network of microvessels giving the CAM in this area a reddish color (average pixel intensity 149 ± 27, Fig. 2a). Average pixel intensities for VEGF-A_{165}b and VEGF-A_{159} were 138 ± 17 and 138 ± 14, respectively. Histological examination of hematoxylin and eosin-stained paraffin sections shows that capillaries are only located in the chorionic epithelium and that the stroma is avascular in PBS controls. VEGF-A_{165}-treated CAMs displayed highly vascularized stroma with a large number of erythrocytes, while CAMs treated with VEGF-A_{165}b or VEGF-A_{159} showed only few vessels (Fig. 2b). These results suggest that the C-terminal domain encoded by exon 8 is required for full biological activity of VEGF-A_{165}. The exact role that exon 8 plays in VEGF-mediated vascularization remains at present unknown.

We next implanted a chamber containing an agarose/heparin matrix loaded with VEGF under the skin of a mouse. As shown in Figure 3, VEGF-A_{165} and VEGF-A_{159} strongly induced angiogenesis in this animal model. VEGF-A_{165}b and $VEGF_{121}$ had only a marginal effect on vessel invasion in this assay. Interestingly, VEGF-A_{165}b

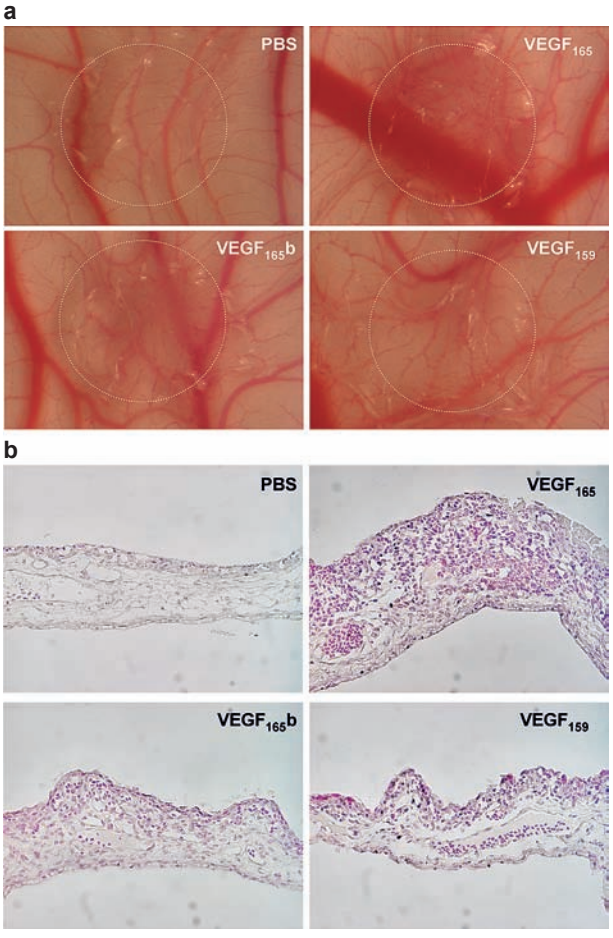

Figure 2. CAM assay. (*a*) CAMs were photographed 48 h after application of either PBS or 1 µg VEGF-A_{165}, VEGF-A_{165}b or VEGF-A_{159}, respectively. Compared with controls and exon 8 mutant proteins, where the vascular network remained unchanged, VEGF-A_{165} promoted highly vascularized CAMs. The dashed circle shows where ligand was applied. (*b*) Hematoxylin and eosin-stained paraffin sections of CAMs.

