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Novel svVEGF isoforms from *Macrovipera lebetina* venom interact with neuropilins

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ABSTRACT

Increased vascular permeability and vasodilation are responses usually elicited by snake envenomation. In this report, we isolated from *Macrovipera lebetina* venom two protein groups designated IC1 (Increasing Capillary1) and IC2 based on their activities on capillary permeability. Mass spectrometry analysis showed that IC1 contained four major proteins of 23,650, 24,306, 24,589 and 24,718 Da, whereas IC2 contained three major proteins of 25,101, 25,194 and 25,298 Da. N-terminal amino-acid sequencing revealed that IC1 and IC2 belong to the snake venom VEGF (svVEGF) family. IC1 and IC2 had a marked specificity for VEGFR-2, with affinities in the nanomolar range. Interestingly, they also bind to NRP1 and NRP2, with affinities in the micromolar range. This is the first report demonstrating that *M. lebetina* encodes several distinct svVEGFs, endowed with a capacity to interact with neuropilins. IC1 and IC2 could be valuable tools to understand the molecular properties of angiogenic factors and their receptors.

Introduction

Snake venoms represent an extraordinary source of biologically active molecules. For instance, it has been shown that several venom proteins are able to induce endothelial cell growth and angiogenesis [1]. These proteins are closely related to the human vascular endothelial growth factor (VEGF), and are capable of strongly increasing capillary permeability, thereby presumably facilitating the access of neurotoxic venom components to their target cells. These snake venom VEGFs (svVEGFs) may be very useful and attractive tools for the study of angiogenesis and the treatment of cardiovascular diseases.

Human VEGF is a family of several isoforms, the most abundant of which is VEGF-A_{165}. This factor mediates its diverse biological activities through two members of the transmembrane receptor-type tyrosine kinase family, called VEGFR-1 (Flt-1) and VEGFR-2 (KDR). Studies have indicated that VEGFR-1 is the dominant VEGF receptor on monocytes and is involved in chemotaxis and tissue factor activation, as well as in signaling and recruitment of stem cells and endothelial precursor cells [2]. VEGFR-2 is considered to be the main signal transducing VEGF receptor for capillary permeability, angiogenesis and mitogenesis of endothelial cells. An additional family of receptors, the neuropilins (NRPs), appears to play a role as VEGF co-receptors in the modulation of binding to other receptors, without being directly active in signaling [2]. For instance, NRP1 enhances the binding of VEGF-A_{165} to VEGFR-2 [3], and regulates angiogenesis through a VEGF-dependent pathway [4].

svVEGFs are disulfide linked homodimers of about 25 kDa with approximately 50% sequence identity with VEGF-A_{165}.

We have previously isolated, from the venom of *Macrovipera lebetina*, a VEGF-like protein that we designated ICPP (Increasing Capillary Permeability Protein), based on its potent ability to increase capillary permeability in mice [5]. ICPP-induced capillary permeability, angiogenesis, endothelial cell mitogenicity, and MAP kinase activation were all mediated through VEGF receptor signaling [6].

In this report, we demonstrate that *M. lebetina* venom in fact contains a variety of proteins belonging to the svVEGF family which preferentially bind to VEGFR-2. This is the first report showing a binding capacity of svVEGF to NRP1 and NRP2.

Materials and methods

Materials. Macrovipera lebetina transmediteranea venom was obtained from live snake specimens, maintained in the serpentarium

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of Institut Pasteur de Tunis (Tunisia). Human VEGF-A_{165}, VEGFR-1/Fc and VEGFR-2/Fc were from ReliaTech (Braunschweig, Germany), rat NRP1/Fc and NRP2/Fc, carrier free VEGF-A_{165}, and goat anti-human NRP2 antibody were from R&D Systems (Minneapolis, MN). Goat anti-NRP, IgG antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit anti-VEGFR-2 antibody was from Cell Signaling Technology, Inc. (Danvers, MA). ^{125}I-sodium was from PerkinElmer Life Sciences, Inc., and the IODO-BEADS® and DSS (Disuccinimidyl Suberate) were from Pierce Biotechnology. NAP 5 Columns and Protein G-Sepharose™ 4 Fast Flow were from GE Healthcare (Uppsala, Sweden).

svVEGFs purification and protein analysis. Fractionation of *M. lebetina* venom was performed by successive chromatographic steps as previously described [5]. The venom samples were applied onto a Superose 12 prep grade column, and the fractions found to induce capillary permeability in mice, were then successively loaded on a Mono Q and Mono S columns (HR5/5), followed by a RP-8 chromatographic step. SDS–PAGE was performed on an 8–25% polyacrylamide gradient gel under reducing or non-reducing conditions according to Laemmli [7]. Protein concentration was estimated by Lowry method [8]. Molecular masses were accomplished by mass spectrometry (MALDI-TOF-MS) on a Voyager DE STR instrument as previously described [6]. The N-terminal amino-acid sequence was determined by Edman degradation after chemically unblocking [9], by an Applied Biosystems Procise 490 sequencer.

Capillary permeability assay. Capillary permeability activities of proteins were tested in white Swiss mice, C57 black mice and Wistar rats using the Miles permeability assay [10]. Experiments on animals were carried out in accordance with the European Community Council Directive (86/609/EEC). Animals ($n = 4$ per group) were injected as previously described [6] with some modifications for rats that were given 1 ml of a 2.5% of the blue dye solution. PBS was used as negative control.

