Identification of the Heparin-Binding Region of Snake Venom Vascular Endothelial Growth Factor (VEGF-F) and Its Blocking of VEGF-A165†

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Received February 3, 2005; Revised Manuscript Received March 27, 2005

ABSTRACT: VEGF-A165 displays multiple effects through binding to KDR (VEGFR-2). Heparin/heparan sulfate-like molecules are known to greatly modulate their interaction. In fact, VEGF-A lacking a C-terminal heparin-binding region exhibits significantly reduced mitogenic activity. We recently found novel heparin-binding VEGFs in snake venom, designated VEGF-Fs, which specifically recognize KDR, rather than other VEGF receptors. VEGF-Fs virtually lack the C-terminal heparin-binding region when compared with other heparin-binding VEGF subtypes, despite their heparin-binding potential. The C-terminal region does not exhibit any significant homology with other known proteins or domains. In this study, we attempted to identify the heparin-binding region of VEGF-F using synthetic peptides. The C-terminal peptide of vammin (one of the VEGF-Fs, 19 residues) bound to heparin with similar affinity as native vammin. We then evaluated the effects of this peptide on the biological activity of VEGF-A165. The C-terminal peptide of VEGF-F exhibited specific blockage of VEGF-A165 activity both in vitro and in vivo. These observations demonstrate that the short C-terminal region of VEGF-F functions fully as the active heparin-binding domain and the corresponding peptide specifically blocks VEGF-A165, thus suggesting that the C-terminal heparin-binding region of VEGF-F recognizes similar heparin/heparan sulfate molecules as VEGF-A165. The present results will provide novel insight into VEGF−heparin interaction and may facilitate the design of new anti-VEGF drugs based on novel strategies.

Vascular endothelial growth factor (VEGF-A165) plays pivotal roles in physiologic and pathologic endothelial proliferative processes (*1*). VEGF-A165 exhibits its multiple biological activities through interaction with two distinct VEGF[1] receptors on vascular endothelial cells: Flt-1 (*fms*-like tyrosine kinase-1, VEGF receptor 1) and KDR (kinase domain-containing receptor, VEGF receptor 2) (*1*, *2*). KDR exhibits stronger ligand-dependent phosphorylation when compared with Flt-1 (*3*) and is the dominant signaling receptor for many of the biological activities of VEGF-A165, such as endothelial growth, vascular permeability, and hypotension (*4*−*8*). VEGF-A165 is an ∼45 kDa homodimeric glycoprotein possessing a 55-residue basic heparin-binding region in its C-terminus (Figure 1A). A C-terminal-deficient form of VEGF-A165 (110 residues) generated by plasmin digestion was not retained on a heparin affinity column and displayed markedly reduced (>100-fold) potency of growth factor activity when compared with intact VEGF-A165, although no significant loss of binding potential to receptors was noted (*9*). These data indicate that the C-terminal portion is critical for the heparin-binding and mitogenic activities of VEGF-A165. It is reported that this region is formed by two subdomains, a two-stranded antiparallel β sheet and a short α helix, and contains four disulfide bridges (*10*). Most of the basic amino acid side chains are localized on one side of this region but are discontinuous in primary structure, and the surface charges strongly suggest that these residues contribute to heparin interaction (*10*).

Exogeneous heparin inhibits autophosphorylation of KDR by VEGF-A165 (*11*) and binding between cell-associated KDR and VEGF-A165 but does not affect interaction with VEGF-A121, a splicing variant of VEGF-A lacking the C-terminal heparin-binding domain (*12*). In contrast to KDR, heparin behaves quite differently with Flt-1; both VEGF-A165 binding and VEGF-A121 binding are potently inhibited by heparin (*13*). These findings suggest that the C-terminal heparin-binding domain of VEGF-A165 is critical for its interaction with cell-associated KDR, while heparin appears to affect receptor function of Flt-1 independently of the heparin-binding potential of ligands.

We recently identified and characterized a novel VEGF subtype, designated VEGF-F, from the venoms of the *Vipera ammodytes ammodytes* (Western sand viper) and *Daboia russelli russelli* (also known as *Vipera r. russelli*, Russell's viper) (designated vammin and VR-1, respectively) (*14*). VEGF-Fs are ∼25 kDa homodimeric heparin-binding proteins that selectively recognize KDR (*14*). They exhibit potent biological activity in vitro and in vivo when compared with VEGF-A165 (*14*). The primary structure of the VEGF-Fs possesses ∼50% identity with that of VEGF-A165, and they have a markedly short C-terminal region when compared with VEGF-A165 (Figure 1A). The C-terminal portion of VEGF-F is composed of 16−17 amino acid residues and is

† This work was supported in part by Scientific Research Grants-in-Aid from the Ministry of Education, Science, and Culture of Japan (T.M.).