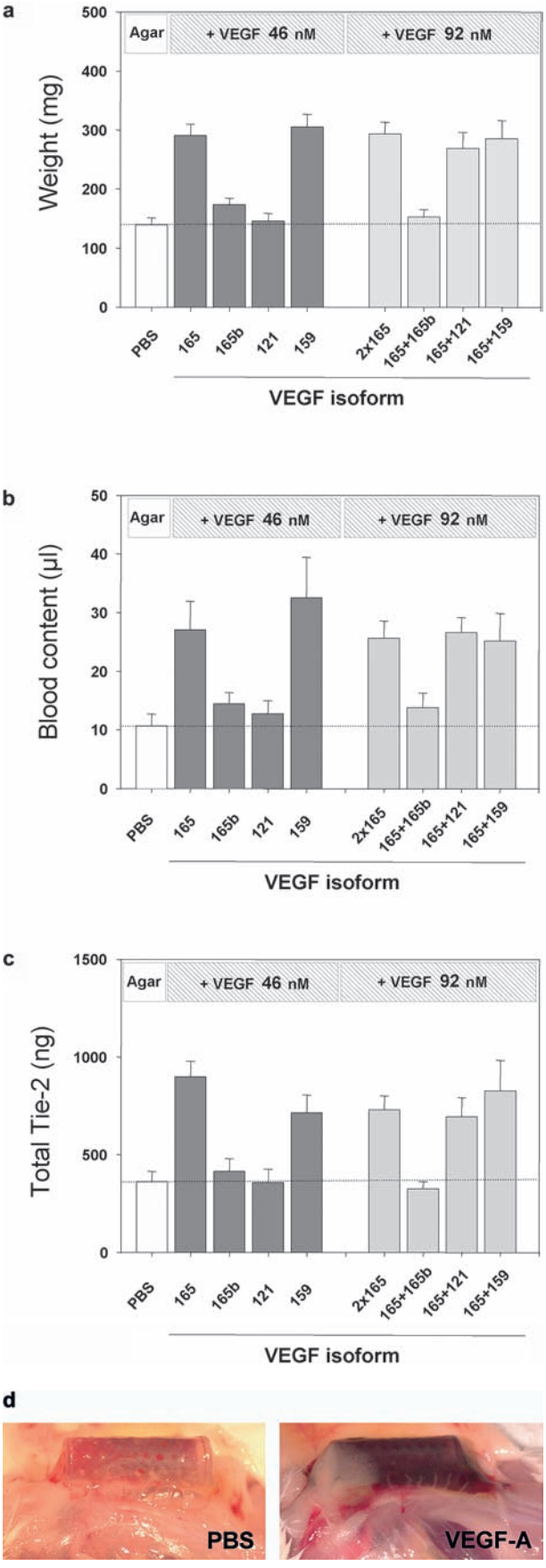

Figure 3. *In vivo* growth factor implant angiogenesis model. Porous chambers containing PBS or VEGF-A (46 nM or 92 nM) in 0.5 ml 0.8% agar containing 20 U/ml heparin were implanted subcutaneously into the flank of a mouse. The angiogenic response was quantified by measuring (*a*) the weight, (*b*) the blood content, and (*c*) Tie-2 expression in the tissue that grows around the implant. Measurements were performed 4 days after implantation. Values are mean ± SEM, $n = 6$. The dashed horizontal line indicates the background level obtained when only PBS buffer was added. (*d*) Representative photographs of two chamber implants, PBS control, and VEGF-A_{165}-treated sample.

antagonized VEGF-A_{165} when both proteins were simultaneously added in equimolar amounts reminiscent of earlier described data [41], while VEGF-A_{159} and $VEGF_{121}$ were not inhibitory. These data indicate that $VEGF_{165}$b is a partial receptor agonist with normal binding capacity for VEGFR-1 and -2 but reduced or qualitatively altered signaling potential.

