C-Terminal Heparin-Binding Peptide of Snake Venom VEGF Specifically Blocks VEGF-Stimulated Endothelial Cell Proliferation

Yasuo Yamazaki Yuko Tokunaga Koji Takani Takashi Morita

Department of Biochemistry, Meiji Pharmaceutical University, Tokyo, Japan

Key Words

Vascular endothelial growth factor · Heparin ·
Heparin-binding peptide · Snake venom ·
KDR-binding protein · Lys49PLA₂

Abstract

Vascular endothelial growth factor-A165 (VEGF-A165) exhibits diverse biological effects through binding to its receptor KDR (VEGFR-2). Heparin-like molecules are known to modulate their interaction. There have been reports that VEGF-A lacking the C-terminal heparin binding region significantly reduced mitogenic activity. Recently, we found novel heparin-binding VEGFs from snake venoms, designated VEGF-Fs, which specifically recognize kinase domain containing receptor (KDR). The C-terminal heparin-binding region is almost completely absent in VEGF-Fs when compared with other heparin-binding VEGFs, despite their heparin-binding potential. In this congress, we report that the C-terminal heparin-binding region of VEGF-F specifically/preferentially interacts with the VEGF-bondable heparin/heparan sulfate, but not with those associated with bFGF or TFPI. We also present the identification of a VEGF receptor-binding protein from the venom of eastern cottonmouth (*Agkistrodon piscivorus piscivorus*). Sequence analysis revealed the isolated KDR-binding protein (designated KDR-bp) is identical to Lys⁴⁹PLA₂, an inactive PLA₂ homologue with strong myotoxicity. KDR-bp binds to the ex-

tracellular domain of KDR with subnanomolar affinity. The interaction between KDR-bp and KDR was blocked by VEGF-A165, and KDR-bp specifically inhibited VEGF-A165-stimulated endothelial cell proliferation, indicating KDR-bp is an antagonistic ligand for KDR. This is the first observation demonstrating that an exogenous factor antagonizes the VEGF receptor, furthermore, it is the first identification of the target molecule of the myotoxic PLA₂ from viper venom.

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VEGF-Blocking Effect of C-Terminal Heparin-Binding Peptide of Snake Venom-Derived VEGF

Vascular endothelial growth factor (VEGF) plays pivotal roles in physiologic and pathologic endothelial proliferative processes [1]. To date, several molecules have been identified as VEGF family proteins: VEGF-A to D, and PlGF from mammals, and viral VEGF (also denoted as VEGF-E) from *Parapoxviruses*. All of these subtypes exhibit distinct receptor selectivity and express diverse biological effects (fig. 1). Recently, we have identified two novel VEGFs in viper venoms and shown them to be specific ligands for KDR (fig. 1) [2]. Because of their structure [3] and strict receptor selectivity [2], we proposed a novel subtype name 'VEGF-F' for these snake venom-derived VEGF (fig. 1).

Fig. 1. VEGFs and their receptor selectivity. Summary of the interaction of several VEGF subtypes with the three tyrosine kinase receptors (Flt-1, KDR, and Flt-4) and a co-receptor (neuropilin-1). The ligand-binding domain of each receptor is shaded in grey.

Vascular endothelial growth factor (VEGF-A165) is a heparin-binding protein that plays a pivotal role in physiologic and pathologic angiogenesis. VEGF-A165 is a ~45-kDa homodimeric glycoprotein composed of two domains: an N-terminal receptor-binding domain and a C-terminal heparin-binding domain. VEGF-A165 binds to its receptor KDR (kinase domain-containing receptor, VEGF receptor 2) and induces a range of biological effects, such as endothelial growth, vascular permeability and hypotension. It is reported that heparin greatly modulates the interaction of VEGF-A165 and its receptor KDR [4]. A C-terminal-deficient form of VEGF-A (110 residues) generated by plasmin-digestion is not retained on a heparin affinity column and displays markedly reduced potency of growth factor activity [5]. In contrast to VEGF-A165, snake venom-derived VEGF (VEGF-F) has a markedly short C-terminal region, despite its heparin-binding potential [2]. This region of VEGF-F is composed of 16–17 amino acid residues and is rich in basic residues. Recently, we have revealed that the heparin-binding region of VEGF-F is located in this region using a synthetic peptide (designated as peptide 1), and furthermore, that this peptide blocks VEGF-A165-stimulated endothelial cell proliferation [6]. In this study, we tested the specificity of peptide 1 for its VEGF-blocking effect.