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[1] Abbreviations: Flt-1, *fms*-like tyrosine kinase-1; KDR, kinase insert domain-containing receptor; VEGF, vascular endothelial growth factor.

10.1021/bi050197d CCC: $30.25
Published on Web 05/27/2005

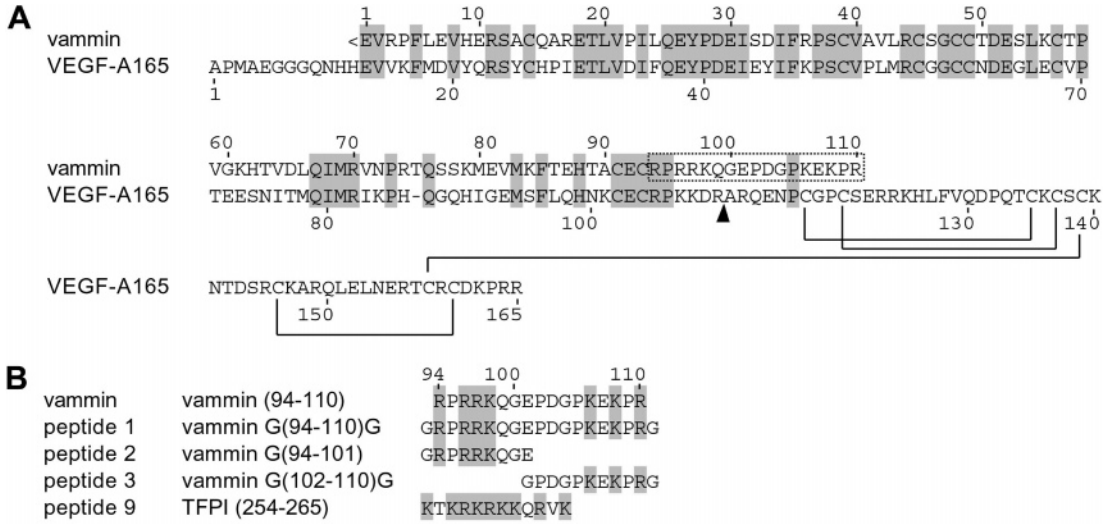

FIGURE 1: Sequence alignment of vammin and VEGF-A165 and sequences of synthetic peptides. (A) Residues identical between vammin and VEGF-A165 are shaded in gray. Disulfide bridges are shown only in the heparin-binding region of VEGF-A165. Numbers on the sequence are amino acid numbers for vammin, and lower numbers correspond to those for VEGF-A165. The C-terminal region of vammin is boxed. The plasmin cleavage site of VEGF-A165 is indicated by an arrowhead. (B) Sequences and designation of synthetic peptides. Basic amino acid residues are highlighted in gray.

rich in basic residues. This region does not include cysteine residues and does not show any significant homology with other proteins or domains, including the C-terminal heparin-binding region of other VEGF subtypes. We herein report that the C-terminal region of VEGF-F fully mediates its heparin-binding activity and specifically inhibits the biological activity of VEGF-A165 in vitro and in vivo. These results indicate that the unique and short C-terminal region of VEGF-F fully functions as an active heparin-binding domain, similarly to other heparin-binding VEGFs, despite their significant structural differences. This is a novel approach for antagonizing VEGF-A165 using a heparin-binding peptide.

EXPERIMENTAL PROCEDURES

Materials. Lyophilized venom of *V. ammodytes ammodytes* and unfractionated heparin were purchased from Sigma (St. Louis, MO). Human umbilical vein endothelial cells (HUVECs) and growth medium (EBM-2 with growth supplements) were obtained from Cambrex (Walkersville, MD). HiTrap heparin HP and COSMOSIL 5C18 AR-300 reversed-phase chromatography columns were from Amersham Biosciences and Nacalai Tesque (Kyoto, Japan), respectively. VEGF-A165 and bFGF were purchased from PeproTech EC (London, U.K.) and Invitrogen (Carlsbad, CA), respectively.

