Molecular cloning and expression of a functional snake venom Vascular Endothelium Growth Factor (svVEGF) from the Bothrops insularis pit viper. A new member of the VEGF family of proteins.¹

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Running title: Cloning of a snake venom Vascular Endothelium Growth Factor

Summary

During the generation of abundant Expressed Sequence Tags (ESTs) from the Viperidae snake Bothrops insularis venom glands, we identified for the first time a cDNA coding for a putative vascular endothelial growth factor-like (VEGF-like) protein. The deduced primary sequence, after complete sequencing of the longest snake venom VEGF (svVEGF) cDNA, displayed similarity with vertebrate VEGFs and with the hypotensive factor (HF) from Vipera aspis venom. Its cDNA was subcloned, expressed in E. coli with a 6X His-tag as an insoluble monomer and purified by a Ni²⁺-affinity chromatography after 8M urea extraction. Antiserum against svVEGF was generated and tested in Western-blot against proteins from snake venoms and cellular extracts. The mature svVEGF appears to be ubiquitous distributed throughout snake venoms and was also confirmed by Northern Blot studies of other related Viperidae species and by cDNA cloning of svVEGF from Bothrops jararaca pit viper. The produced recombinant protein, dimerizes after refolding processes and was biologically characterized, showing ability to increase vascular permeability. These results established that svVEGF is a novel and important active toxin during the early stages of bothropic snake bite envenoming and represents a new member of the VEGF family of proteins.
Introduction

The diversity of proteins, enzymes and biologically active peptides found in snake venoms influences the wide range of biological processes destabilized during envenoming. In *Viperidae* snakes, these effects can be categorized in local damage or systemic injury. Local damage results mainly from enzymes such as phospholipases A$_2$ which produces myonecrosis and edema (1) and metalloproteinases that destroys the endothelium, components of vascular wall and extravascular matrix, causing vascular permeability disorders. The systemic effects are those related to the disruption of the hemostatic equilibrium of blood coagulation and hemorrhage caused by other metalloproteinases, serinoproteinases, disintegrins and type C lectins (2). Another common effect observed in these envenomings is hypotension, first known to be a consequence of bradykinin potentiating peptides (BPPs)$^2$, described and cloned in *B. jararaca* venom (3,4). These peptides inhibit angiotensin converting enzyme (ACE EC3.4.15.1) and enhances bradykinin action, generated by proteolytic cleavage of host proteins by venom proteases. Kallikrein-like enzyme is one of them, which promotes kinins release from kininogen, increasing capillary permeability (5). Despite the well-characterized action mechanisms of BPPs, it was established that other factors present in the venoms also act as hypotensive agents. For instance, the presence of natriuretic peptides was described by protein chemistry and cDNA cloning studies (4, 6-8).
In *Vipera aspis* venom, a hypotensive factor (HF) was described as being a potent non-lethal decreasing pressure protein (9). The identity of this toxin and of ICPP (10), a related polypeptide from *Vipera lebentina*, remained unknown until its amino acid sequence was uncovered by Edman sequencing, showing a strong similarity with vascular endothelium growth factor (VEGF) (10,11). The biological properties of HF, besides the hypotension, includes the angiogenic effects and the vascular permeability increasing capability of typical VEGFs (11).

The VEGF family of proteins is composed by several members, including the VEGF, first known as vascular permeability factor (VPF) (12), the placental growth factor (PIGF) and platelet-derived growth factor (PDGF). They are dimeric proteins composed of two identical or structurally related subunits linked by two inter-chain disulfide bridges. VEGFs act through binding to specific tyrosine-kinases receptors VEGFR-1 (flt-1), VEGFR-2 (KDR/flk-1) and/or VEGFR-3 (flt-4), which are mainly expressed in endothelial cells (see reviews in 13-15). Human VEGF gene is composed of 8 exons that can be alternatively spliced resulting in five C-terminal truncated isoforms of monomeric proteins named by their length in amino acids of the mature protein, VEGF$_{121}$, VEGF$_{145}$, VEGF$_{165}$, VEGF$_{189}$ and VEGF$_{206}$ (13, 16). The shorter forms are released into the blood. The longer ones stay adsorbed to the extracellular matrix of cell surfaces through heparin-like proteoglycans molecules that binds to the basic amino acid residues localized at the carboxi-terminus of the longest VEGF forms (17). The distribution of VEGF occurs throughout vertebrates, being found and cloned in zebrafish, *Xenopus*, chick and many mammals. Other VEGF related molecules has also been reported: VEGF-B (18), VEGF-C (19) and VEGF-D (20). Some virus genomes have been reported to code a VEGF-like protein (21, 22).
During the analysis of preliminary ESTs generated from a cDNA library of venom glands from the snake *B. insularis*, an endemic *Viperidae* snake restricted to Queimada Grande Island in the São Paulo State coast (Brazil), we identified many independent clones coding for a VEGF-like protein. A cDNA library from *B. jararaca*, a pit viper snake responsible for the majority of snake envenoming accidents in Brazil, was screened and also revealed cDNAs coding for this factor. This is the first report of VEGF-like cDNA sequences cloned from snake venom glands, here named svVEGF. In addition, *B. insularis* svVEGF was expressed in *E. coli* and the recombinant protein was biologically characterized. An antiserum was also produced and used to detect svVEGF in other snake venoms. 3.
Experimental Procedures