VEGF-A_{165}b and VEGF-A_{159} show reduced binding to HS and do not bind to neuropilin-1. VEGF-A_{165} interacts with HSPG proteoglycans such as glypicans [50–53]. This interaction has been proposed to be mediated by the exon 7-encoded domain [54]. Our initial attempts to purify VEGF-A_{165}b by heparin-affinity chromatography were not successful, and we therefore constructed expression vectors encoding N-terminally His_6 tagged proteins. Heparin binding of all VEGF-A variants was quantitatively determined on a HiTrap heparin HP column equilibrated with 20 mM Tris and 0.15 M NaCl at pH 7. The bound material was eluted by applying a linear NaCl gradient from 0.15 to 1.2 M. Of the VEGF-A_{165}, 55% was bound to the heparin column and eluted at a salt concentration of 0.4 M, while VEGF-A_{121}, the most acidic VEGF-A splice variant, did not bind heparin at all. At 0.15 M salt VEGF-A_{165}b and VEGF-A_{159} only weakly interacted with heparin with 14% and 21%, respectively, of the material bound to the column. In the absence of NaCl, all proteins containing the exon 7, but lacking the exon 8 sequence, weakly bound to the column, while $VEGF_{121}$ did not bind heparin at all (data not shown). These data show that VEGF-A lacking exon 8 shows drastically reduced affinity for heparin, suggesting that the C-terminal exon 8-encoded sequence is essential for interaction with HS. HS binding does not, therefore, solely depend on sequences encoded by exon 7 as described before.

The longer isoforms of VEGF family proteins also bind to neuropilins, a class of cell surface receptors involved in many biological processes [55]. This interaction has been shown to be mediated by the exon 7 domain of VEGF-A [56]. We next determined binding of the various VEGF-A isoforms to recombinant neuropilin-1. As shown in Figure 4a, VEGF-A_{165} bound to neuropilin-1, while the variants lacking exon 8 showed no binding. Similarly, VEGF-A_{165}, but not VEGF-A_{165}b, VEGF-A_{159} and VEGF-A_{121},

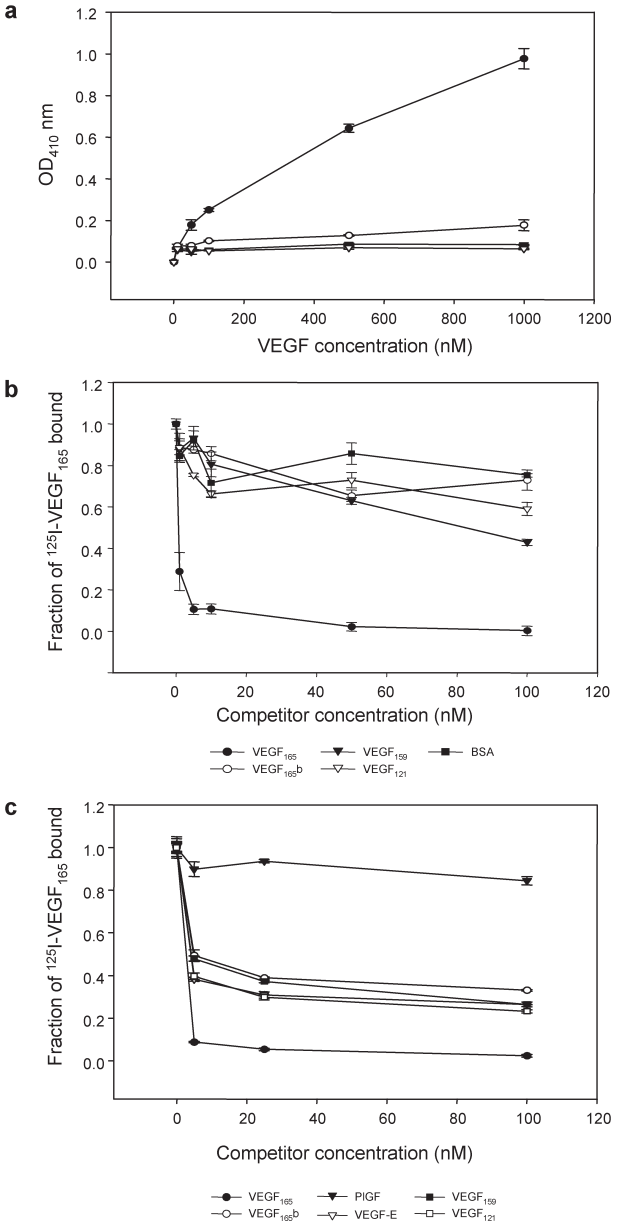

Figure 4. VEGF-A mutants lacking the exon 8 encoded sequence do not bind neuropilin-1. (*a*) Various VEGF-A isoforms were added in increasing concentrations to recombinant neuropilin-1 immobilized on ELISA plates. Values are means ± SD, $n = 3$. (*b*) Competition of ^{125}I-labeled VEGF-A_{165} binding to recombinant neuropilin-1 immobilized on ELISA plates by various VEGF-A isoforms. Values are means ± SD, $n = 3$. (*c*) Competition of ^{125}I-labeled VEGF-A_{165} binding to PAE cells expressing VEGFR-2 and neuropilin-1 by various VEGF-A isoforms, values are means ± SD, $n = 3$.

