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Discovery of a novel vascular endothelial growth factor (VEGF) with no affinity to heparin in *Gloydius tsushimaensis* venom

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ABSTRACT

Strong vascular permeability enhancing activity was found only in the venom of *Gloydius tsushimaensis*, in Tsushima island, Japan, when examined together with the venoms of *G. blomhoffii* snakes in several areas of Japan and of *G. ussuriensis* in South Korea. The active protein purified by using Superdex 75 and Mono Q columns showed no affinity to heparin, and migrated on SDS-PAGE with molecular weights of 26 and 13 kDa under nonreducing and reducing conditions, respectively, showing that it exists as homodimer. Its N-terminal amino acid sequence was highly homologous to those of snake venom vascular endothelial growth factors (VEGFs). The sequence of this protein, named GtVF, was inferred from the one base-substituted two cDNAs (438 bp) obtained *via* the 3′ RACE. The phylogenetic analysis suggested the presence of a new type of snake venom VEGFs including GtVF with no affinity to heparin in addition to the known three types of snake venom VEGFs with high affinity to heparin. Since the vascular permeability enhancement by GtVF was inhibited by the antibody against kinase insert domain-containing receptor (KDR), the vascular permeability enhancing activity of GtVF arises through KDR but without heparin binding.

Abbreviations: sv, snake venom; PLA_2, phospholipase A_2; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; VHD, VEGF homology domain; Flt-1, fms-like tyrosine kinase-1; KDR, kinase insert domain-containing receptor; UTR, untranslated region; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate; CBB, Coomassie Brilliant Blue; DTT, dithiothreitol; 2D, two-dimensional; IEF, isoelectric focusing; IPH, immobilized pH gradient; PBS, phosphate-buffered saline; RACE, rapid amplification of cDNA end; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; *Bi*, *Bitis gabonica*; *G*, *Gloydius*; *Hs*, *Homo sapiens*; *P*, *Protobothrops*; *Pm*, *P. mucrosquamatus*; *Tf*, *Trimeresurus flavoviridis*.

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1. Introduction

Snake venoms are complex mixtures of pharmacologically active proteins and polypeptides. Some of these proteins exhibit enzymatic properties such as phospholipases A_2, metalloproteases and serine proteases (Gutierrez and Lomonte, 2013; Markland and Swenson, 2013; Serrano, 2013), whereas others are considered to be nonenzymatic factors. Over the last few decades, many nonenzymatic proteins have been identified and characterized in snake venoms. They are classified into several structural or functional protein families such as C-type lectins and related proteins, cysteine-rich secretory proteins (CRISPs) and anticoagulant proteins as summarized in some reviews (Calvete, 2013; Gutierrez and Lomonte, 2013; Kini and Fox, 2013; Markland and Swenson, 2013; McCleary and Kini, 2013; Morita, 2005).

In mammals, it is known that vascular endothelial growth factor (VEGF) plays a central role in regulation of vasculogenesis and angiogenesis (Ferrara and Davis-Smyth, 1997) and that there are five families of VEGFs, that is, VEGF-A, VEGF-B, VEGF-C, VEGF-D and PlGF (placental growth factor) (Koch et al., 2011). VEGF-A, which is the first discovered VEGF, is a homodimeric glycoprotein and is physiologically expressed as nine splicing isoforms with 121–206 amino acid residues (Yamazaki and Morita, 2006). $VEGF_{165}$ is the most physiologically abundant isoform of VEGF-A. After a VEGF-like potent hypotensive factor, named HF, was first isolated from *Vipera aspis aspis* (Aspic viper) venom (Komori et al., 1999), VEGFs became very important nonenzymatic proteins in snake venoms (Gasmi et al., 2000; Junqueira de Azevedo IL et al., 2001; Yamazaki et al., 2003; Takahashi et al., 2004; Yamazaki and Morita, 2006).

Now there are seven VEGF families by adding viral VEGF (VEGF-E) (Lyttle et al., 1994; Ueda et al., 2003) and snake venom VEGF (VEGF-F) as exogenous members to five mammalian families described above. VEGF-Fs from Viperidae snake venoms are distinct from mammalian VEGFs because there are structural differences in their receptor-binding domains (Suto et al., 2005; Yamazaki et al., 2003).

