Identification of Vascular Endothelial Growth Factor Receptor-binding Protein in the Venom of Eastern Cottonmouth

Vascular endothelial growth factor (VEGF) and its receptor KDR (kinase insert domain-containing receptor) are central regulators of blood vessel formation. We herein report a KDR-binding protein we have isolated in the venom of eastern cottonmouth (Agkistrodon piscivorus piscivorus). Sequence analysis revealed the isolated KDR-binding protein (designated KDR-bp) is identical to Lys49 phospholipase A2 (Lys49PLA2), an inactive PLA2 homologue with strong myotoxicity, in which Lys49 substitutes Asp49, a key residue for binding the essential cofactor Ca2+. KDR-bp binds to the extracellular domain of KDR with subnanomolar affinity. KDR-bp also binds to a lesser extent with Flt-1 and IgG but not to other receptors with similar immunoglobulin-like domain structures such as platelet-derived growth factor receptor α. The interaction between KDR-bp and KDR was blocked by VEGF165 and KDR-bp specifically inhibited VEGF165-stimulated endothelial cell proliferation, indicating KDR-bp is an antagonistic ligand for KDR. Lys49PLA2s from another snake venom were found to exhibit similar receptor binding properties to KDR-bp. This is the first report to demonstrate that an exogenous factor antagonizes VEGF and its receptor system. Our observation offers further insight into the as yet unknown molecular mechanism of myotoxic activity of snake venom Lys49PLA2s. Furthermore, KDR-bp would make a valuable tool for studying the structure and function of KDR, such as that expressed on skeletal muscle cells.

Vascular endothelial growth factor (VEGF)1 and its receptor system play a central role in the angiogenic process (1). The physiological significance of this system has been established in several reports which show that genetic deficiency of either ligand or receptor results in embryonic lethality because of failed vasculature development. VEGF165 binds to two well characterized receptors on endothelial cells, namely fms-like tyrosine kinase-1 (Flt-1, VEGF receptor 1) and kinase insert domain-containing receptor (KDR, VEGF receptor 2). Flt-1 and KDR have highly homologous features related to the receptor tyrosine kinase family; both receptors are comprised of a seven-tandem extracellular immunoglobulin-like domain, a transmembrane domain, and a cytoplasmic tyrosine kinase domain. Several studies show the second and third domains are ligand-binding domains in both receptors (2, 3), indicating similar ligand-receptor binding. VEGF165 is an antiparallel homodimer, covalently linked by two disulfide bridges. It has been shown that one VEGF165 dimer binds two molecules of receptor, indicating the dimer ligand causes receptor dimerization following receptor activation (4).

To date, several trials have attempted to find antagonizing molecules for VEGF-VEGF receptor interaction (5), including monoclonal antibodies, soluble receptors, peptides, siRNA, and several small synthetic inhibitors. The only physiological inhibitor of this interaction reported is tissue inhibitor of metalloproteinase-3 (TIMP-3) (6). TIMP-3 directly binds to the extracellular domain of KDR and blocks the angiogenic activity of VEGF165 with subnanomolar affinity (6).

Snake venoms are a plentiful source of several interesting molecules possessing unique biological activities that are never seen in mammals. Recently, two novel VEGFs, designated VEGF-F, have been isolated and characterized from viper venoms (7). These snake venom-derived VEGFs exhibit strict receptor selectivity compared with other known VEGF subtypes; they specifically recognize KDR but do not recognize any other known VEGF receptors (7). The crystal structures of both proteins display similar but markedly distinct features from other known VEGFs, including the structure of receptor-binding loop and the surface potential (8). In this study, we report that myotoxic basic phospholipase A2 (Lys49PLA2) obtained from the venom of eastern cottonmouth binds the extracellular domain of KDR with subnanomolar affinity and results in the blockage of VEGF165. Lys49PLA2s are widespread in the venom of several vipers and are structurally classified as group II PLA2s, although the molecular mechanism of their myotoxicity remains unknown (9, 10). Despite significant homology to active PLA2s, Lys49PLA2s are inactive phospholipolytic enzymes because the Lys49 substitutes Asp49, a key residue for binding of the essential Ca2+-cofactor (11). This is the first report demonstrating that the exogenous factor antagonizes the VEGF receptor by binding to the exterior face of the receptor and, furthermore, is the first identification of the target molecule of myotoxic basic phospholipase A2 in viper venom.