cDNA Library Construction - One specimen of *B. insularis* was collected from Ilha da Queimada Grande in São Paulo State coast (Brazil). The snake was milked to stimulate the production of mRNAs in the venom glands. After 5 days, the animal was sacrificed by CO₂ and the pair of venom glands was dissected and kept in liquid nitrogen until use. For total RNA extraction, it was used the Trizol reagent (Gibco-BRL Life Technologies) according to the manufacturer protocol. A column of oligo-dT cellulose (Amersham-Pharmacia Biotech) was used for mRNA purification. The cDNAs were synthesized from 5 μg of mRNA using the Superscript Plasmid System for cDNA Synthesis and Cloning (Gibco-BRL Life Technologies), linked to EcoRI adapters (Amersham-Pharmacia Biotech), selected by size (350-600 pb and up 600 pb) in agarose gel electrophoresis and directionally cloned in pGEM11Zf+ plasmid (Promega) at EcoRI/NotI sites. *E. coli* DH5α cells were transformed with the cDNA library plasmids and then plated on a 2YT agarose plate containing 100 μg/ml of ampicillin (23).

svVEGF Sequence Identification - ESTs were generated from *B. insularis* library. Random colonies were inoculated in the same liquid culture medium, grown overnight and the
plasmidial DNA purified and sequenced on a ABI 377 using BigDye2 dideoxyterminators (Applied Biosystems). The primers used to sequence were M13 forward and M13 reverse. Generated ESTs were compared to GenBank nucleotide and protein databases using Blastn and Blastx programs at NCBI to identify putative gene products. Complete DNA sequence was obtained by primer-walking and deposited at GenBank under accession number AY033151. Sequence analysis was performed by using Vector NTI Suite software (Informax).

**Northern Blot Assay** - Total RNAs from several tissues and snakes were isolated as described above (cDNA library construction). 10 µg of total RNAs were fractionated on denaturant 1% agarose/formaldehyde gel, transferred to a nylon membrane (Genescreen, NEN) and fixed by baking at 80°C for 2 hours. The probe was obtained by digestion of clone GH027 by EcoRI and HindIII restriction enzymes. The svVEGF fragment was isolated after agarose gel electrophoresis and labeled by random primer extension, using digoxigenin (DIG) labeled dNTPs (Boehringer). The membrane was incubated with denatured DIG-labeled probe diluted in hybridization buffer (50% formamide, 5 X SSC, 5 X Denhardt’s, 0,1% N-laurilsarcosine and 100µg/ml salmon sperm DNA) at 42°C for 16h. The membrane was washed twice with 2X SSC / 0,1% SDS at 20°C for 5 minutes and twice with 0,1X SSC / 0,1% SDS at 65°C for 15 minutes. Detection was performed with anti-digoxigenin-alkaline-phosphatase conjugate according to manufacturer protocol and interrupted after 20 minutes of colorimetric reaction.

