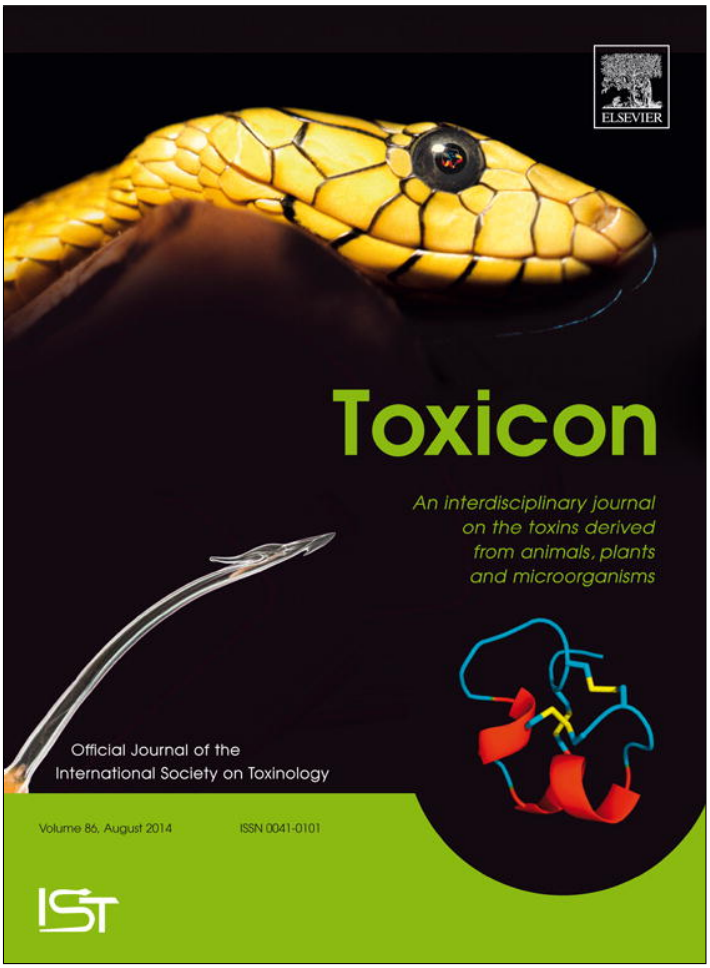

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Discovery of a novel vascular endothelial growth factor (VEGF) with no affinity to heparin in *Gloydius tsushimaensis* venom

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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,