Toxin Purification. Vammin was purified as described previously (*14*).

Peptide Synthesis and Purification. All peptides were synthesized with a peptide synthesizer using F-moc strategy. After deblocking and cleavage from resin, the peptide (40 mg) was dissolved in 10 mL of 50 mM Tris-HCl, pH 8.0, and fractionated on a HiTrap heparin HP column (10 mL) using a linear gradient of 10 column volumes of NaCl up to 1 M. Peptide-containing fractions were then applied to a 5C18 reversed-phase HPLC column. Purified peptides were freeze-dried and subjected to amino acid and peptide sequence analyses.

Amino Acid Analysis and Peptide Sequencing. Peptide samples were hydrolyzed by treatment with a mixture of 5.7 M HCl and 1% phenol vapor in tubes sealed under a vacuum at 110 °C for 24 h. After evaporation, hydrolysates were analyzed on a Hitachi model L-8500 amino acid analyzer. Amino acid sequence analysis was performed using an Applied Biosystems protein sequencer (Models 473A and 477) and a Shimadzu PPSQ-21A protein sequencer (Shimadzu, Japan).

Analytical Heparin Affinity Chromatography. A HiTrap heparin HP column (10 mL) was preequilibrated with buffer containing 50 mM Tris-HCl, pH 8.0, using AKTA Explorer 10S (Amersham Biosciences). Protein or peptide was loaded onto the column, washed with 5 column volumes of the same buffer, and eluted using a linear gradient of 0−1.0 M NaCl (10 column volumes). The column effluent was monitored at 230 nm. NaCl concentration for elution of each peptide was calculated on the basis of the conductivity of the eluted peak.

Endothelial Cell Proliferation Assay. HUVECs were plated into 96-well tissue culture plates at a density of 5000 cells/well in EBM-2 containing growth supplements. After 6 h, the culture medium was replaced with serum-reduced medium (EBM-2 containing 1% fetal bovine serum without growth supplements), and cells were incubated for a further 18−20 h, after which VEGF-A165 or vammin (1 nM) in the presence or absence of synthetic peptide was added, and cells were incubated for 3 days. Tetra Color One cell proliferation assay reagent (Seikagaku Corp., Tokyo, Japan) was then added, and cells were incubated for 2 h. Results are expressed as percentage of control: the proliferation rate induced by each growth factor in serum-reduced medium was given a value of 100% while that of unstimulated cells was given a value of 0%.

Measurement of Arterial Blood Pressure. Male Wistar rats ($n = 3-5$, 150−220 g) were used in this in vivo study. After anesthesia with an intraperitoneal injection of carbamic acid ethyl ester (1 mg/g), a polyethylene tube was inserted into the carotid artery and connected to a pressure transducer

(model P10EZ; Becton Dickinson, Franklin Lakes, NJ). The systolic, diastolic, and mean arterial pressures were measured on a recorder connected to a carrier amplifier (model AP-621; Nihon Koden, Tokyo, Japan). After confirmation of blood pressure stability by injecting saline (600 μL), the peptide sample (600 μL) was administered into the right femoral vein. Subsequently, VEGF-A165 or vammin (600 μL, 0.1 μg/g) was injected into the left femoral vein.

RESULTS

Synthesis of the C-Terminal Peptide of VEGF-F and Estimation of Heparin-Binding Potential. The heparin-binding potential of VEGF-A165 is directly associated with its biological activity mediated by KDR. As several basic residues are clustered in the C-terminal region of VEGF-F, we anticipated this region to be involved in the heparin-binding activity of VEGF-F. We first synthesized the C-terminal region of vammin (corresponding to residues 94−110; 17 residues), designated peptide 1, using a peptide synthesizer and F-moc strategy (Figure 1B). The peptide was purified by two-step chromatography; heparin affinity chromatography followed by reversed-phase chromatography. The sequence and quantity of synthetic peptide were confirmed by peptide sequencing and amino acid analysis, respectively. The heparin-binding potential was assessed by analytical affinity chromatography. Purified peptide was applied to a HiTrap heparin column and eluted with a linear gradient of NaCl. Peptide 1 was eluted with a slightly higher but similar concentration of NaCl as vammin (Figure 2 and Table 1). These results indicate that the heparin-binding activity is completely mediated by the C-terminal region of vammin. The slight increase in the heparin affinity of peptide 1 may be the result of differences in accessibility to the immobilized heparin molecule between peptide 1 and the C-terminal region of native vammin. Because basic amino acid residues are clustered in the N- and C-terminal parts of peptide 1, we synthesized two additional peptides that correspond to the N- or C-terminal parts of peptide 1, designated peptides 2 and 3, respectively (Figure 1B). Peptide 2 also bound to the heparin column, exhibiting similar activity as peptide 1 (Figure 2, Table 1). In contrast, peptide 3 was not retained after continuous washing with NaCl-free buffer, indicating substantially lower affinity to heparin (Figure 2 and Table 1). These data clearly indicate that the basic residues clustered in the N-terminal portion of peptide 1 primarily mediate the heparin-binding function.