**Recombinant Protein Expression** - A DNA fragment encoding the mature svVEGF was amplified by PCR using clone GH027 as template. The svVEGF forward primer (svVEGF-M 5’
tgcgcgcaagtgaatctctagagga 3’) was designed and contained a XhoI restriction site and the nucleotide sequence coding the first seven amino acids of the putative mature protein (Fig. 1). The reverse primer used was SP6 (5’ attaggtgacactatag 3’) which anneals to the SP6 promoter located in the pGEM11Zf+ vector. The PCR product was cloned into pGEM-T plasmid (Promega). Plasmidial DNA containing the mature svVEGF insert was digested with XhoI and HindIII enzymes. The excised insert was subcloned in the same sites of pAE expression vector 4 , a plasmid derived from pRSET (Invitrogen) and pET 3-His (24) plasmids, resulting in the pAE-msvVEGF construct. The correct cloning was confirmed by sequencing. The recombinant mature svVEGF (rmsvVEGF) was expressed with additional eight amino acids fusioned at N-terminus, including a 6XHis-tag. For the production of rmsvVEGF, transformed E. coli BL21 (DE3) cells were inoculated in 100 ml of LB/amp and grown overnight at 37 °C. In the next day, the culture was diluted with 2 X YT medium to 1 L and grown until the OD at 600 nm reached 0.6. At this moment, IPTG was added to a final concentration of 0.5 mM and the induced culture was incubated for additional 2h. The cells were then collected by centrifugation. Bacterial pellet was resuspended in TIN buffer (100 mM Tris-HCl pH 8,0; 5 mM imidazole and 300 mM NaCl) and disrupted by french-pressure. Inclusion bodies were harvested by centrifugation, washed with 2M urea in TIN buffer. rmsvVEGF was extracted with extraction buffer (100 mM Tris-HCl pH 8,0; 300 mM NaCl, 8 M urea and 5 mM imidazole) for 12 hours and clarified by centrifugation. The supernatant was loaded onto a Ni²⁺-sepharose column (Amersham-Pharmacia). The column was washed with 10 volumes of extraction buffer, followed by 10 volumes of wash buffer (extraction buffer + 60 mM imidazole) and rmsvVEGF was eluted with elution buffer (extraction buffer + 1 M imidazole). Fractions of 1 ml were collected and analyzed.
in SDS-PAGE.

Protein refolding was achieved essentially as described (25). Ten mg of unfolded rmsvVEGF, determined by Coomassie Blue (26), were pooled, diluted to a final concentration of 0.5 mg/ml in 20 mM Tris pH 8.0, 6M guanidine hydrochloride, 10 mM DTT and incubated for 3 hours, at room temperature. 20 ml of this solution was dialyzed against 250 ml of 20 mM Tris-HCl pH 8.0, 2M urea, containing 2 mM reduced glutathione (GSH) and 0.5 mM oxidized glutathione (GSSG) for 24 hour at 4°C. The dialysis buffer was changed to 500 ml of 20 mM Tris pH 8.0 and kept dialyzing overnight at 4°C. The insoluble material was removed by centrifugation and the soluble refolded protein was extensively dialyzed against PBS. The mixture of monomers and dimers was separated by heparin-sepharose chromatography as described (11). The protein mixture was adsorbed on the resin in PBS, washed by PBS and the refolded rmsvVEGF dimers were eluted with PBS containing 300 mM NaCl.

**rmsvVEGF Antiserum Generation** - Ten male Swiss mice (20g/animal) were immunized with 15 µg of unfolded rmsvVEGF. The protein was injected i.p. in 500 µl of PBS buffer containing 10% (w/v) Al(OH)₂ used as adjuvant. After 15 days, the animals were injected with a booster dose of additional 15 µg of the protein. In the 30th day, they were bled from periorbital artery and the serum extracted by centrifugation after clotting. The serum title was followed by ELISA.

**Western-Blotting** - Samples of snake venoms obtained from Herpetology Department, Instituto Butantan, cellular extracts and recombinant rmsvVEGF were resolved in 15% SDS-
PAGE in both reducing or non-reducing conditions and blotted onto nitrocellulose membranes. The membranes were stained with Ponceau S, blocked with 5% non-fat milk, incubated with the anti-rmsvVEGF serum at 1:500 dilution in PBS/0.5% Tween 20 for 2 hours, washed and incubated with horse-peroxidase-conjugated anti-mouse IgG (Sigma) for 1 hour. The blot was revealed using DAB (LifeTechnologies) after metal ion enhancement with CoCl$_2$.