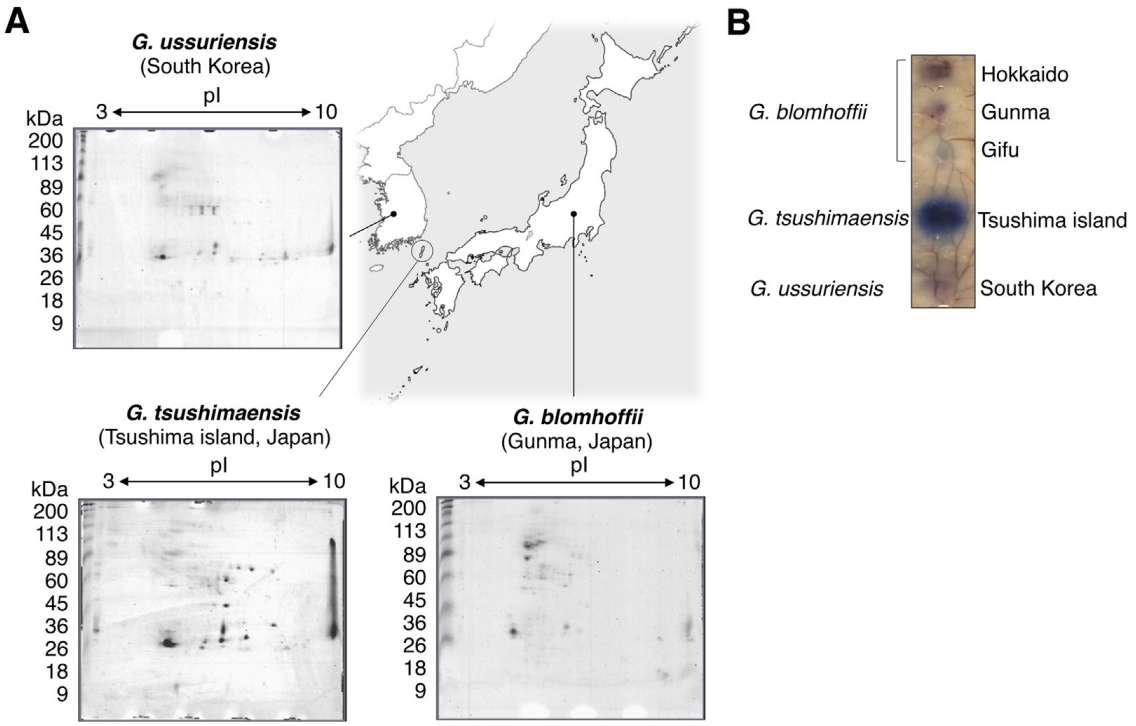

Fig. 1. 2D-gel electrophoresis (A) and Miles assay (B) of the venoms of *Gloydius* genus snakes, *G. blomhoffii*, *G. tsushimaensis* and *G. ussuriensis*. (A) Precast strips for isoelectric focusing (IEF) (7 cm, linear pH 3-10 gradient) were used. Gels were run under the identical conditions and scanned on an image analyzer system as described in "Materials and Methods." (B) Blue spots of Evans blue indicate that the dye leakage from plasma into skin occurred most strongly for *G. tsushimaensis* venom but much less strongly for the venoms of other *G. blomhoffii* snakes and of *G. ussuriensis*. Each venom, 0.5 μg/100 μl, is injected into the skin. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Rockford, IL, USA). The protein obtained with vascular permeability enhancing activity was named GtVF.

2.3. Two-dimensional (2D) gel electrophoresis

The 2D gel electrophoresis was conducted as follows. The freeze-dried crude venom (approx. 300 μg) was dissolved in 370 μl of rehydration solution (8 M urea, 4% CHAPS, 100 mM DTT) containing 0.4% Bio-Lyte (pH 3-10 linear). Precast immobilized pH gradient (IPG) strips for isoelectric focusing, 7 cm IPG gel strips (Bio-Rad, Hercules, CA, USA) were used for separation of proteins in the 1st dimension. After 12 h of rehydration in the sample solution, isoelectric focusing (IEF) was carried out at 20 °C with a PROTEAN IEF system (Bio-Rad) using the following protocol: 500 V for 15 min, 4000 V for 2 h and 4000–20,000 V (gradient) for 1 h. Following IEF, the strips were reduced and alkylated by sequential incubation with 2% DTT and 2.5% iodoacetamide in the equilibration buffer (6 M urea, 40 mM Tris–HCl pH 8.8, 2% sodium dodecyl sulfate (SDS) and 20% (v/v) glycerol). To accomplish the 2nd dimension, the previously reduced and alkylated strips were washed in SDS-polyacrylamide gel electrophoresis (PAGE) running buffer and directly applied on 10–20% polyacrylamide gradient gels on a PROTEAN II system (Bio-Rad). Electrophoresis was carried out at constant current (25 mA per gel). The gels were stained with 0.1% (w/v) Coomassie Brilliant Blue (CBB) in 40% (v/v) methanol and 10% (v/v) acetic acid and destained with the same solution without CBB. The gel spots were scanned with GS-800 calibrated densitometer on an image analyzer system (Bio-Rad) and analyzed with PDQuest software (Bio-Rad).

2.4. N-terminal amino acid sequencing

The sample was prepared according to the protocol of AproScience (Tokushima, Japan). Briefly, the purified sample was separated by 15% SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane (Sigma) using Owl semi-dry transfer apparatus with 3 mA/cm^2. After staining the membrane with Simply Blue (Life Technologies, Grand Island, NY, USA) and destaining with 45% methanol in 7% acetic acid and 90% methanol, the band corresponding to molecular weight 13 kDa was excised and transferred into tube and soaked with 60% methanol twice, 90% methanol once and washed with ultrapure water, and incubated with pyroglutamyl aminopeptidase (Takara, Shiga, Japan) in the buffer attached for 5 h at 50 °C and then the N-terminal amino acid sequence was analyzed at the contracted research company, AproScience. Protein sequence databases were searched using BLAST.

2.5. Miles assay

Vascular permeability enhancing activity was measured by means of Miles assay (Miles and Miles, 1952). The experiment was done according to the criteria of animal experiments of Kumamoto University Animal Experiment Committee and under its permission of the Committee. Guinea pigs (350–450 g body weight, both sexes) were anesthetized with an intramuscular injection of ketamine