Surface plasmon resonance analysis. The VEGFR-1/Fc, VEGFR-2/Fc, NRP1/Fc and NRP2/Fc chimeras were covalently coupled, through their solvent-accessible primary amine groups, to the CM5 sensor chip, using a Biacore 2000 instrument and the Amine Coupling Kit (Biacore), according to manufacturer's instructions. Just prior to injection, NRP1/Fc, NRP2/Fc, VEGFR-1/Fc and VEGFR-2/Fc were diluted to a concentration of 20 nM in 10 mM sodium acetate (pH 5.2 for neuropilins and pH 4.5 for VEGF-R1/R2). VEGFR-1/Fc, VEGFR-2/Fc, NRP1/Fc and NRP2/Fc were immobilized to 2100, 2900, 3200 and 3300 resonance units (RU $\approx$ pg mm^{-2}), respectively. Binding assays were performed at 25 °C in PBS. Concentrations from 4 nM to 4 μM of IC1 or IC2 were injected at a flow rate of 30 μl/min on the VEGFR-1, VEGFR-2, NRP1 and NRP2 surfaces, as well as on an unmodified CM5 reference surface. Control experiments were performed using VEGF-A_{165} (0.1–20 nM). Regeneration was performed with 1 M $MgCl_2$ for 1 min. The equilibrium dissociation constant (K_d) values were calculated from the steady-state SPR responses. The association and the dissociation profiles were further analyzed by BIAevaluation 4.1 software (Biacore), using single-exponential functions of time (Langmuir monovalent binding model) or equations taking into account the dimeric nature of the svVEGFs and VEGF-A_{165} (bivalent analyte model).

Cell culture. Parental Porcine Aortic Endothelial Cells (PAEC) and PAEC expressing VEGFR-2 (PAEC VEGFR-2) were kindly provided by Dr. Lena Claesson-Welsh (University of Uppsala, Uppsala, Sweden). PAEC NRP1 and NRP2 were established as previously described [3,11]. PAECs were grown in Ham's F-12 medium containing 10% FBS and L-glutamine/penicillin G/streptomycin sulfate.

Binding and cross-linking studies of ^{125}I-IC1 and ^{125}I-IC2 to stable PAEC VEGFR-2, NRP1 or NRP2 lines. IC1, IC2 and VEGF-A_{165} were iodinated using the IODO-BEADS® method as previously described [3]. Specific activities of about 10,000–35,000 cpm/ng, for IC1 and IC2, and 50,000–80,000 cpm/ng for VEGF-A_{165}, respectively, were obtained.

For the competition binding experiments, PAEC VEGFR-2, NRP1 and NRP2 cells were incubated with ^{125}I-VEGF-A_{165} (5 ng/ml) in binding buffer at increasing concentrations of cold VEGF-A_{165}, IC1 or IC2 as previously described [12]. The experiment was repeated in duplicate at least twice. IC_{50} values were calculated from the graphs using the Origin 5.0 software (Microcal, Inc., Northampton, MA).

For the cross-linking experiments, cells were washed with ice-cold PBS and incubated with the radioligands (^{125}I-VEGF-A_{165} at 25 ng/ml, ^{125}I-IC1 at 100 ng/ml, or ^{125}I-IC2 at 100 ng/ml, respectively) in the same experimental conditions described [12]. The cross-linked complexes were immunoprecipitated overnight at 4 °C with anti-VEGFR-2, anti-NRP1 or anti-NRP2 antibodies, respectively, or the correspondent isotype controls as described [12].

Results

Purification of the snake venom VEGFs

Snake *M. lebetina* venom was fractionated by successive size exclusion and ion exchange chromatographies [5] (Fig. 1A). Two fractions S3 and S5, with high capillary permeability activity, eluted from a Mono S column successively at 0.14 and 0.18 M NaCl, were then investigated by reverse phase C8 chromatography. One peak from each fraction was potent in capillary permeability: IC1 from fraction S3 (Fig. 1B) and IC2 from fraction S5 (Fig. 1C). IC1 and IC2 appeared to be homogeneous as judged by further analysis by RP-18 HPLC (Supplementary Fig. S1). Electrophoretic analysis by SDS–PAGE under reducing and non-reducing conditions revealed that, like ICPP, IC1 and IC2 were composed of disulfide linked dimers and had the same electrophoretic pattern (Fig. 1B and C, inlet).

Protein analysis of IC1 and IC2

IC1 and IC2 were analyzed by MALDI-TOF spectrometry. Spectra showed that each of them contained different molecular species. IC1 revealed four peaks of 23650.02, 24306.46, 24589.65 and 24718.60 Da (Supplementary Fig. S2), with differences of 128.95, 285.14 and 656.46 Da between them. Assuming that these proteins are dimeric, these differences are being consistent with the differences by at least three amino acids, explaining why they were not separated [13]. The same phenomenon was observed with IC2 which shows three well represented peaks at 25101.12, 25194.98 and 25298.81 Da. The main peak of 25101.12 Da could correspond to ICPP, previously isolated [6], since its mass matches with that of ICPP.

The N-terminal sequence of the first 13 amino-acid residues of IC1 (EVRPFPEVYERIA) showed that it shared 84.6% and 53.8% similarity (76.9% and 46.1% identity), with ICPP and human VEGF-A_{165}, respectively (Fig. 1D). Three differences between IC1 and ICPP at positions 7, 10 and 12 where noticed, suggesting that these differences could be genetically encoded and that IC1 are not a proteolytic processing variant of ICPP.

Effects on capillary permeability

The activities of IC1 and IC2 on capillary permeability were tested in three different animals: white mice, black mice and rats. Although IC1 and IC2 seemed to have similar effects on white and black mice, IC2 increased capillary permeability in rats approximately 2-fold more than IC1 (2.25 ± 0.50 cm^2 versus 1.06 ± 0.41) (Fig. 2).