The C-Terminal Peptide of VEGF-F Inhibits VEGF-A165-Induced Endothelial Proliferation. We next examined whether heparin-binding potential is involved in the biological activity of VEGF-F, as is the case with VEGF-A165. In the presence of high concentrations of unfractionated heparin, both vammin- and VEGF-A165-stimulated endothelial cell growth was completely abolished (Figure 3), thus indicating that the growth factor activity of VEGF-F is also mediated by its heparin-binding function.

We speculated that if the C-terminal heparin-binding region of vammin recognizes the same or similar heparin/heparan sulfate structures, it competes with VEGF-A165 on the endothelial surface and inhibits the biological activity of VEGF-A165. The effects of peptide 1 on endothelial cell proliferation activity of vammin or VEGF-A165 were thus

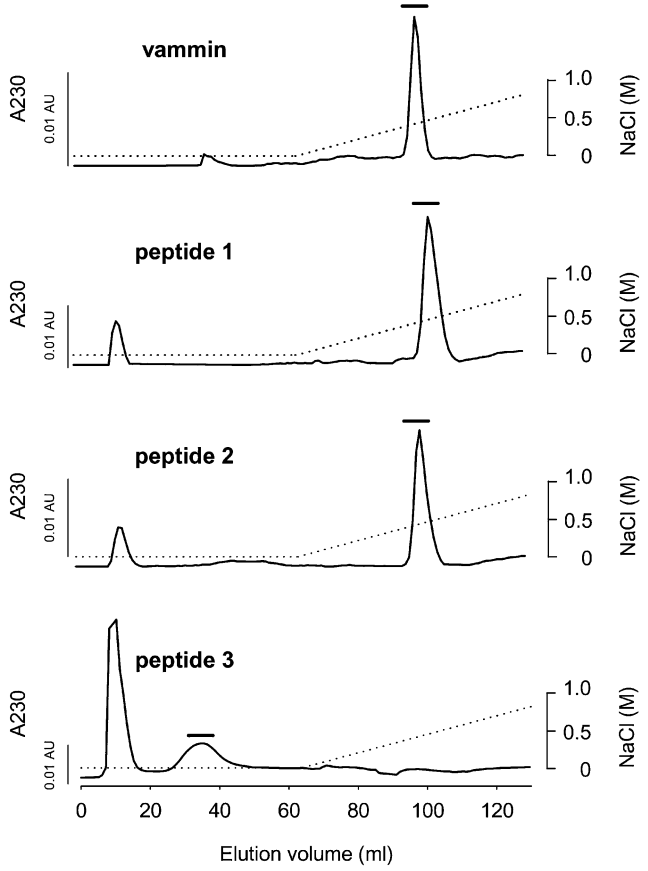

FIGURE 2: Evaluation of heparin affinity using analytical affinity chromatography. Purified vammin, peptide 1, peptide 2, or peptide 3 was loaded onto a HiTrap heparin HP column and eluted with a linear gradient of NaCl up to 1.0 M (dashed line). The column effluent was monitored at 230 nm. Eluted positions of vammin and peptides are indicated by bold lines. The first peak in each chromatogram was confirmed not to contain any peptide.