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Identification of VEGF Receptor-binding Protein

EXPERIMENTAL PROCEDURES

The “Experimental Procedures” section is provided in the supplemental material.

RESULTS AND DISCUSSION

Identification of KDR-binding Protein in 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 (12), such as RVV-X, while others include factor X-binding protein, which binds the Gla domain of factor X and prevents blood coagulation (13). From this point of view, we speculated that some snake venoms might contain VEGF receptor antagonists, in contrast to previously identified snake venom VEGF-like proteins. We screened 29 species of snake venom using an index of blocking activity for the interaction between KDR-IgG and vammin, a snake venom-derived KDR-specific VEGF from the venom of Viper ammodytes ammodytes. As a result, we detected strong inhibitory activity in the venom of A. piscivorus piscivorus (eastern cottonmouth) with an IC$_{50}$ of $\sim 10$ nM, in addition to previously identified VEGF-containing venoms, while the venom of Pseudechis australis, an elapid snake, did not affect this assay at all, even at $200 \mu$g/ml (supplemental Fig. 1). By using two successive chromatographic runs, we isolated the inhibitory protein to give a single chain, highly basic protein with a relative molecular mass of 60.8 kDa, hereinafter named KDR-binding protein (KDR-bp) (Fig. 1A). Sequence analysis of N-terminal and enzymatically digested fragments revealed that the peptide sequence of KDR-bp is identical to Lys$_{49}$-phospholipase A$_2$ (Lys$_{49}$PLA$_2$), an inactive phospholipase A$_2$ homologue with potent myotoxic activity (GenBank™ accession number: PSSNAM; Fig. 1B).

KDR-bp Binds to the Extracellular Domain of VEGF Receptors but Not Those of PDGF or FGF Receptors—To evaluate the affinity of the isolated protein to KDR, kinetic parameters were measured using the Biacore system. KDR-bp bound to both immobilized KDR-IgG and the extracellular domain of KDR (KDR-D1–7) with similar affinities ($K_d$ of $1 \times 10^{-8}$ M), while no specific interaction was observed with active Asp$_{49}$PLA$_2$ from Trimeresurus flavoviridis venom (Tf PLA$_2$), despite the structural similarity (Fig. 2A and Table I). We also tested the binding ability of KDR-bp to other VEGF receptors, Flt-1 (14), neuropilin-1 (15), and Flt-4 (16, 17). Specific binding was observed with Flt-1-IgG and the extracellular domain of the Flt-1 (Flt-1D1–6)–immobilized surface, although the binding affinity was $\sim 10$ times smaller than that of KDR ($K_d = 7.4 \times 10^{-8}$ M, Fig. 2B). In contrast to Flt-1, only slight interaction was observed with neuropilin-1-IgG and the Flt-4-IgG-immobilized surface (Fig. 2C). As the extracellular region of VEGF receptors, including KDR, Flt-1, and Flt-4, is composed of seven tandem immunoglobulin-like domains, we considered the possibility that KDR-bp recognizes a common structural feature of immunoglobulin superfamily proteins, resulting in apparent binding ability to receptor-immunoglobulin chimeric proteins, such as neuropilin-1-IgG and Flt-4-IgG. We evaluated the binding ability to human IgG and the extracellular domains of other growth factor receptors, which are composed of tandem immunoglobulin-like domains that are similar to VEGF receptors. KDR-bp bound to immobilized-human IgG to a smaller extent ($K_d = 1.75 \times 10^{-6}$ M) but did not bind to the extracellular domain of PDGF receptor $\alpha$ at all (supplemental Fig. 2). KDR-bp bound only slightly to PDGF receptor $\beta$-IgG and FGF receptor 1a-IgG, with a comparative extent to human IgG, suggesting binding to the IgG portion of chimeric proteins (supplemental Fig. 2). In all binding experiments, specific interaction was only observed with KDR-bp (Lys$_{49}$PLA$_2$), while we could not detect any specific interaction with TF PLA$_2$ (Fig. 2 and supplemental Fig. 2). These results strongly indicate that Lys$_{49}$PLA$_2$ from the venom of eastern cottonmouth is a selective ligand for VEGF receptors, especially KDR.