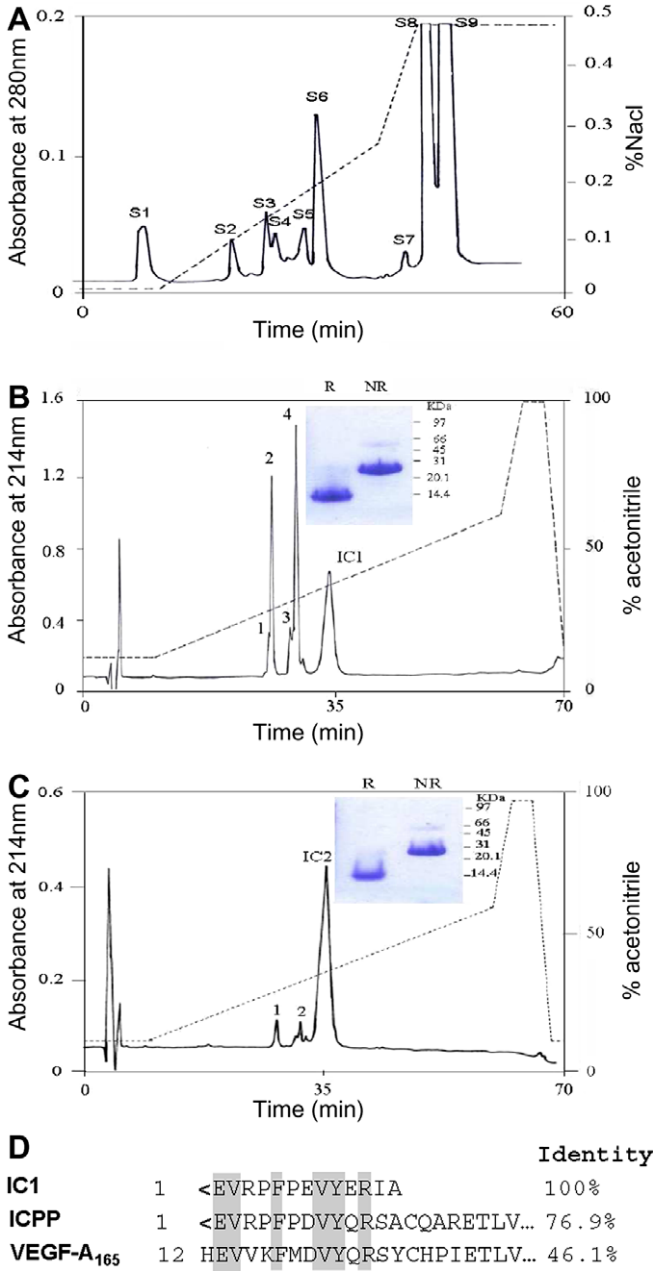

Fig. 1. Purification and structural characterization of *Macrovipera lebetina* venom VEGFs. (A) The fraction obtained after the gel filtration and anion exchange chromatography steps (data not shown) was applied to a cation exchange Mono S HR5/5 column eluted in 40 min at 1 ml/min with a gradient of 0–0.3 M NaCl. (B, C) Isolation of IC1 from S3 and IC2 from S5 by HPLC. Solvents A and B were 0.1% TFA in water and 0.1% TFA in acetonitrile, respectively. Proteins were eluted from the C8 column in 60 min at a flow of 0.8 ml/min by a linear gradient of 10–60% of solvent B. IC1 and IC2 were detected at 214 nm and collected manually at 33 min and 34 min, respectively. Inlet: purified fraction (2 μg) was analyzed by SDS–PAGE in reducing (R) and non-reducing (NR) conditions. (D) Alignment and comparison of the N-terminal sequence of IC1 with those of ICPP and human VEGF-A_{165}. Residues identical between IC1, ICPP and human VEGF-A_{165} are shaded in gray.

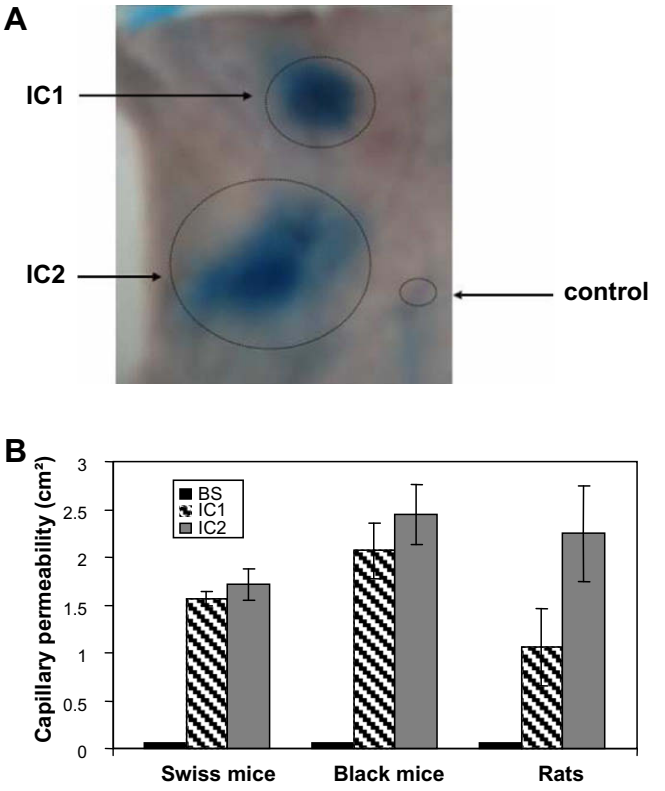

Fig. 2. Capillary permeability activity of IC1 and IC2 on different rodent species. One microgram of IC1 or IC2 was injected intradermally on the back of animals (Swiss mice, C57black/6 mice or Wistar rats). (A) Differential effect of IC1 and IC2 on the penetration of blue dye-complexed albumin in the back of a Wistar rat (photo). (B) Estimation of the areas of the circled blue spots. Data are expressed as the mean ± SD of triplicate experiments. BS: Buffered saline control (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