Table 1: Heparin-Binding Potential of Synthetic Peptides[a]

		NaCl (M)
vammin		0.33
peptide 1	vammin G (94−110)G	0.36
peptide 2	vammin G (94−101)	0.34
peptide 3	vammin G (102−110)G	<0.01
peptide 9	TFPI (254−265)	0.73

[a] The heparin-binding potential was estimated on the basis of the required NaCl concentration for elution from the heparin column.

investigated. Peptide 1 completely inhibited both vammin- and VEGF-A165-stimulated endothelial proliferation with similar IC_{50} values (320 and 280 μM, respectively) (Figure 4A), which suggests that vammin and VEGF-A165 recognize similar heparin/heparan sulfate structures. We also tested peptides 2 and 3 on VEGF-A165-stimulated endothelial cell growth. Relatively weak inhibition was observed in peptide 2-treated cells when compared with peptide 1, while peptide 3 had an even smaller effect (Figure 4B), thus showing good agreement with the heparin-binding affinity data. These results strongly suggest that the basic amino acids in peptide 2 primarily mediate the heparin/heparan sulfate-binding function of VEGF-Fs but are not sufficient for binding and that the C-terminal portion (peptide 3 region) also contributes to the interaction.

Peptide 1 Specifically Inhibits Proliferation of Vascular Endothelial Cells. To investigate the specificity of peptide

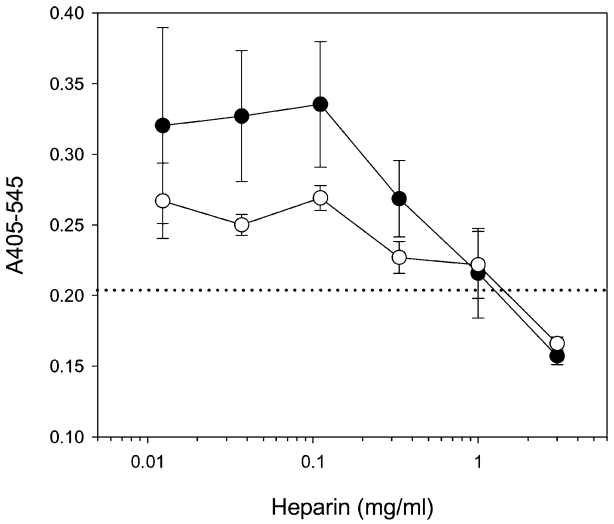

FIGURE 3: Heparin inhibits both vammin- and VEGF-A165-induced endothelial proliferation. HUVECs (5000 cells/well) were seeded onto a 96-well culture plate and incubated for 6 h. The medium was then replaced with serum-reduced medium, and plates were incubated for 18 h. The indicated concentrations of unfractionated heparin were added, and cells were then exposed to 1 nM vammin (closed circles) or VEGF-A165 (open circles). After 3 days of exposure, the proliferation rate was evaluated using a WST-8-based method.

1, we assessed its inhibitory effects against proliferation induced by basic fibroblast growth factor (bFGF), which is a well-characterized heparin-dependent growth factor (*15*). Peptide 1 exhibited effects on VEGF-A165-induced proliferation but not on bFGF-induced proliferation (Figure 5A). Furthermore, we tested the inhibitory effects of a synthetic peptide designed on the basis of the heparin-binding sequence of tissue factor pathway inhibitor (TFPI), which inhibits blood coagulation proteases (*16*). Although the TFPI peptide (residues 254−265, peptide 9) displayed high affinity for heparin (Table 1), it weakly blocked VEGF-A165-induced endothelial cell growth when compared to peptide 1 (Figure 5B). These combined data indicate that peptide 1 specifically/preferentially interacts with the VEGF-bondable heparin/heparan sulfate structure.

Peptide 1 Blocks Hypotensive Activity of VEGF-A165. We next evaluated the inhibitory activity of these peptides in vivo. Intravascular administration of vammin (0.1 μg/g) induces rapid and potent hypotension when compared with VEGF-A165 injection (*14*). Pretreatment with peptide 1 (3 μg/g) completely blocked the vammin-induced hypotensive effect, but only slight blockage was observed with VEGF-A165-stimulated hypotension (data not shown). A higher dose of peptide 1 (30 μg/g) was required to completely inhibit VEGF-A165-induced hypotension (Figure 6), indicating that peptide 1 is able to block the biological activity of VEGF-A165 even in vivo. Blockage of the hypotensive effects of VEGF-A165 was also observed in peptide 2 preinjected rats, but peptide 3 had no effect (Figure 6). These in vivo data showed good agreement with the in vitro data using cultured endothelial cells (Figure 4).