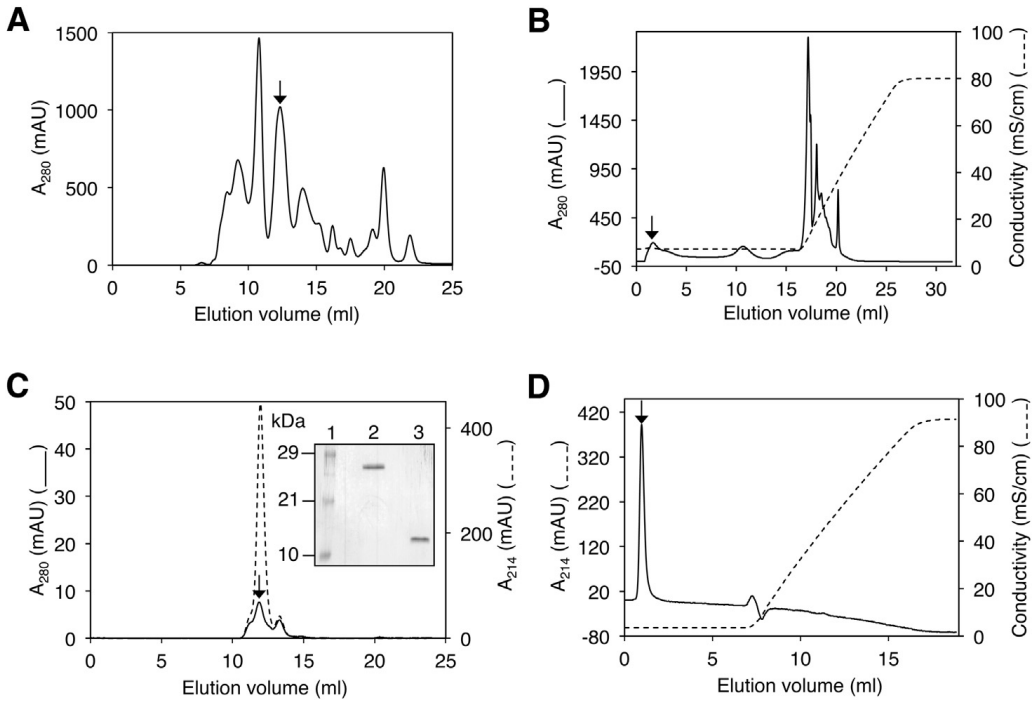

Fig. 2. Column chromatographies of GtVF. (A) *G. tsushimaensis* venom was chromatographed on a Superdex 75 column using an ÄKTA purifier system with 50 mM Tris–HCl (pH 7.4) containing 50 mM NaCl at a flow rate of 0.5 ml/min. Elution was monitored by absorbance at 280 nm and the active fraction indicated by arrow was collected and concentrated with Nanosep 3K device. (B) The active fraction from (A) was loaded on a Mono Q HR5/5 column with 50 mM Tris–HCl (pH 7.4) and eluted with 30 min linear gradient of 50–800 mM NaCl at a flow rate of 0.5 ml/min. The active flow-through fractions were collected and concentrated with Nanosep 3K device. (C) The active fraction from (B) was chromatographed on a Superdex column twice. The second elution profile is shown here. The active fractions indicated by arrow were collected and concentrated with Nanosep 3K device. The inset shows SDS-PAGEs of the sample under the nonreducing (2) and reducing conditions (3) and of molecular weight markers (1). The protein bands were visualized with silver staining (D). The active fraction from (C) was loaded on a Hitrap-Heparin column with 50 mM Tris–HCl buffer (pH 8.0) and eluted by increasing NaCl concentration up to 0.8 M.

(80 mg/kg body weight). Thirty mg/kg body weight of Evans blue (2.5% solution in 0.6% saline) was administered intravenously, followed by an intradermal injection of 50 μl of test sample (dissolved in 10 mM Tris-b saline, pH 7.3) into the clipped flank of the guinea pig. After 10 min, animals were sacrificed. For quantification of dye leakage, the blue spots were excised and incubated in 2 ml of formamide at 60 °C for 48 h. The amounts of extracted dyes were determined by measuring the absorbance at 620 nm as described previously (Imamura et al., 1994).

2.6. 3′-RACE for cloning GtVF cDNAs

Total RNA was extracted from *G. tsushimaensis* venom gland using the RNeasy Maxi Kit (QIAGEN, Hilden, Germany). 3′-Rapid amplification of cDNA ends (RACE) ready first-strand cDNAs were synthesized using the SMARTer™ RACE cDNA Amplification Kit (Takara) according to the manufacturer's instructions. 3′- RACE PCR was conducted for synthesized cDNAs with universal primer and gene-specific primer 5′-TTCTGAGCAGCTGTGAAGCCAGGAG-3′, which was designed based on the highly conserved 5′ UTR of snake venom VEGF cDNAs. The amplified DNA fragments were electrophoresed, gel-purified, cloned into T-vector pMD19 (Simple) (Takara) and sequenced using CEQ8000 (Beckman Coulter).