In vitro binding properties to the VEGF-A_{165} receptors

To determine the specificity patterns and binding characteristics of IC1 and IC2, we performed real-time surface plasmon resonance (SPR) assays. Fig. 3 showed that both IC1 and IC2 displayed a marked specificity for VEGFR-2 with virtually identical equilibrium dissociation constants (K_d) in the nanomolar range. These affinities were 10-fold lower than that of VEGF-A_{165} for VEGFR-2 (Fig. 3C and Table 1). IC2 had a barely detectable binding on VEGFR-1 (Fig. 3A), with an affinity 10,000-fold lower than for VEGFR-2 (Table 1).

Interestingly, we found that IC1 and IC2 bind to both NRP1 and NRP2 (Fig. 3B and D) with affinities in the micromolar range (Table 1). IC2 bound to both NRP1 and NRP2 approximately 2-fold stronger than IC1 (Fig. 3 and Table 1). The SPR association and dissociation profiles could not be correctly described assuming a simple monovalent (Langmuir) svVEGF/receptor binding mechanism. This could be in part due to the non-oriented covalent strategy of immobilization of the NRP/Fcs [14] or to the intrinsic heterogeneity (polymorphism) of each IC protein group.

Competition of ^{125}I-VEGF-A_{165} with IC1 or IC2 for binding to PAEC VEGFR-2, NRP1 and NRP2

To confirm results obtained with SPR assays, we investigated the ability of IC1 and IC2 to compete with ^{125}I-VEGF-A_{165} for binding to PAEC stably expressing VEGFR-2, NRP1 or NRP2. Cold VEGF-A_{165} was used as a positive control. Results showed that VEGF-A_{165} (IC_{50} = 1.7 ± 0.3 nM) was slightly more effective than IC2 (IC_{50} = 2.9 ± 0.6 nM) and IC1 (IC_{50} = 3.9 ± 0.4 nM) in competing for ^{125}I-VEGF-A_{165} binding (Fig. 4A and Table 2). On the contrary, VEGF-A_{165} was significantly more effective than IC1 and IC2 in displacing ^{125}I-VEGF-A_{165} from the PAEC NRP1 cell surface (Fig. 4B). At high concentration (4 μM) of IC1 and IC2, only 50% and 70%, respectively, of the total ^{125}I-VEGF-A_{165} were displaced with IC_{50}s of 4.1 μM and 2.95 μM, respectively (Table 2). Inhibition with VEGF-A_{165} reached a saturation at about 20 nM (IC_{50} = 1.5 ±