DISCUSSION

In this study, we demonstrated that the C-terminal region of VEGF-F functions as a heparin-binding domain and specifically inhibits the biological activity of VEGF-A165

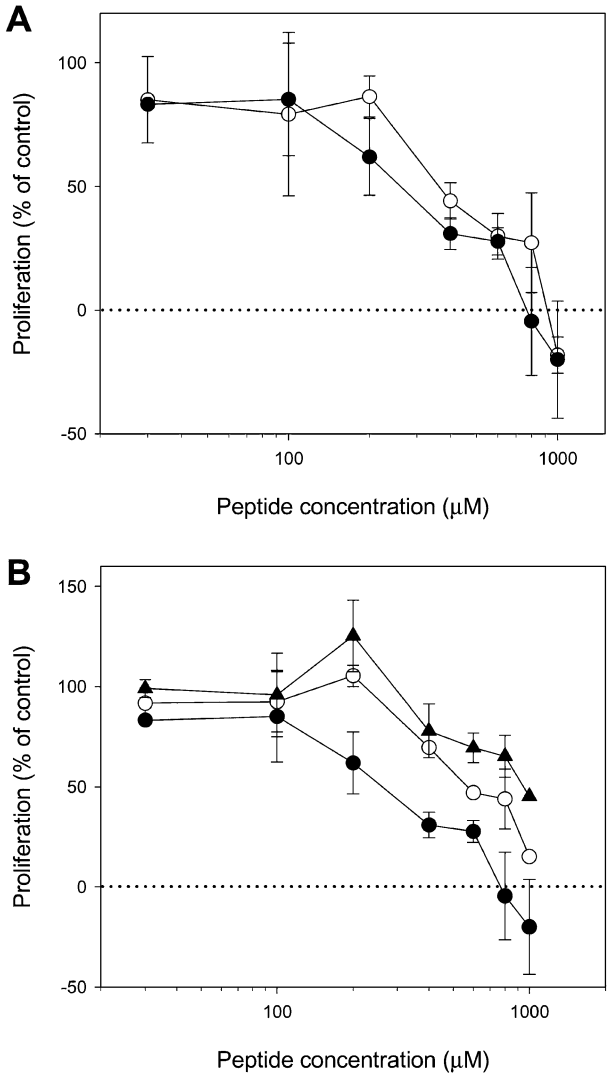

FIGURE 4: The C-terminal heparin-binding peptide of VEGF-F blocks VEGF-A165-stimulated endothelial proliferation. (A) The C-terminal peptide of VEGF-F (peptide 1) inhibited both vammin- (open circles) and VEGF-A165-induced (closed circles) proliferation. Endothelial cells were exposed to vammin or VEGF-A165 for 3 days in the presence of the indicated concentrations of peptide 1. (B) Effects of peptide 1 (closed circles), peptide 2 (open circles), and peptide 3 (closed triangles) on VEGF-A165-induced endothelial proliferation.

both in vitro and in vivo. Our data also indicate that a heparin/heparan sulfate-like molecule participates in the endothelial proliferation activities of both VEGF-F and VEGF-A165. The primary sequence of this region does not share any significant homology with any known protein or domains, thus indicating a unique heparin-binding domain. It is known that some of the VEGF family proteins, such as VEGF-A189 (*17*), VEGF-B167 (*18*), and PlGF-2 (*19*), also bind to heparin via their C-terminal region. All of these are homologous and have a relatively long C-terminal heparin-binding region (40−85 residues) when compared with VEGF-F (16−17 residues). The data presented here strongly indicate that the short heparin-binding domain of VEGF-F is sufficient for its biological activity to induce endothelial cell proliferation. Snake counterparts corresponding to VEGF-A have recently been identified (*20*, *21*). These observations strongly suggest that snake venom VEGFs are specialized for venom glands. In this regard, we may presume