Screening of Bothrops jararaca cDNA Library for VEGF cDNA - A cDNA library from $B. jararaca$ venom glands (27) was constructed as described above for $B. insularis$ cDNA library. The svVEGF cDNA from $B. jararaca$ venom gland was isolated by PCR using the forward svVEGF-M primer and the SP6 primer described above (Recombinant Protein Expression Section). These primers would hybridize respectively to the svVEGF cDNA and to the SP6 promoter at pGEM11Zf(+) plasmid used to construct the library. The amplified DNA was further subcloned in pGEM-T (Promega) and completely sequenced as described above. To obtain the complete cDNA sequence containing the 5'$\text{UTR}$ and the signal peptide, a PCR was performed using $B. jararaca$ cDNA library as a template and the svVEGF reverse primer (5’ agctgtcaagaggtcttc 3’, Fig. 1) and the T7 promoter which would hybridize to the cDNA and to pGEM11Zf(+) plasmid, respectively. The amplified fragment was further subcloned and completely sequenced. All the sequences were assembled with ContigExpress software (Informax) to derive the complete $B. jararaca$ svVEGF cDNA sequence. The complete $B. jararaca$ svVEGF sequence was confirmed after sequencing of at least 3 independent clones and is available in GenBank under accession number AY033152.
Vascular Permeability Assays - The refolded dimeric form of rmsvVEGF was assayed for its ability to induce vascular permeability in male Swiss mice (18-22g), obtained from Instituto Butantan. Briefly, 50 µl of PBS or equivalent volume of PBS containing different concentrations of protein were injected intradermally into mice backs. Immediately after, 200 µl of 1% Evan’s Blue solution was injected intravenously through orbital plexus. The dye binds to plasma albumin and is used as a macromolecular tracer during microvascular permeability increasing (28). At varying intervals of time after injections, animals were sacrificed and the dorsal epithelium dissected and photographed to visualize the leakage of the dye into the extravascular space.

Results

Identification and Characterization of Snake Venom VEGF by cDNA Cloning - A cDNA library was constructed from the venom glands of *B. insularis* in order to characterize abundant ESTs from this specialized tissue. The ESTs obtained from 5’ and/or 3’ ends were searched with Blastn and Blastx programs (29), for similar sequences in DNA and protein databases. From the first 163 clones analyzed, 4 clones revealed significant similarity at amino acid level with VEGFs from different sources. *Danio rerio* (zebrafish) VEGF
165 showed the highest score, followed by mammalians VEGFs. Since the size of the longest cDNA, accessed by restriction analysis were about 1.2 kb, two primers (svVEGF-f and svVEGF-r) were designed based on both ESTs ends to extend and close the sequence. The complete nucleotide sequence (Fig. 1) was 1200 bp and revealed an ORF of 146 amino acids with an ATG start codon at position 186 and
the TGA stop codon at position 624. The cDNA possesses an unusual superimposed double polyadenylation signal (AATAAATAAA) located 11 bp upstream of the poly A+ tail. The deduced svVEGF polypeptide has a predicted signal peptide composed by the first 24 amino acids, according to Nielsen’s algorithm (30) and is in accordance with the N-terminal amino acid sequence alignment of HF protein characterized from *V. aspis* snake venom (11) (Fig. 2). A molecular mass of 13.8 kDa and pI of 8.19 were predicted for the putative mature protein.

Figure 2 shows the sequence alignment and comparison of deduced svVEGF with HF protein, VEGFs and PIGFs. The mature 122 amino acid sequence presents 68.4% of identity with HF protein from *Vipera aspis* and around 50% with other vertebrate VEGFs, being more similar to *Danio rerio* (64.2%) than to human VEGFs (60.0%). PIGFs and VEGF-B are less similar to svVEGFs, but showed some conserved structural features. The main characteristic of VEGF/PIGF family is the presence of the three intra-chain and the two inter-chain disulfide bridges. svVEGF possesses all the 8 cysteine residues involved in the disulfide bridges formation, thus suggesting its ability to dimerize through the formation of the same intra and inter disulfide bonds. The deduced signal peptide is shorter (Fig. 2) and the putative mature protein displays the hydrophobic residues Val2, Phe5, Met6 which have been shown to participate in an amphipathic α-helix that stabilize the dimerization of human VEGF (31). The methionine at position 3 would participate in that process in the place of the valine in this same position in VEGF121. Val3 was also shown to be an important amino acid residue in the dimerization of human VEGF. Except for Glu7, none of the other charged residues are observed in the N-terminus. The C-terminus is different from that of HF protein and longer than the
shortest isoforms of VEGF/PIGF. The glycosylation site presented in some VEGFs (Fig. 2) is absent in svVEGF.