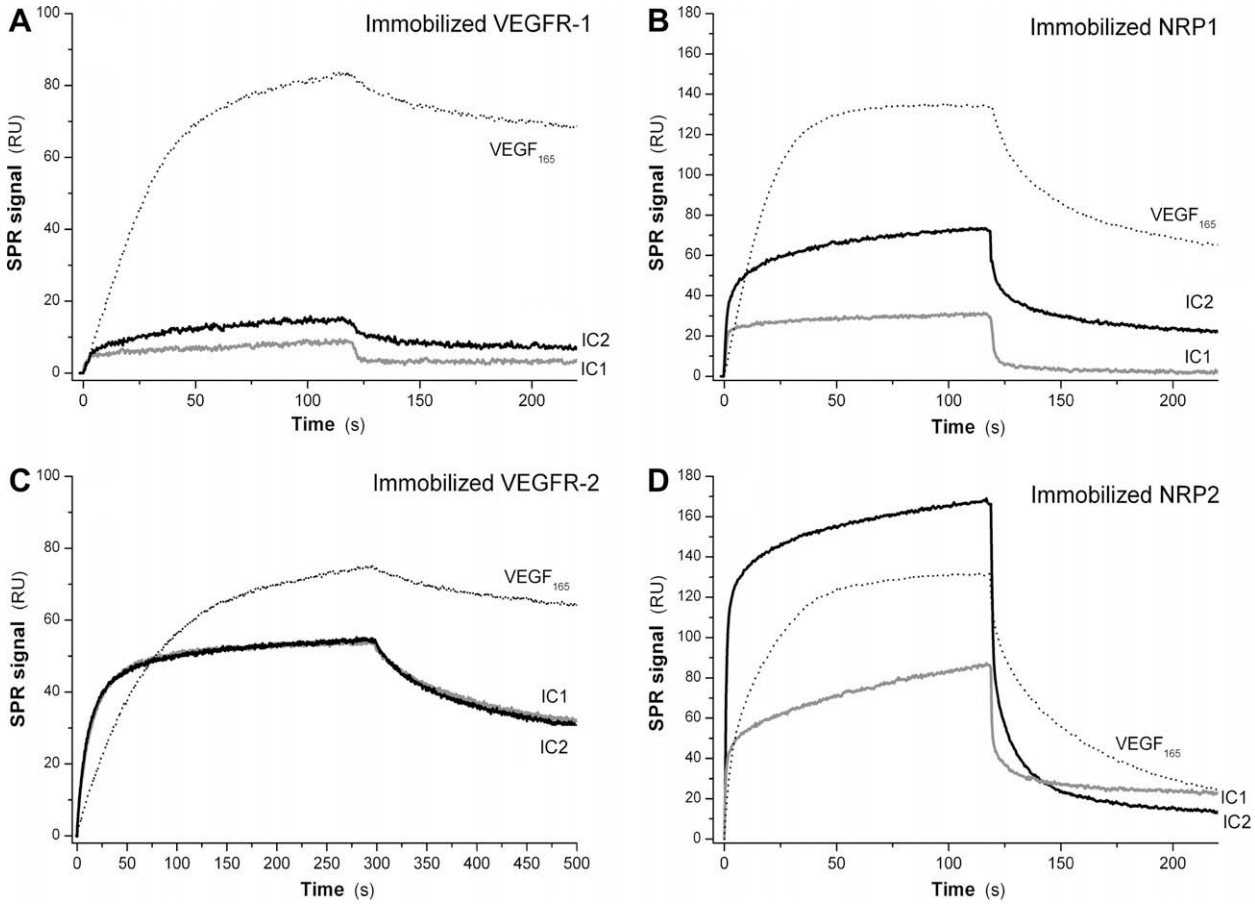

Fig. 3. *Macrovipera lebetina* venom VEGFs interact differentially with VEGF receptors. Real-time SPR association and dissociation profiles corresponding to the injections of IC1 (gray lines), IC2 (bold black lines) or VEGF-A_{165} (dashed lines) over immobilized VEGF receptors. (A) IC1 (2 μM), IC2 (2 μM) or VEGF-A_{165} (5 nM) over VEGFR-1. (B) IC1 (2 μM), IC2 (2 μM) or VEGF-A_{165} (20 nM) over NRP1. (C) IC1 (125 nM), IC2 (125 nM) or VEGF-A_{165} (5 nM) over VEGFR-2. (D) IC1 (2 μM), IC2 (2 μM) or VEGF-A_{165} (20 nM) over NRP2.

Table 1
Equilibrium dissociation constants (K_d) of the interactions between *Macrovipera lebetina* venom VEGFs (IC1 and IC2) or human VEGF-A_{165} in solution, and immobilized VEGF receptors (VEGFR-1, VEGFR-2, NRP1 or NRP2).

Immobilized VEGF receptors	VEGF in solution		
	IC1	IC2	VEGF-A_{165}
VEGFR-1	N.D.	21 (±8) × 10^{-6} M	9.1 (±3.2) × 10^{-11} M
VEGFR-2	1.5 (±0.6) × 10^{-9} M	1.6 (±0.5) × 10^{-9} M	1.5 (±0.5) × 10^{-10} M
NRP1	4.8 (±1.3) × 10^{-6} M	2.5 (±0.5) × 10^{-6} M	3.8 (±0.5) × 10^{-9} M
NRP2	2.0 (±1.1) × 10^{-6} M	8.1 (±1.4) × 10^{-7} M	9.7 (±1.8) × 10^{-9} M

0.7 nM). On PAEC NRP2, VEGF-A_{165} was still the best competitor (IC_{50} = 2.6 ± 1.1 nM) (Fig. 4C and Table 2). However, on these cells, the IC_{50} for IC2, (42.0 ± 10.9 nM) was significantly lower than that on PAEC NRP1, while the IC_{50} for IC1 (4.1 μM) was in the same range to its IC_{50} on PAEC NRP1 (Table 2).

Cross-linking of ^{125}I-IC1 and ^{125}I-IC2 to PAEC NRP1 and NRP2

In order to confirm the interaction of svVEGFs with VEGF co-receptors, we investigated the ability of IC1 and IC2 to bind both NRP1 and NRP2 expressed on endothelial cells. Fig. 4D showed that ^{125}I-IC2 formed complexes with PAEC NRP1 or NRP2 which were immunoprecipitated by the respective NRP1 and NRP2 antibodies, but not by corresponding control IgGs (Fig. 4D, upper). Interestingly, NRP2 was immunoprecipitated at a significantly lower level than NRP1 (Fig. 4D). However similar results were obtained using ^{125}I-VEGF-A_{165} as a positive control (data not shown). The differences between the NRP1 and NRP2 band intensities might therefore be due to different levels of expression of the two NRPs in transfected PAEC, rather than to differences in affinities. As for IC1, while complexes with PAEC NRP1 were readily observed, although in lower amounts than for IC2, the band for NRP2 was below the threshold of detection (Fig. 4D, lower). Finally, as expected, we observed that IC1 and IC2, as well as VEGF-A_{165}, formed complexes with PAEC VEGFR-2 (data not shown).

Discussion

Snake venom VEGFs (svVEGFs) play a crucial role in the dissemination of the venom toxins in diverse preys. Previous studies suggested that the venom of *M. lebetina* contains only one svVEGF called ICPP [6,15]. This protein mediates its activities through VEGFR-2 and possibly VEGFR-1 [6].

In the present study, we showed that the venom of *M. lebetina* in fact contains several others VEGFs. In deed, we identified two distinct protein groups IC1 and IC2 based on their capillary permeability activity with molecular masses ranging from 23,650 to 25,298 Da. Attempts to isolate each individual protein from the two protein groups according to their molecular masses (gel filtration), their charges (anion and cation exchange chromatography) and their hydrophobicity (RP-18 HPLC) did not succeed. This was also the case for others peptides from the same venom [13].