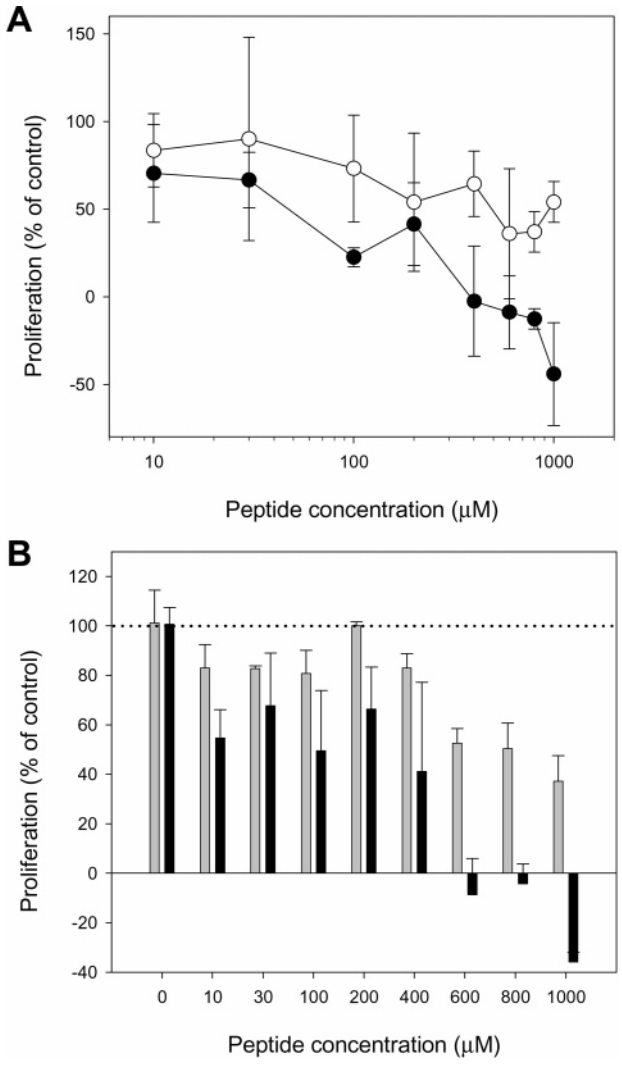

FIGURE 5: Peptide 1 specifically inhibits VEGF-A165-induced cell growth. (A) Peptide 1 did not affect bFGF-induced endothelial cell proliferation. HUVECs were exposed to VEGF-A165 (closed circles) or bFGF (open circles) in the presence of peptide 1. (B) Peptide 1 exhibits potent inhibition of VEGF-A165-stimulated proliferation when compared to the heparin-binding peptide of TFPI (peptide 9). HUVECs were stimulated with VEGF-A165 in the presence of peptide 1 (black column) or peptide 9 (gray column).

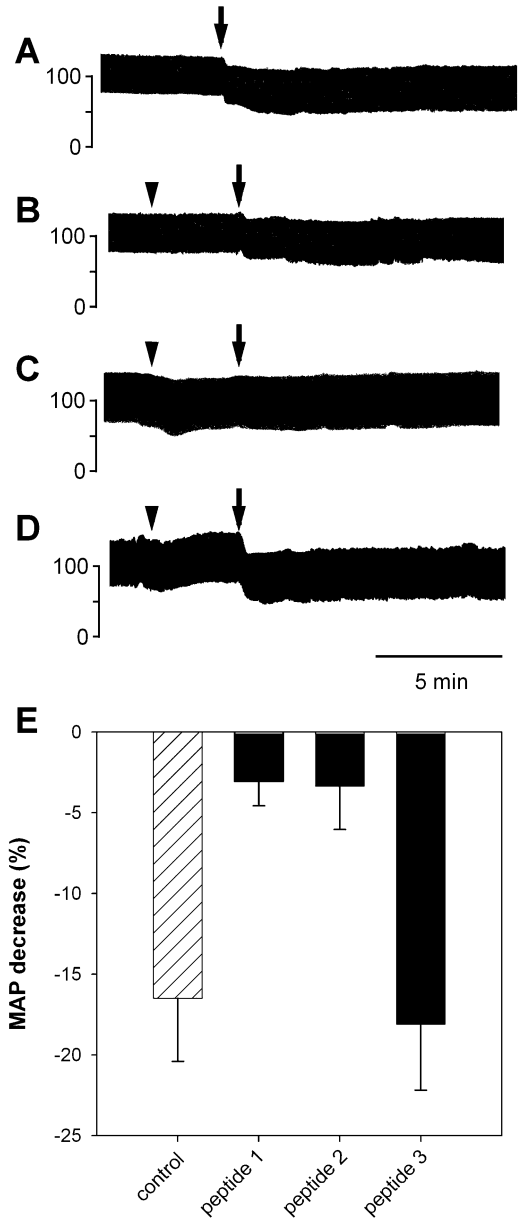

FIGURE 6: Peptide 1 blocks VEGF-A165-induced hypotension in vivo. After pretreatment with synthetic peptides (arrowheads), VEGF-A165 (0.1 μg/g, arrow) was injected into the left femoral vein. (A) Recording trace of rat arterial blood pressure after intravenous injection of 0.1 μg/g VEGF-A165. (B) Peptide 1 pretreatment. (C) Peptide 2. (D) Peptide 3. (E) Maximum decrease in mean arterial blood pressure (MAP): control, VEGF-A165 alone ($n = 3$); peptide 1 ($n = 3$); peptide 2 ($n = 4$); peptide 3 ($n = 3$) pretreatment.

that VEGF-Fs acquired unique and specialized heparin-binding domains for their toxic activity, in addition to strict receptor selectivity, in their evolutional process.