Production of Recombinant Mature svVEGF (rmsvVEGF) - To determine its biochemical and biological properties, rmsvVEGF was expressed in E. coli. A cDNA fragment coding the mature protein was amplified by PCR, cloned in pGEM-T system (Promega) and subcloned in the T7 based promoter pAE plasmid \(^4\). The final construct encodes a N-terminally fused sequence containing a 6Xhis-tag and two amino acid residues derived from Xho I restriction site. The 17kDa rmsvVEGF monomer was clearly visible in the extracts of induced or uninduced E. coli BL21 (DE3) cells transformed with pAE-msvVEGF for two hours by IPTG (Fig. 3a). Overnight inductions were also efficient in rmsvVEGF expression, producing up to 50 mg of the recombinant protein. The protein was expressed in the cells as inclusion bodies. The protein in the inclusion bodies was solubilized by urea and further purified by Ni\(^{++}\)-chelating affinity chromatography, resulting in 30 mg of purified monomers (Fig. 3a).

Since VEGFs are active just in the dimerized forms through intra- and inter-disulfide bonds (32), several attempts to refold the monomers were done. The best results were achieved by using oxidizing/reducing pair of 2mM GSH and 0,5mM GSSG, before urea removing as essentially described (25). Although some protein loss occurs due to precipitation after urea remove, this procedure could dimerize about 20% of monomeric mature svVEGF, producing a final yield of 2 mg of refolded protein from 10 mg of monomers (Fig. 3b). To purify the refolded dimeric rmsvVEGF from monomeric forms, we raised the possibility that rmsvVEGF could bind to heparin-sepharose like HF protein and some VEGF forms. The binding to heparin-sepharose
is not a general rule for VEGFs. Human VEGF$_{121}$ does not bind to heparin-sepharose whereas VEGF$_{189}$ binds strongly and VEGF$_{165}$ binds with intermediary affinity, being eluted with 0.9 M NaCl (33). In the case of rmsVVEGF, our results showed that it has a relatively weak affinity for heparin-sepharose, being eluted with 0.3 M NaCl (Fig. 3b).

Expression of svVEGF in Other Snake Venoms and Tissues - Anti-rmsnVEGF antiserum was generated in mice, against the unfolded rmsVVEGF. This antiserum was able to recognize in Western-blot the reduced (17 kDa) monomeric form of rmsVVEGF in reducing SDS-PAGE condition (lane 1, Fig. 4), but not the refolded (30 kDa) dimeric form in SDS-PAGE in non reducing condition (data not shown).

To investigate the biological distribution of svVEGF, Western-bLOTS were performed with samples of some Viperidae and Elapidae snake venoms. These include the venoms from B. insularis, B. jararaca, B. jararacussu and Crotalus durissus terrificus (Viperidae) as well as the Micrurus corallinus (Elapidae) venom.

A major immunoreactive band of 14 kDa was detected in the B. insularis venom extract (lane 3, Fig. 4). The molecular mass is in according to that predicted from the amino acid sequence deduced from cDNA cloning. A major immunoreactive band of 15 and 14.5 kDa was also detected in the B.jararaca and B.jararacussu venom, respectively (lanes 4 and 5, Fig. 4). In the later venom, a minor band of 16 kDa was also detected. C. durissus terrificus venom also displayed a 16.0 kDa band (lane 6, Fig. 4). The Elapidae snake, Micrurus corallinus, presents a 16.5 kDa immunoreactive component in its venom (lane 7, Fig. 4). These results clearly show that svVEGFs are widely distributed among the snake venoms. In addition, svVEGFs are present
in the venoms with different molecular masses. This may be due to the differences in the primary amino acid sequences among the svVEGFs, by alternative splicing or to their post-translational modifications. It is interesting to note that in the B. insularis venom gland extract, a band of 14 kDa was detected (lane 8, Fig. 4). In contrast, extracts of the B. insularis heart tissue, did not present any immunoreactive band (lane 9, Fig. 4). It was shown that heart, lung, kidney and other organs from adult vertebrates are typical VEGF expressing tissues (34). This result suggests that the svVEGF is a snake venom component and its expression is specific to the snake venom glands. These results were also confirmed by Northern-blot studies. A 1.3 kb transcript was detected in the venom glands of B. insularis, B.jararaca and C. durissus terrificus, but not in the B. insularis lung, carotid artery, pancreas and maxillary muscle tissues (Fig. 5).