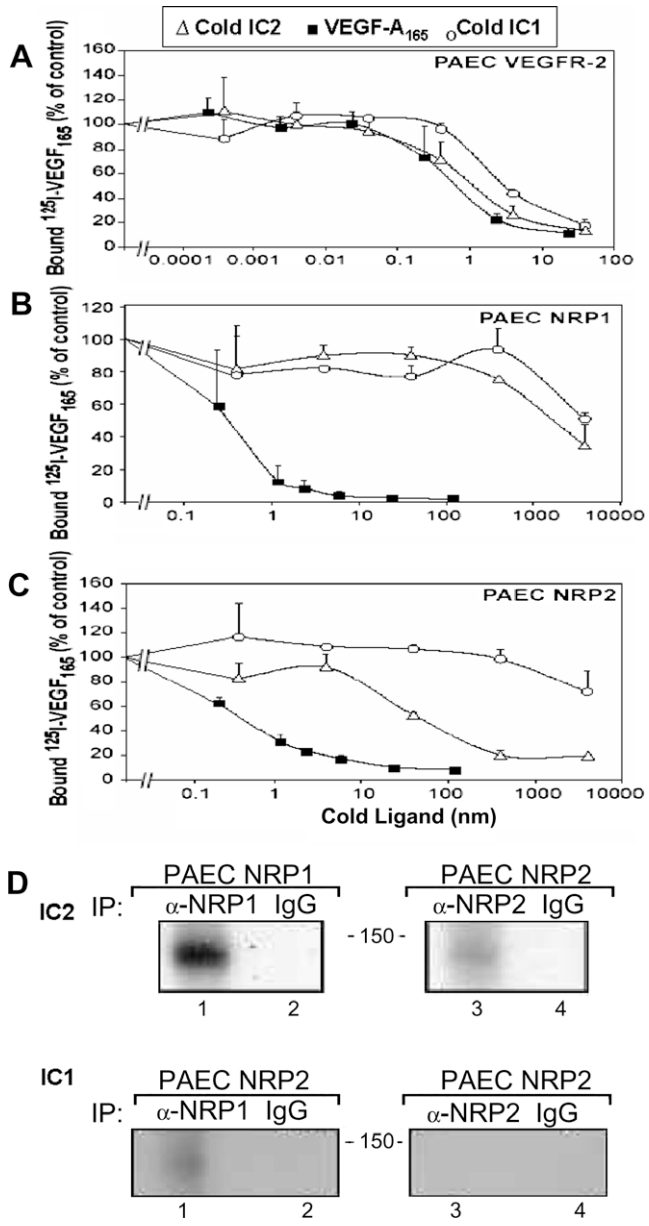

Fig. 4. Competition and cross-linking experiments on endothelial cells. PAEC VEGFR-2 (A), NRP1 (B) and NRP2 (C) were incubated in a 48-well plate with ^{125}I-VEGF-A_{165} (5 ng/ml) at increasing concentrations of cold VEGF-A_{165} (■), cold IC2 (Δ) or cold IC1 (○) for 1 h at 4 °C. After lysis, the cell-associated radioactivity was measured in a γ-counter. The results are expressed as percentages of cell-bound radioligand. (D) PAE cells transfected with NRP1 or NRP2 were incubated with ^{125}I-IC2 (100 ng/ml) (upper) or ^{125}I-IC1 (100 ng/ml) (lower). The cross-linked complexes were immunoprecipitated with anti-NRP1, -NRP2 or isotype control IgG antibodies and resolved by SDS–PAGE on a 7.5% acrylamide gel. The corresponding autoradiographies are shown.

The existence of molecular svVEGF polymorphism has been reported in different snake venoms by several groups. Komori et al. [16] postulated that these could be due to protease cleavage during the process of purification or storage. Other groups attributed these variations to either differences in the primary amino-acid sequences, alternative splicing or post-translational modifications [17]. More recently, Boldrini-França et al. [18] identified seven expressed sequence tags classified as svVEGFs from *Crotallus durissus collilineatus* venom gland cDNA library. In our case, N-terminal sequencing of IC1 reveals 13 amino acids that differ from that of ICPP by three residues showing that it consists of distinct isoforms.

Table 2
IC_{50}s corresponding to the inhibition of ^{125}I-VEGF-A_{165} binding to PAEC VEGFR-2, NRP1 and NRP2 by VEGF-A_{165}, IC1 and IC2.

Ligands	IC_{50}		
	PAEC VEGFR-2	PAEC NRP1	PAEC NRP2
VEGF-A_{165}	1.7 (±0.3) × 10^{-9} M	1.5 (±0.7) × 10^{-9} M	2.6 (±1.1) × 10^{-9} M
IC1	3.9 (±0.4) × 10^{-9} M	4100 (±1000) × 10^{-9} M	4100 (±300) × 10^{-9} M
IC2	2.9 (±0.6) × 10^{-9} M	2950 (±450) × 10^{-9} M	42.0 (±10.9) × 10^{-9} M

Our SPR experiments showed that the *M. lebetina* venom IC1 and IC2 are capable of forming very stable complexes with VEGFR-2 ($t_{1/2} = \ln 2/k_{off} \approx 900$ s), similar to vammin from *Vipera ammodytes ammodytes* [19]. At high concentrations, a faint VEGFR-1 binding could be detected for IC2 and none for IC1 (Fig. 3A). However, it is very unlikely that such a weak binding at these high concentrations has any biological significance. Contrary to svVEGFs from the Viperinae sub-family, VEGFs from the Crotalinae subfamily have been shown to bind preferentially to VEGFR-1 [20,21]. IC1 and IC2 were also able to interact with NRP1 and NRP2 at a comparable concentration range of that of Tuftsin (TKPR), an analog of VEGF-A_{165} exon8 [22]. This novel binding capacity of IC1 and IC2 to neuropilins could reflect genuine individual characteristics of the svVEGFs of *M. lebetina* arising from evolutive divergence.