We, first, examined whether the heparin-binding potential is involved in the biological activity of VEGF-F. In the presence of high concentrations of unfractionated heparin, both vamin (a VEGF-F from *Vipera ammodytes ammodytes* venom)- and VEGF-A165-stimulated

endothelial cell proliferation was completely abolished. These results indicate growth factor activity of VEGF-F is also mediated by its heparin-binding function. In order to investigate the specificity of peptide 1, its inhibitory effects against proliferation induced by bFGF, a well-characterized heparin-dependent growth factor [7], was assessed. Peptide 1 exhibited effects on VEGF-A165-induced proliferation, but not on bFGF-induced proliferation. Furthermore, the inhibitory effect of a synthetic peptide whose design was based on the heparin-binding sequence of tissue factor pathway inhibitor (TFPI) [8] was tested. Although the TFPI peptide displayed high affinity for heparin, it only weakly blocked VEGF-A165-induced endothelial cell growth when compared to peptide 1. These data indicate that peptide 1 specifically/preferentially interacts with the VEGF-bondable heparin/heparan sulfate structure [6].

Identification of a VEGF Receptor-Binding Protein from the Venom of Eastern Cottonmouth

Several snake venoms contain activator and inhibitor toxins that both target the same molecule: some vipers possess blood coagulation factor X activators, such as RVV-X, while others include factor X-binding protein (X-bp). X-bp binds the Gla domain of factor X and prevents blood coagulation. These activator and inhibitor combinations are also seen in the toxins that target plate-

let receptors. From this point of view, we speculated that some snake venoms might contain VEGF receptor antagonists, in combination with the previously identified snake venom VEGF-F.

Initially, we screened 29 species of snake venom using an index of blocking activity for the interaction between KDR-IgG and vamin (a snake venom-derived KDR-specific VEGF). As a result, strong inhibitory activity was detected in the venom of the eastern cottonmouth (*Agkistrodon piscivorus piscivorus*) from the United States with an IC₅₀ of ~10 µg/ml. In addition, previously identified VEGF-containing venoms (*Vipera ammodytes ammodytes* and *Daboia russelli russelli*) also exhibited inhibitory activity, while the venom of *Pseudechis australis*, a elapid snake, did not affect this assay at all, even at 200 µg/ml. These results strongly suggest that eastern cottonmouth venom contains a specific component that blocks vamin-KDR interaction. By using two successive chromatographic runs, the inhibitory protein was isolated as a single-chain, highly basic protein with a relative molecular mass of 16.8 kDa (designated KDR-binding protein; KDR-bp). Sequence analysis revealed the peptide sequence of KDR-bp to be identical to Lys⁴⁹PLA₂ (GenBank™ accession number: PSSNAM), an inactive PLA₂ homologue with potent myotoxic activity. The molecular mechanism of its myotoxicity is poorly understood [9, 10].

In order to evaluate the affinity of the isolated protein to KDR, kinetic parameters were measured using the Biacore system. KDR-bp bound to the extracellular domain of KDR with a K_d of subnanomolar, while no specific in-

teraction was observed with active Asp⁴⁹PLA₂ from Habu snake (*Trimeresurus flavoviridis*) venom (Tf PLA₂). The binding of KDR-bp to other VEGF receptors, Flt-1, and neuropilin-1 was also tested. Specific binding was observed with the extracellular domain of the Flt-1, although the binding affinity was ~10-fold smaller than that of KDR. In contrast to Flt-1, only slight interaction was observed with neuropilin-1-IgG. The binding ability to the extracellular domain of PDGF receptor α, which is composed of tandem immunoglobulin-like domains (similar to VEGF receptors) was also evaluated. KDR-bp did not bind to PDGF receptor α at all. In all binding experiments, specific interaction was only observed with KDR-bp (Lys⁴⁹PLA₂), while no specific interaction with Tf Asp⁴⁹PLA₂ could be detected.

As expected from the screening in the initial study, KDR-bp and KDR-IgG interaction was inhibited by VEGF-A165, indicating KDR-bp shares the binding epitope on KDR with VEGF-A165. We next evaluated the effect of KDR-bp on the biological activity of VEGF-A165. KDR-bp specifically and completely attenuated the endothelial cell growth stimulated by VEGF-A165 without toxic effect on cell viability. These observations indicate KDR-bp is an antagonistic ligand for KDR. This is the first report to demonstrate an exogenous factor that antagonizes VEGF and its receptor system, furthermore, it is the first identification of the molecular target of snake venom derived myotoxic PLA₂ [11]. KDR-bp would make a valuable tool for studying the structure and function of KDR, such as expressed on skeletal muscle cells.

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