Previous studies have shown that blockage of VEGF-A165 results in the inhibition of angiogenesis and tumor development in vivo (*1*). To date, several research groups have attempted to generate blocking peptides against the VEGF−VEGF receptor pathway (*22*−*27*), although few experiments targeting the C-terminal heparin-binding region of VEGF-A165 have been reported (*28*). We herein demonstrated that the C-terminal heparin-binding region of VEGF-F blocks the biological activity of VEGF-A165. This is a novel approach for antagonizing VEGF-A165 using a heparin-binding peptide.

Peptide 1 blocked both vammin- and VEGF-A165-stimulated cell growth but had no effect on bFGF, while peptide 9 (C-terminal heparin-binding sequence of TFPI) showed slight blockage (Figure 5). The basic polymeric structure of heparin/heparan sulfate is an alternating repeat sequence of disaccharide units, which can be modified by sulfation or acetylation (*29*). This structural variability is the basis for the wide range of heparin-binding proteins. In this regard, our observations strongly suggest that peptide 1 recognizes a specific structure within heparin/heparan sulfate, possibly similar to the structure that interacts with VEGF-A165, although structural determination of the bound heparin/heparan sulfate is required. Peptide 3 binds to a heparin column with much weaker affinity when compared with peptides 1 and 2, but it was only slightly effective in the in vitro endothelial proliferation assay. As described above, heparin (heparan sulfate) molecules are made up of repeated hexuronic acid and D-glucosamine disaccharide units (*29*). Each saccharide residue can be modified at multiple sites,

which results in the generation of a wide variety of modified disaccharide units. Furthermore, it is known that the disaccharide compositions between heparin and heparan sulfate are considerably different (*30*). Taking these facts into consideration, peptide 3 may interact with only small populations of heparin units or specific sequences within heparan sulfate. In addition, peptide 1 blocked the hypotensive activity of VEGF-A165 and vammin with different potency (see Results section). It is therefore possible is that the C-terminal region (peptide 1 region) of VEGF-F may greatly contribute to its potent hypotensive activity when compared with VEGF-A165; however, further studies are required.

Due to the lower heparin affinity of peptide 3 when compared with peptide 1, we estimated that the N-terminal region of peptide 1 mainly contributes to its heparin interaction. Four conserved proline residues are found in the C-terminal sequence. Proline residues typically have considerable effect on peptide and protein structure, for example, in turn or helix formation. These residues are also conserved in VR-1 and other snake venom VEGFs (*31*, *32*), except for svVEGFs from the venom of *Bothrops* species (*33*), thus suggesting some role in conformation and heparin interaction. In an effort to better understand the structure−function relationship of this unique heparin-binding peptide, we attempted to determine the solution structure of peptide 1 by NMR spectroscopy; however, we were not able to define a complete structure. CD spectral data suggested that this peptide forms a potential tertiary structure in trifluoroethanol. Because this short peptide is rich in repeating residues (arginine, lysine, and proline), it may be impossible to define the solution structure using nonlabeled peptides. In addition, we recently determined the crystal structures of vammin and VR-1 (*34*). In both crystals, we were not able to define the complete structure of the C-terminal heparin-binding region. These data suggest that the C-terminal heparin-binding region of VEGF-Fs forms multiple highly flexible structures in aqueous solution.

In summary, we demonstrated that the heparin-binding site of VEGF-F is located in its C-terminal short region; furthermore, a synthetic peptide of this region specifically blocks the biological activity of VEGF-A165 both in vitro and in vivo. Because this heparin-binding peptide has a relatively small structure (perhaps recognizes penta- or hexasaccharide structures) and specific inhibitory activity against VEGF-A165, it may be a valuable seed for designing new anticancer drugs that target VEGF−heparin interaction.

ACKNOWLEDGMENT

The authors thank Dr. Mineko Yamaguchi for helpful comments on endothelial cell proliferation assay and Ms. Sanae Tashiro for technical assistance.

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BI050197D