efficiently blocked binding of VEGF-A_{165} to neuropilin-1 in competitive ELISA (Fig. 4b). These data were confirmed on PAE cells expressing VEGFR-2 together with neuropilin-1. As shown in Figure 4c, only exon 8 containing VEGF-A completely competed binding of $VEGF_{165}$. Our data therefore suggest that sequences encoded by exon 7 are not sufficient for HS and neuropilin-1 binding to VEGF-A and thus reveal a role for the exon 8 sequence in these interactions.

VEGF-A_{165}b shows delayed and attenuated signal output through VEGFR-2. We next assessed the signaling properties of VEGF-A_{165} and VEGF-A_{165}b using a phospho-specific antibody against pY1175 of VEGFR-2 as shown in Figure 5a. Receptor stimulation by VEGF-A_{165}b was delayed, less robust and more transient than with VEGF-A_{165}. One of the primary signaling pathways activated by VEGFR-2 is signaling via MAP kinases such as ERK-1 and -2 that regulate cell migration, survival, mitogenesis and cell differentiation. Earlier results suggest that the extent and the kinetics with which receptor tyrosine kinases activate MAP family kinases are of pivotal importance for the biological output elicited by a particular ligand [57–59]. We, therefore, studied the time course of ERK-1 and -2 activation in two types of endothelial cells derived from pig aorta (PAEC) and bovine aorta (BAEC). We found robust and sustained activation of ERK-1 and -2 by VEGF-A_{165}, while VEGF-A_{165}b promoted only weak and blunted activation (Fig. 5b). The predominant MAP kinase activated in endothelial cells was ERK-2. In cells treated with VEGF-A_{165} ERK-2 activity was two- to threefold lower than in controls treated for 15 min with fetal calf serum (lanes F in Figure 5b) and was up to tenfold lower in cells treated with VEGF-A_{165}b (Fig. 5b). A quantification of a series of experiments is given in Table 1. In all experiments VEGF-A_{165}b showed significantly lower activity at all time points. From these data, we conclude that the C-terminal domain of VEGF-A encoded by exon 8 determines the kinetics and the strength of signal output by VEGFR-2.

Discussion

The discovery of new VEGF-A splice variants by Bates and coworkers has raised interesting questions concerning the role of the C-terminal domain encoded by exon 8 of VEGF-A_{165} in receptor activation. The biological activity of this splice variant was initially described in tumor cells expressing VEGF-A_{165}b, which showed drastically reduced malignancy *in vivo* when compared with VEGF-A_{165} expressing cells [40, 41]. Putative exon 9 variants of VEGF-A can be deduced from the mouse, human, dog, bovine, monkey, quail and zebrafish DNA sequence [41] (and our own unpublished work). The exon 9 encoded sequence is not conserved among these species, while the rest of the protein sequence is highly conserved. This indicates that the specific biological function of VEGF-A_{165}b might predominantly derive from the absence of exon 8 and not from the acquisition of new functionalities encoded by exon 9. The phenotype of VEGF-A_{159}, a mutant that was made to assess whether exon 9 is directly