Crystal structure of Tuftsin with the B domain of NRP1 demonstrates that exon8 contributes to the binding of VEGF-A_{165} to NRPs [23]. Interestingly, the C-terminus of ICPP (EKPR) [6], the main component of IC2, reveals high homology to VEGF exon8 (CDKPRR). This suggests that the interaction of IC2 with NRP1 is mediated in part by its C-terminal residues. Although the biological relevance of this interaction is not yet well understood, two possibilities can be proposed: either neuropilins can act as co-receptors for svVEGFs, enhancing signal transduction via VEGFR-2 (as in the case of VEGF-A), or the interaction with neuropilins results in the triggering of other unknown biological functions during envenomation.

In accordance with SPR results, competition binding experiments showed that IC1, IC2 and VEGF-A_{165} share similar binding properties to VEGFR-2. However, VEGF-A_{165} was a better ligand for neuropilins. IC2, while being a weak competitor of ^{125}I-VEGF-A_{165} for binding to NRP1, appears to be more effective competitor for binding to NRP2 (Table 2). The expression pattern of the two neuropilins in the vascular system is compartmentalized. NRP1 is specifically expressed in arteries, which stimulation modulates the immune system [24], and is involved in axonal guidance [25], endothelial permeability [26] and inflammation [27]. NRP2 is on the contrary, specifically expressed in veins and lymphatics [28,29]. Thus, the interaction capacity and selectivity of IC2 for NRP2 may have interesting therapeutic implications.

Interestingly, IC1 and IC2 also showed slightly different specificity patterns when assaying their effect on capillary permeability in different rodent species. Yamazaki et al. [30] have recently published a large overview of the diversity of the svVEGF family among different species, and studied the mechanism by which the polymorphism is generated. The novelty of our study is that we show that svVEGF polymorphism occurs not only among species, but also within a same species, most probably through gene duplication and diversification. Future studies should allow a better understanding of the mechanism of diversity generation among the proteins composing snake venoms and should also be helpful for the design of peptidomimetic inhibitors of angiogenesis in cancer therapy. Moreover, these VEGF-mimetic molecules could be useful to stimulate revascularization in ischemic diseases, since

they could promote endothelial cell survival and migration through their interaction with neuropilins.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.08.068.