Cloning of svVEGF from B. jararaca - Since the western-blot and northern-blot results indicated an ubiquitous distribution of svVEGF in snake venoms and no other nucleotide sequence of snake VEGFs had been described, we decide to clone the svVEGF cDNA from B. jararaca for comparison to B. insularis svVEGF. B.jararaca snake bite represents the main cause of snake envenoming accidents in Brazil (35). The entire cDNA sequence of B. jararaca svVEGF was obtained after PCR, using the B.jararaca venom gland cDNA library (27) as a template and the oligos described in Experimental Procedures. All the sequences were clustered and the B. jararaca svVEGF cDNA sequence was obtained by superposing the sequenced fragments.

The nucleotide sequence of Bothrops jararaca svVEGF (supplement, Fig. I ) is quite similar to B. insularis svVEGF cDNA sequence, differing in just few nucleotides in the coding region and some gaps and insertions sequences at 5’ and 3’ UTR. The amino acid sequence is
also very similar between the two botropic svVEGFs (Fig. 2), presenting 93% of identity. Comparing HF protein from Vipera aspis to, svVEGFs from B. insularis and B. jararaca, they display 68% of identity and should represent orthologous forms of svVEGFs. The cloning and sequencing of B. jararaca svVEGF also confirms the ubiquitous expression and presence of svVEGF in the snake venoms. The molecular mass of B. jararaca svVEGF would be similar, if not identical, to B. insularis svVEGF in the SDS-PAGE. Both proteins possess a predicted 122 amino acid mature svVEGF. However, in the western-blots studies shown in figure 3, B. jararaca svVEGF molecular mass is slightly higher than B. insularis svVEGF as determined by SDS-PAGE. The reasons for this discrepancy are unknown.

**Biological activity of svVEGF** – An increase in vascular permeability is one of the earliest biological effects of the VEGF action in vivo. This activity was investigated by Miles assay (28) using refolded rmsvVEGF. Refolded rmsvVEGF was injected intradermally in the mice backs. As a control, equivalent volume of PBS was injected below the site of refolded rmsvVEGF inoculation in the same animal. The ability of rmsvVEGF to produce blue spots is due to leaking of systemic Evan’s Blue (see Experimental Procedures) and is clearly observed in figure 6. The dose dependent effect was observed in mice injected with different doses of refolded rmsvVEGF (Fig. 6A). The extrusion of the albumin dye complex was detected as early as 5 minutes after inoculation of rmsvVEGF and did not increase after 15 minutes (Fig. 6B).
Discussion

During the sequencing of abundant ESTs from the *B. insularis* venom gland cDNA library, we have characterized a cDNA coding for a protein with high identity to vertebrate VEGFs, including human VEGFs. This is the first nucleotide sequence reported for a VEGF derived from the snake venom gland. The major goal of this EST project, is to define the transcripts that characterize this specialized tissue in *B. insularis*. *B. insularis* is an endemic snake restricted to the Island of Queimada Grande. In contrast to other bothropic snakes, its diet consists mainly of birds and some invertebrates (36). For this reason, *B. insularis* venom might present some specific toxins selected over time to better capture this kind of prey. As a
consequence, we would expect to describe novel transcribed sequences from *B. insularis* venom glands of seminal importance for the venom gland physiology and function. In addition, this approach would also lead to the description of new putative toxins. The characterization of svVEGF is an example of such reverse biology effort (7). The svVEGF protein is a new venom component described in *B. insularis* and possesses several of the vertebrate VEGF features, including the eight cysteines in the conserved positions. These cysteines are responsible for the inter- and intra-molecular disulfide bridges characteristic of this family of proteins. The presence of svVEGF in other snake venoms investigated here was also shown through Western-blot (Fig. 4), Northern-blot (Fig. 5) and cDNA cloning and sequencing of *B. jararaca*svVEGF (Fig. 2 and Supplemental data). Recently, a heparin-binding hypotensive factor (HF) with vascular permeability activity and mitogenic activity on endothelial cells was structurally characterized as a VEGF-like protein in the venom of *V. aspis aspis* by complete amino acid sequencing by Edman degradation (11). A similar protein (ICPP) was also partially characterized from the venom of *V. lebetina* with vascular permeability activity that also resembles the VEGF family of proteins (10). Our data extend these results and help to establish that VEGFs are venom components distributed not only in *Viperidae* but also in *Elapidae* snake venoms. Furthermore, the Western- and Northern-Blot studies showed that the expression of svVEGF is specific to the venom glands and may represent a distinct VEGF transcript/protein from other VEGF-expressing tissues like heart, lung and kidney. Like HF protein, the refolded rmsvVEGF from *B. insularis* also possesses a weak affinity for heparin-sepharose resin, being step-eluted with 0,3 M NaCl in PBS. We would expect that binding to heparan-molecules occurs under physiological conditions *in vivo* and such kind of interaction would play some role in the activity of svVEGFs.
This notion is supported by the results showing that the mitogenic activity of HF protein was inhibited by an excess of heparin molecules in bovine aortic endothelial cells (11). The cell-associated heparan molecules in the membrane would facilitate the presentation of svVEGFs to their receptors (37). We do not know which receptor mediates the effect of svVEGFs or if they bind to the VEGFR-1 (flt-1) (38), VEGFR-2 (flk-1/KDR) (39), VEGFR-3 (flt4) (19) or to neuropilin –1 (40). The three first proteins are tyrosine kinase receptors that bind different VEGF forms. Neuropilin-1 functions as an axon guidance factor receptor for the collapsin/semaphorin family of proteins (41,42) and enhances both the binding of VEGF165 to VEGFR-2 and VEGF165-mediated chemotaxis (40), suggesting that neuropilin-1 may be a co-receptor for VEGFR-2.