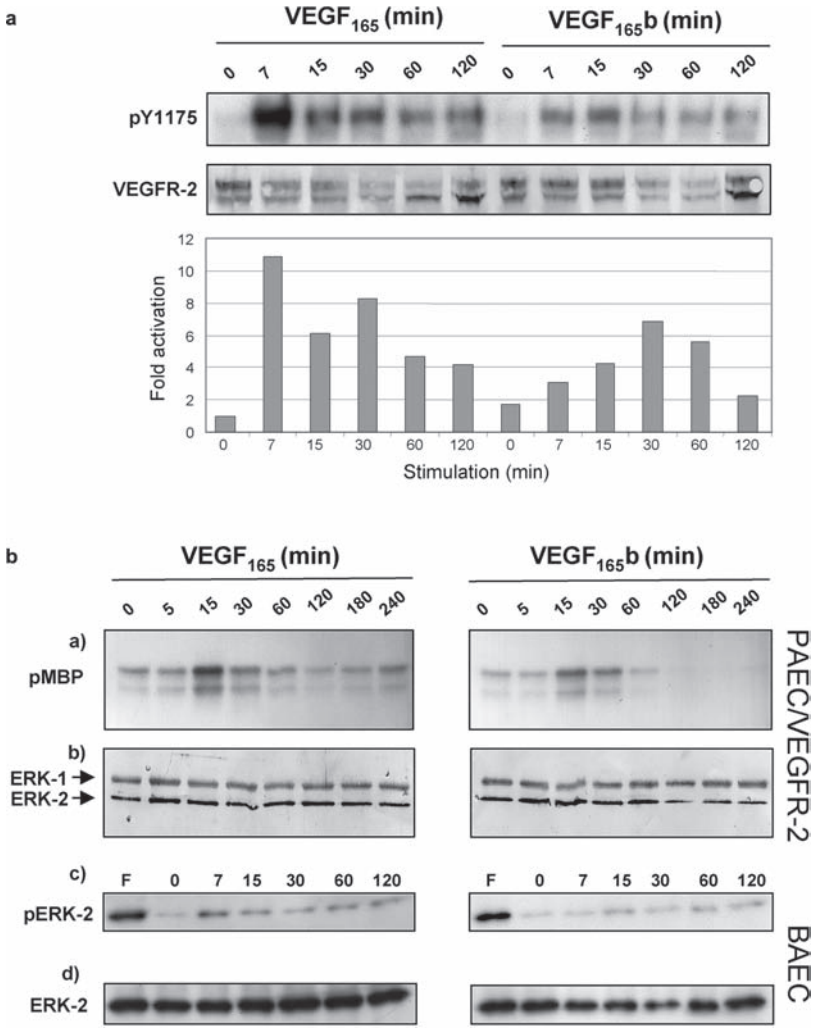

Figure 5. Time course of VEGFR-2 and ERK kinase activation. (*a*) Activation of VEGFR-2 was determined with a phospho-specific antibody recognizing pY1175 in cells stimulated with 50 ng/ml VEGF-A. Top panel, Western blot treated with p1175Y antibody; middle panel, Western blot showing the total amount of immunoprecipitated VEGFR-2; graph, data quantification (relative activity corrected for total amount of protein). (*b*) Time course of ERK-1 and -2 activation in cells stimulated with 50 ng/ml VEGF-A. a) and b), ERK activity determined in PAE cells expressing VEGFR-2, c) and d), similar experiment performed with BAEC cells. a) autoradiograph of an *in vitro* ERK-1 and -2 MBP phosphorylation assay; b) Western blot showing the total amount of immunoprecipitated ERK-1 and -2 used for the phosphorylation assay; c) Western blot showing ERK-2 activity determined with a phospho-specific antibody, d) Western blot showing total amount of ERK-2 determined with pan ERK-1/2 antibody. Lanes marked with F in c) and d) show activity of cells stimulated for 15 min. with 10% FBS. Lysates prepared from the same amount of cells were loaded on each lane of the gels. Representative experiments are shown, statistical analysis from several experiments for ERK activation is given in Table 1.