KDR-bp Inhibits VEGF$_{165}$-KDR Interaction and Blocking of VEGF$_{165}$—As expected from the screening in the initial study, purified KDR-bp competitively blocked both VEGF$_{165}$ and vammin binding to KDR-IgG with an IC$_{50}$ of $2 \times 10^{-6}$ M (supplemental Fig. 3). Similarly, KDR-bp and KDR-IgG interaction was inhibited by VEGF$_{165}$ (Fig. 3), indicating that KDR-bp...
shares the binding epitope on KDR with VEGF<sub>165</sub>, or alternatively, blocks allosterically by binding to a region distinct from the VEGF<sub>165</sub>-binding site, such as the fourth immunoglobulin-like domain, which is essential for VEGF<sub>165</sub>-induced KDR dimerization.

We next evaluated the effect of KDR-bp on the biological activity of VEGF<sub>165</sub>. KDR-bp specifically and completely attenuated the endothelial cell growth stimulated by VEGF<sub>165</sub> without toxic effect on cell viability but did not affect fetal serum (5%)-induced cell growth (Fig. 4 and supplemental Fig. 4). These observations indicate that KDR-bp is an antagonistic ligand for KDR.

**Influence of KDR Binding Ability on Biological Properties of Lys<sup>49</sup>PLA<sub>2</sub>s**—Snake venom PLA<sub>2</sub>s are a widespread toxin in most venomous snakes. Several Viperidae snakes possess Lys<sup>49</sup>PLA<sub>2</sub>s in their venoms, which are inactive PLA<sub>2</sub> homologues, because of substitution of Asp<sup>49</sup>, a key residue for the binding of essential Ca<sup>2+</sup>-cofactor, by Lys<sup>49</sup>, and which exhibit strong myotoxic activity (9, 10). To date, a large number of myotoxic PLA<sub>2</sub>s from several viper venoms have been isolated and structurally characterized; however, the molecular mechanism of myotoxicity of these inactive PLA<sub>2</sub>s is poorly understood. We have reported herein that snake venom Lys<sup>49</sup>PLA<sub>2</sub> is an antagonistic ligand for KDR. This result provides further insight into the molecular mechanism of snake venom myotoxicity. It has been reported that heparin could attenuate the myotoxic activity of Lys<sup>49</sup>PLA<sub>2</sub>s and that their heparin binding ability and myotoxic activity are completely mediated by the C-terminal portion (9, 18, 19). The KDR-bp (Lys<sup>49</sup>PLA<sub>2</sub>) we have identified interacted with heparin with a K<sub>d</sub> of 4.5 × 10<sup>−9</sup> M (supplemental Fig. 5) and has shown that its myotoxic site is located in the C-terminal region (18). Heparin is known to greatly modulate VEGF-VEGF receptor interaction, particularly that of VEGF<sub>165</sub>-KDR (20, 21). In addition, Lys<sup>49</sup>PLA<sub>2</sub> were shown to form a Ca<sup>2+</sup>-independent homodimer in aqueous solution and to exhibit dimerization-dependent myotoxic activity (22). For these aspects, both KDR-bp and VEGF<sub>165</sub> have very similar ligand properties, despite their opposite biological effects.