The receptor(s) responsible for mediating vascular permeability is unknown. However, the fact that all of the VEGF family members which have this activity bind to VEGFR-2, suggests that this receptor (and neuropilin-1?) may be involved (14). In addition, a mutant of VEGF-C which can not bind VEGFR-2 loses the ability to induce vascular permeability (43). Nevertheless, the description of a VEGF mutant that can not bind to VEGFR-2 but induces vascular permeability supports the existence of a novel receptor not identified so far (14). Since the rmsvVEGF described here possesses vascular permeability activity, it is possible that this response would be mediated by VEGFR-2 or by a novel receptor. Further studies on svVEGF receptors would be necessary to clarify this question. It is interesting that the vascular permeability activity induced by HF protein (5 ng) is more than ten times higher than its mitogenic effects on endothelial cells (half-maximal response around 125 ng/ml). For this reason, the increase in capillary permeability can be considered the primary action of svVEGFs, including the svVEGFs described here, the HF and ICCP proteins (10,11).
The presence of VEGF-like molecules in the venom of the snakes (named here as svVEGF) is not a surprise if we suppose that during evolution, toxins were selected over the years to block, inhibit or mimic natural ligands and enzymes of their preys. The functions of these toxins are not restricted to immobilize and kill the preys, but also to facilitate the venom spread and distribution, so the toxins may have a local and/or a systemic action. The vascular permeability induced by VEGFs is rapid, transient and reversible. The VEGFs are the most potent vascular permeability factors known. On a molar basis, they are at least 1000 times more active than histamine (44,45). Local vasodilatation, vascular permeability increasing and hypotension are usually responses elicited by snake bite envenomations. In botropic snake accidents, these responses are evoked by the kinins released from circulating kininogen by kallikrein-like enzymes in the venom (46), by BPPs that inhibit the angiotensin converting enzyme, inhibiting the conversion of angiotensin I to angiotensin II (3) and by natriuretic peptides (4,6). We can add to this list, the svVEGF. The rmsvVEGF induced a dose response increase in capillary permeability. Although we did not test the hypotensive action or the mitogenic response in endothelial cells by rmsvVEGF, it is conceivable that svVEGF possesses these same kind of activities like the HF protein, a VEGF-like protein characterized from the V. aspis aspis venom. The combination of these molecules during the envenoming should severe the local and systemic response in the prey. These molecules (BPP, natriuretic peptides and kallikrein-like enzymes) are not restricted to snake venoms, but can be found in spider, scorpion and ornithorhynchus venoms (47-49). It is interesting that in the Loxosceles deserta spider, VEGF-like molecules was not described in the venom, but the expression of VEGF mRNA was induced in keratinocytes by L. deserta venom that could account for the vasodilatation, edema
and erythema observed following *L. deserta* spider envenomation (50). All these data indicate the importance of VEGF-like molecules (exogenous or endogenous) as mediators of vascular permeability induced by these animal envenomings.