Table 1. Kinase activity of ERK-1/-2 determined by MBP phosphorylation assays after stimulation with 50 ng/ml VEGF-A.[a]

ERK activity	% activity	SD (n = 3)
15 min	38	12
30 min	27	2
60 min	29	6
120 min	42	2
180 min	24	18

[a] Shown are the relative activities of VEGF-A_{165}b compared with VEGF-A_{165} determined in HUVE and PAE cells expressing VEGFR-2. Activity of VEGF-A_{165} at each time point was arbitrarily set to 100% ($p < 0.09$ in all series).

responsible for the biological activity of VEGF-A_{165}b, or whether the absence of exon 8 was sufficient, was, however, not clear. In some assays, VEGF-A_{159} was similar to VEGF-A_{165}b, in others it behaved like VEGF-A_{165}. Replacing exon 8 by exon 9 resulted therefore in a more drastic phenotype than exon 8 deletion. Without detailed structural information for VEGF-A_{165}b such data are difficult to interpret. So far only the structures of a C-terminally truncated form of VEGF-A [60] and of the exon 7 and 8 encoded heparin binding domain have been solved [61]. This latter NMR structure shows that cysteine 146 forms an intramolecular disulfide bond with cysteine 160, restricting the flexibility of this domain and stabiliz-

ing a patch of basic amino acids exposed on the surface of this protein. The functional significance of this disulfide bond is further documented by a VEGF-A mutant in which cysteine 146 was changed to a serine residue [62] and which had reduced activity. Taken together, our data show that the sequence encoded by exon 8 is part of the heparin and neuropilin-1 binding domains and/or is mandatory for their proper folding. VEGF-A mutants lacking cysteine 160 may assume a more flexible structure, resulting in reduced affinity for HSPG and preventing binding to neuropilin-1. Additional mutagenesis of exon 8 will help to elucidate the exact role that this domain plays in VEGF-A mediated receptor activation.

All VEGF-A variants lacking exon 8 studied here bound VEGF receptors 1 and 2 with the same affinity as VEGF-A_{165}. On the other hand, mutants lacking exon 8 only weakly bound HS and did not bind to neuropilin-1 at all. Expression in endothelial cells of neuropilin-1, a plexin family protein that also interacts with semaphorins and plays an essential role in both neural guidance and vessel development, has been shown to enhance binding of VEGF-A_{165} to VEGFR-2 [37, 53, 63]. This was attributed to the b1b2 domain of neuropilin-1 that interacts with the exon 7 and 8 encoded C terminus of VEGF-A_{165} [64]. The importance of neuropilin-1 for the development of functional vessels is well documented in knockout animals [65]. Using *in vivo* angiogenesis animal models we show that VEGF-A_{165}b has reduced biological activity and even inhibits angiogenic signaling when used together with VEGF-A_{165}.

VEGF-A_{165}b also elicited significantly weaker signal output through VEGFR-2 and ERK kinases. This further documents that binding to HSPG and neuropilin-1 is not only required for the deposition and storage of VEGF-A_{165} in the ECM and on neuropilin-1-expressing cells, but that these interactions have also a direct impact on receptor signaling. VEGF-A_{165} leads to robust and sustained activation of VEGFR-2, while signaling via VEGFR-2 by the exon 9 variant is weak, shows delayed kinetics, and is only transient. Such differences might result from the distinct structure of ligand/receptor complexes, and suggest that only VEGF-A_{165} is capable of fully activating the receptor and its downstream signaling pathways. Co-ligation of VEGFR-2 with HSPG and/or neuropilin-1, resulting in delayed receptor desensitization by phosphatases and attenuated receptor degradation similar to recently published work on HSPG/VEGFR-2 complexes is a possible explanation for this phenomenon [66].

Coordinated expression of soluble and ECM-bound VEGF-A are indispensable for the development of functional vascular networks. Neuropilin-1-expressing cells might further support this process by sequestering VEGF-A, thereby contributing to the ordered deposition of this protein in the vascular bed. Based on our data we propose that HSPGs and/or neuropilin-1 fulfill an additional role by directly modulating VEGF-mediated receptor activation. Exon 8 VEGF-A splice variants show features that are typical for partial receptor agonists; they bind VEGF receptors with identical affinity as fully functional ligands yet elicit attenuated signal output. The biological role that VEGF-A isoforms lacking exon 8 play *in vivo* during vessel development remains at present unclear and must be addressed in transgenic animals.

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