References

[1] A. Gasmi, J. Pouysségur, New proteins from snake venom: attractive tools for studying angiogenesis, in: R.V. Zubar (Ed.), New Angiogenesis Research, Nova Science Publishers, 2005, pp. 116–131.
[2] N. Ferrara, H.P. Gerber, J. LeCouter, The biology of VEGF and its receptors, Nat. Med. 9 (2003) 669–676.
[3] S. Soker, S. Takashima, H.Q. Miao, G. Neufeld, M. Klagsbrun, Neuropilin-1 expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor, Cell 92 (1998) 735–745.
[4] P. Lee, K. Goishi, A.J. Davidson, R. Mannix, L. Zon, M. Klagsbrun, Neuropilin-1 is required for vascular development and is a mediator of VEGF-dependent angiogenesis in zebrafish, Proc. Natl. Acad. Sci. USA 99 (2002) 10470–10475.
[5] A. Gasmi, F. Abidi, N. Srairi, A. Oijatayer, H. Karoui, M. Elayeb, Purification and characterization of a growth factor-like which increases capillary permeability from *Vipera lebetina* venom, Biochem. Biophys. Res. Commun. 268 (2000) 69–72.
[6] A. Gasmi, C. Bourcier, Z. Aloui, N. Srairi, S. Marchetti, C. Gimond, S.R. Wedge, L. Hennequin, J. Pouysségur, Complete structure of an increasing capillary permeability protein (ICPP) purified from *Vipera lebetina* venom. ICPP is angiogenic via VEGF receptor signaling, J. Biol. Chem. 277 (2002) 29992–29998.
[7] U.K. Laemmli, Cleavage of structural proteins during the assembly of head of bacteriophage T4, Nature 277 (1970) 680–685.
[8] O.H. Lowry, N.H. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[9] I. Kawasaki, H.A. Itano, Methanolysis of the pyrrolidone ring of amino-terminal pyroglutamic acid in model peptides, Anal. Biochem. 48 (1972) 546–565.
[10] A.A. Miles, D.L. Wilhelm, Enzyme like globins from serum reproducing the vascular phenomena of inflammation: I. An activable permeability factor and its inhibitor in guinea pigs serum, Br. J. Exp. Pathol. 36 (1955) 71–81.
[11] D.R. Bielenberg, Y. Hida, A. Shimizu, A. Kaipaine, M. Kreuter, C.C. Kim, M. Klagsbrun, Semaphorin 3F, a chemorepulsant for endothelial cells, induces a poorly vascularized, encapsulated, nonmetastatic tumor phenotype, J. Clin. Invest. 114 (2004) 1260–1271.
[12] E. Geretti, A. Shimizu, P. Kurschat, M. Klagsbrun, Site-directed mutagenesis in the B-neuropilin-2 domain selectively enhances its affinity to VEGF165, but not to semaphorin 3F, J. Biol. Chem. 282 (2007) 25698–25707.
[13] R. Barbouche, N. Marrakchi, P. Mansuelle, M. Krifi, E. Fenouillet, H. Rochat, M. El Ayeb, Novel anti-platelet aggregation polypeptides from *Vipera lebetina* venom: isolation and characterization, FEBS Lett. 392 (1996) 6–10.
[14] P. England, F. Bregegere, H. Bedouelle, Energetic and kinetic contributions of contact residues of antibody D1.3 in the interaction with lysozyme, Biochemistry 36 (1997) 164–172.
[15] A. Bazaa, N. Marrakchi, M. El Ayeb, L. Sanz, J.J. Calvete, Snake venomics: comparative analysis of the venom proteomes of the Tunisian snakes *Cerastes cerastes*, *Cerastes vipera* and *Macrovipera lebetina*, Proteomics 5 (2005) 4223–4235.
[16] Y. Komori, T. Nikai, T. Taniguchi, K. Masuda, H. Sugihara, Vascular endothelial growth factor VEGF-like heparin-binding protein from the venom of *Vipera aspis aspis* (Aspic viper), Biochemistry 38 (1999) 11769–11803.
[17] I.L. Junqueira de Azevedo, S.H. Farsky, M.L. Oliveira, P.L. Ho, Molecular cloning and expression of a functional snake venom vascular endothelium growth factor (VEGF) from the *Bothrops insularis* pit viper. A new member of the VEGF family of proteins, J. Biol. Chem. 276 (2002) 39836–39842.
[18] J. Boldrini-França, R.S. Rodrigues, F.P. Fonseca, D.L. Menaldo, F.B. Ferreira, F. Henrique-Silva, A.M. Soares, A. Hamaguchi, V.M. Rodrigues, A.R. Otaviano, M.I. Homsi-Brandeburgo, *Crotallus Durissus collilineatus* venom gland transcriptome: analysis of gene expression profile, Biochimie 91 (2009) 586–595.
[19] Y. Yamazaki, K. Takani, H. Atoda, T. Morita, Snake venom vascular endothelial growth factors (VEGFs) exhibit potent activity through their specific recognition of VEGFR-2 (VEGF receptor 2), J. Biol. Chem. 278 (2003) 51985–51988.
[20] H. Takahashi, S. Hattori, A. Iwamatsu, H. Takizawa, M. Shibuya, A novel snake venom vascular endothelial growth factor (VEGF) predominantly induces vascular permeability through preferential signaling via VEGF receptor-1, J. Biol. Chem. 279 (2004) 46304–46314.
[21] Y.L. Chen, H.I. Tsai, T.M. Hong, S.H. Tsai, Crotalid venom vascular endothelial growth factor has preferential affinity for VEGFR-1. Characterization of *Protobothrops mucrosquamatus* venom VEGF, Thromb. Haemost. 93 (2005) 331–338.
[22] M.A. von Wronski, N. Raju, R. Pillai, N.J. Bogdan, E.R. Marinelli, P. Nanjappan, K. Ramalingam, T. Arunachalam, S. Eaton, K.E. Linder, F. Yan, S. Pochon, M.F. Tweedle, A.D. Nunn, Tuftsin binds neuropilin-1 through a sequence similar to that encoded by exon 8 of vascular endothelial growth factor, J. Biol. Chem. 281 (2006) 5702–5710.
[23] C.W. Vander Kooi, M.A. Jusino, B. Perman, D.B. Neau, H.D. Bellamy, D.J. Leahy, Structural basis for ligand and heparin binding to neuropilin B domains, Proc. Natl. Acad. Sci. USA 104 (2007) 6152–6157.
[24] P.H. Romeo, V. Lemarchandel, R. Tordjman, Neuropilin-1 in the immune system, Adv. Exp. Med. Biol. 515 (2002) 49–54.
[25] Q. Schwarz, J.M. Vieira, B. Howard, B.J. Eickholt, C. Ruhrberg, Neuropilin 1 and 2 control cranial gangliogenesis and axon guidance through neural crest cells, Development 135 (2008) 1605–1613.
[26] P.M. Becker, J. Waltenberger, R. Yachechko, T. Mirzapoiazova, J.S. Sham, C.G. Lee, J.A. Elias, A.D. Verin, Neuropilin-1 regulates VEGF-mediated endothelial permeability, Circ. Res. 96 (2005) 1257–1265.
[27] M.R. Saban, J.M. Backer, M.V. Backer, J. Maier, B. Fowler, C.A. Davis, C. Simpson, X.R. Wu, L. Birder, M.R. Freeman, S. Soker, R.E. Hurst, R. Saban, VEGF receptors and neuropilins are expressed in the urothelial and neuronal cells in normal mouse urinary bladder and are upregulated in inflammation, Am. J. Physiol. Renal Physiol. 295 (2008) 60–72.
[28] Y. Herzog, C. Kalcheim, N. Kahane, R. Reshef, G. Neufeld, Differential expression of neuropilin-1 and neuropilin-2 in arteries and veins, Mech. Dev. 109 (2001) 115–119.
[29] A. Eichmann, L. Yuan, D. Moyon, F. Lenoble, L. Pardanaud, C. Breant, Vascular development: from precursor cells to branched arterial and venous networks, Int. J. Dev. Biol. 49 (2005) 259–267.
[30] Y. Yamazaki, Y. Matsunaga, Y. Tokunaga, S. Obayashi, M. Saito, T. Morita, Snake venom vascular endothelial growth factors (VEGF-Fs) exclusively vary their structures and functions among species, J. Biol. Chem. 284 (2009) 9885–9891.