**References**


16. Poltorak, Z., Cohen, T., Sivan, R., Kandelis, Y., Spira, G., Vlodavsky, I., Keshet, E. and


Footnotes:
For page 1:

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The nucleotide sequences of svVEGF cDNAs from *B. insularis* and *B. jararaca* described here were deposited in the GenBank database under accession numbers AY033151 and AY033152, respectively.

2. Abbreviations used are: BPP, bradykinin potentiating peptide; VEGF, vascular endothelial growth factor; svVEGF, snake venom VEGF; rmsvVEGF, recombinant mature svVEGF; PIgf, Placental growth factor; PDGF, platelet derived growth factor; HF, hypotensive factor; EST, expressed sequence tag; DTT, dithiothreitol; IPTG, Isopropylthio-β-galactoside; ELISA, enzyme linked immunosorbent assay; GSH, reduced glutathione; GSSG, oxidized glutathione.

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3. The anti-rmsvVEGF antiserum is available on request.

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4. Ramos et al., unpublished results.
**Figure legends**

Fig. 1. **Complete sequence of *Bothrops insularis* svVEGF cDNA and its deduced protein.** The signal peptide is underlined by dots and the polyadenilation signal is between parenthesis. Arrow corresponds to N-terminal primer used together with SP6 from the pGEM11Zf(+) vector to amplify and subclone the coding region into the expression vector. Continuous underlines indicate the sequence region from where the primers were designed to complete the sequence by primer walking.

Fig. 2. **Alignment of the deduced svVEGF amino acid sequences characterized from *B. insularis* and *B. jararaca* venom gland cDNA libraries with HF protein from *V. aspis* aspis and VEGFs/PlGFs.** Identical amino acid residues with high score in BLOSUM 62 matrix are denoted in black and conserved ones in gray. The intrachain and interchain disulfide bridges are marked. The box indicate the N-glycosilation site found in some VEGFs/PlGFs. The N-terminus amino acid position of the mature VEGFs is located at position +1. VEGFs121: *Danio rerio* (ac: AF059661), *Homo sapiens* (ac: AF214570). VEGF-B: *Homo sapiens* (ac: P49765), PlGFs: *Mus musculus* (ac: P49764) and *Rattus norvegicus* (ac: A56125).

Fig. 3. **Analysis of recombinant mature svVEGF in 15% SDS-PAGE.** A) Extracts of *E. coli* BL21(DE3) transformed with pAE vector without insert and induced by IPTG for 2h (Ct); pAE-msvVEGF not induced (NI); pAE-msvVEGF induced for 2h (IN). Inclusion bodies (IB) were solubilized in 8M urea and adsorbed on a Ni$^{2+}$-sepharose column. Samples from Ni$^{2+}$-
affinity chromatography flow-through (FT) and the elute (EL) after 1M imidazole were shown. B) Samples after refolding (Re) and heparin-sepharose (Hep) purification of rmsVVEGF were analyzed with (+β) or without (-β) β-mercaptoetanol. The arrows indicate the monomeric and * the dimeric forms of rmsVVEGF.


Fig. 5. svVVEGF transcripts from different snakes and tissues by northern-blot analysis. A) Total RNAs (10 µg) from the venom glands: 1. B. insularis 2. B.jararaca, 3. C. durissus terrificus and other B. insularis tissues: 4. lung, 5. carotid artery, 6. pancreas and 7. maxillary muscle, were fractionated on a 1% formaldehyde agarose gel, transferred to a nylon membrane and probed with DIG labeled svVVEGF cDNA from clone GH027. B) The gel was stained with ethidium bromide before transferring to nylon membrane.

Fig. 6. In vivo vascular permeability increasing induced by rmsVVEGF in male Swiss mouse. Evan’s blue dye was injected through orbital artery immediately after intradermally injection of 50 µl of rmsVVEGF solution or PBS (control) in the mice backs. The spots represent extrusion of the dye. A) Dose-response of rmsVVEGF in 15 minutes. B) Time-response of 25 ng of rmsVVEGF.
Figure 1. Junqueira de Azevedo et al., 2001
Figure 2: Junqueira de Azevedo et al.
Figure 3. Junqueira de Azevedo et al.
Figure 4. Junqueira de Azevedo et al.
Figure 5. Junqueira de Azevedo et al.
Figure 6. Junqueira de Azevedo et al.
Molecular cloning and expression of a functional snake venom Vascular Endothelium Growth Factor (svVEGF) from the Bothrops insularis pit viper. A new member of the VEGF family of proteins

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