Lys<sup>49</sup>PLA<sub>2</sub>s from the venom of the Habu snake (T. flavoviridis), basic protein I and basic protein II, were shown to induce apoptosis in HL-60 promyelomonocytic leukemia cells but not in TFPLA<sub>2</sub>, an active PLA<sub>2</sub> derived from same venom.同样

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**TABLE I**

| KDR-bp from | Association | Dissociation | Rate constants |
|-------------|-------------|-------------|---------------|
|             | k<sub>a</sub> | k<sub>diss</sub> | k<sub>diss</sub>/k<sub>a</sub> |
| A. p. piscivorus | 3.44 | 3.30 | 0.96 |
| KDR-IgG | 3.41 | 3.42 | 1.0 |
| KDR-D1–7 | 0.11 | 0.84 | 7.6 |
| IgG | 0.046 | 8.04 | 175 |
| Basic protein I from T. flavoviridis | 3.03 | 2.74 | 0.90 |
| KDR-D1–7 | 0.21 | 1.51 | 7.2 |

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**FIG. 3.** VEGF<sub>165</sub> blocks KDR-bp-KDR interaction. KDR-IgG (5 nm) and indicated concentration of VEGF<sub>165</sub> was incubated for 60 min on a KDR-bp-immobilized plate. KDR-IgG was detected by peroxidase-conjugated anti-human IgG.

**FIG. 4.** KDR-bp blocks the biological activity of VEGF<sub>165</sub>. Serum-starved cultured HUVECs (3,000 cells/well) were incubated with various concentrations of KDR-bp in the presence of VEGF<sub>165</sub> (1 nM) or fetal calf serum (5%). After 3 days cultivation, proliferation rate was evaluated by Tetra Color One system (Seikagaku Corp., Tokyo, Japan). Results are expressed as percentage of control: the proliferation rate induced by VEGF<sub>165</sub> or fetal calf serum (5%) was given a value of 100%, while that of unstimulated cells was given a value of 0%.

Lys<sup>49</sup>PLA<sub>2</sub>-induced apoptosis was observed on a lymphoblasticoid cell line CRL-8062 (23). It is well established that KDR is functional on some VEGF-producing leukemias, including HL-60 cells, resulting in the formation of a VEGF/KDR autocrine loop, which mediates their proliferation and survival (24, 25). Taking our findings into consideration, Lys<sup>49</sup>PLA<sub>2</sub>-mediated apoptosis in leukemic cells could be explained by its KDR-blocking property. In fact, both basic protein I and basic protein II interacted with the extracellular domain of KDR with similar affinities compared with KDR-bp (Table 1). These data also suggest that KDR-binding ability is a general characteristic of Lys<sup>49</sup>PLA<sub>2</sub>s in several snake venoms. However, it is unclear whether the binding to IgG is a biologically important event in envenoming or not. Several animals, such as hedgehogs and mongoose, have toxin-neutralizing proteins in their blood. An anti-myotoxic protein, DM64, was isolated from the serum of southern opossum (Didelphis marsupialis), which is composed of five immunoglobulin-like domains. DM64 forms...
a noncovalent complex with snake venom-derived myotoxic PLA$_2$ (myotoxin II/Lys49PLA$_2$ from Bothrops asper venom) and neutralizes its myotoxicity (26). These facts suggest that snake venom Lys49PLA$_2$ may recognize a specific structural motif present in some of the immunoglobulin superfamily proteins.

At the present, we could not confirm the reactive site of KDR-bp on KDR binding. Crystal structural studies reveal that the overall structure of Lys49PLA$_2$ is quite similar to that of Asp$_{49}$PLA$_2$ (27). In addition, it is reported that the replacement of Lys49 of Lys49PLA$_2$ to Asp does not reduce its myotoxic activity at all (11). Considering these facts, we speculate that KDR binding properties of Lys49PLA$_2$ are also not directly dependent on the amino acid at position 49, although it is unknown whether KDR binding ability is involved in its myotoxicity or not.

In this study, we identified Lys$_{49}$PLA$_2$s from the venom of eastern cottonmouth as an antagonistic ligand for KDR with subnanomolar affinity and also identified Lys$_{49}$PLA$_2$s from another snake venom. In addition to its unique activity, KDR-bp is a major component of eastern cottonmouth venom (it can be obtained at a yield of 10% of venom proteins); for these aspects, it is far superior to other VEGF-antagonizing proteins (28).

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