In response to various VEGFs, three VEGF tyrosine kinase receptors have been identified, that is, fms-like tyrosine kinase-1 (Flt-1 also known as VEGFR-1) (de Vries et al., 1992; Quinn et al., 1993; Shibuya et al., 1990), KDR (VEGFR-2) (Quinn et al., 1993; Terman et al., 1992) and flt-4 (VEGFR-3) (Joukov et al., 1996; Lee et al., 1996; Stacker et al., 1999). Although their roles in signaling have not been fully elucidated, KDR appears to mediate three major actions of VEGF, that is, vascular permeability, cell survival and cell division (Yamazaki and Morita, 2006). Most of snake venom VEGFs exhibited highly specific binding to KDR essentially with an equal affinity as $VEGF_{165}$ does but with no binding to other VEGF receptors (Yamazaki et al., 2009).

Several Crotalinae snakes inhabit Japan. *Protobothrops* (formerly *Trimeresurus*) genus snakes inhabit the southwestern islands of Japan and *Gloydius* (formerly *Agkistrodon*) genus snake, *G. blomhoffii*, distributes in the entire islands of Japan except Tsushima island and the southwestern islands. Intriguingly, the endemic original species, *G. tsushimaensis*, inhabits Tsushima island which is a small island located between Kyushu island, Japan, and Korean peninsula. Our studies done over a few decades showed that the major components of snake venoms such as phospholipase A_2 (PLA_2) isozymes and serine protease isozymes have evolved in an accelerated manner to acquire their diverse physiological activities (Ohno et al., 2003; Deshimaru et al., 1996). We also found that interisland evolution has occurred among PLA_2 isozymes of *P. flavoviridis* snakes in the southwestern islands of Japan, namely, Amami-Oshima, Tokunoshima and Okinawa (Chijiwa et al., 2000; Murakami et al., 2009). Such regional changes of PLA_2 isozymes from snake venoms is of great interest from the viewpoint of molecular diversification in response to their native environments.

Thus, the comparison was made for the venom compositions in *Gloydius* genus snakes from several different areas in Japan and Korea in order to see whether the similar regional change has occurred in *Gloydius* genus snake venoms. Then, we found fairly strong vascular permeability enhancing activity in *G. tsushimaensis* venom but not in the venoms of *G. blomhoffii* from three specified areas in Japan and of *G. ussuriensis* in South Korea. It became evident that the vascular permeability enhancing activity in *G. tsushimaensis* is due to VEGF with no affinity to heparin and acting through the aid of KDR similarly as known for other snake venom VEGFs.

2. Materials and methods

2.1. Materials

Crude venoms from *G. blomhoffii*, *G. ussuriensis* and *G. tsushimaensis* were provided by The Japan Snake Institute, Gunma, Japan. Both recombinant mouse VEGF-A and mouse VEGFR-2/Flk-1 affinity purified polyclonal Ab, Goat IgG were purchased from R & D.

2.2. Purification of GtVF

Crude *G. tsushimaensis* venom (5 mg) was applied on a Superdex 75 column previously equilibrated with 50 mM Tris–HCl (pH 7.4) containing 50 mM NaCl and eluted with the same buffer at a flow rate of 0.5 ml/min on an ÄKTA purifier system, GE Healthcare Ltd., UK. This chromatographic procedure was repeated four times to obtain enough amount of the protein for further purification. The active fraction pooled were concentrated and desalted with Nanosep centrifugal 3K device (Pall Life Sciences, Ann Arbor, MI, USA). The solution was then loaded on a Mono Q HR5/5 column (GE Healthcare) equilibrated with 50 mM Tris–HCl (pH 7.4) and eluted with a 30-min linear gradient of 50–800 mM NaCl at a flow rate of 0.5 ml/min (Fig. 2B). The flow-through fraction showing vascular permeability enhancing activity was concentrated by Nanosep 3K device. The solution was chromatographed on a Superdex 75 column with 50 mM Tris–HCl (pH 7.4) containing 100 mM NaCl and the active fractions were collected and concentrated by Nanosep 3K device. The solution was again eluted on a Superdex 75 column (Fig. 2C). The protein concentration was determined by BCA™ protein assay kit (Pierce,