The novel sequences reported in this work were deposited in the DDBJ (DNA data bank of Japan) under the following accession numbers: *GtVF1*, AB829336 and *GtVF2*, AB829897.

3. Results and discussion

3.1. Comparison of the venom compositions of G. blomhoffii snakes from several areas of Japan and of Gloydius genus snakes from the Far East

Based on the facts that the venom compositions of *P. flavoviridis* snakes in Amami-Oshima, Tokunoshima and Okinawa islands are subtly different and it brought about novel evolutionary findings (Chijiwa et al., 2000; Murakami et al., 2009), we examined whether the regional changes are observed in the venom compositions of *G. blomhoffii* snakes which are the most common venomous snakes in Japan. *G. blomhoffii* snakes in Hokkaido (the most northern island) are larger in size and their skin patterns are quite different from those of Gunma and Gifu of the main-island (Honshu) of Japan. The venom compositions of *G. blomhoffii* snakes of these areas were compared from the spot patterns of 2D gel electrophoresis of the crude venoms (data not shown). Although it appeared that the expression levels of some proteins are variable between Hokkaido and Gifu (Gunma), clear regional variation has not been observed in their venom compositions. The phenomena may suggest that the far isolated environments are the prerequisite for occurrence of the regional change in venom composition. Thus, the areas for survey were expanded to the Far East. Comparison was made for the 2D electrophoretic profiles of the venom proteins of *G. tsushimaensis* of Tsushima island and *G. ussuriensis* of South Korea beside that of *G. blomhoffii*

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1     1 ATG GCT GCA TAC CTG CTG GCA GTT GCC ATC CTC TTC TGC ATC CAG GGC TGG CCA TCA GGG 60
1'    1 Met Ala Ala Tyr Leu Leu Ala Val Ala Ile Leu Phe Cys Ile Gln Gly Trp Pro Ser Gly 20
2     1 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... 60
2'    1  *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *  20

1    61 ACA GTG CAG GGA CAA GTG ATG CCC TTT ATG GAA GTG TTC AAG CGC AGC GTC TGC CAG ACC 120
1'   21 Thr Val Gln Gly Gln Val Met Pro Phe Met Glu Val Phe Lys Arg Ser Val Cys Gln Thr 40
2    61 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... 120
2'   21  *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *  40

1   121 AGG GAG ACG CTA GTG CCC GTC CTC GAA GAG CAC CCC AGT GAA ATT GCC GAC CTC TTC AAG 180
1'   41 Arg Glu Thr Leu Val Pro Val Leu Glu Glu His Pro Ser Glu Ile Ala Asp Leu Phe Lys 60
2   121 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... 180
2'   41  *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *  60

1   181 CCC TCC TGT GCC ACC GTG TTG CGA TGC GGC GGC TGC TGC AGC GAC GAA AGC CTC GCG TGC 240
1'   61 Pro Ser Cys Ala Thr Val Leu Arg Cys Gly Gly Cys Cys Ser Asp Glu Ser Leu Ala Cys 80
2   181 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... 240
2'   61  *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *  80

1   241 ACC GCT GTG GGA AAG CGC TCC GTC GGT CGG GAG ATC TTG CGG GTG GAT CCT CGC AAG GGG 300
1'   81 Thr Ala Val Gly Lys Arg Ser Val Gly Arg Glu Ile Leu Arg Val Asp Pro Arg Lys Gly 100
2   241 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... 300
2'   81  *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *  100

1   301 ACT TCG AAG ATA GAG GTG ATG CAA TTC ACG GAG CAC ACA GAC TGT GAA TGC AGG CCT CGA 360
1'  101 Thr Ser Lys Ile Glu Val Met Gln Phe Thr Glu His Thr Asp Cys Glu Cys Arg Pro Arg 120
2   301 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... 360
2'  101  *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *  120

1   361 TCA AGA AGC GGG GTG GAC AGC GGG AAG CGC AAG AGG AAC CCA GAG GAA GGG GAG CCG AGA 420
1'  121 Ser Arg Ser Gly Val Asp Ser Gly Lys Arg Lys Arg Asn Pro Glu Glu Gly Glu Pro Arg 140
2   361 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... 420
2'  121  *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *  140

1   421 GCC AAG TTC CCC TTT GTC TGA                                                   441
1'  141 Ala Lys Phe Pro Phe Val xxx                                                   146
2   421 ... ... ... ... C.. ... ...                                                   441
2'  141  *   *   *   *  Leu  *  xxx                                                   146
```

Fig. 3. The complete nucleotide sequences of *G. tsushimaensis*, *GtVF1* (1) and *GtVF2* (2), and their deduced amino acid sequences, GtVF1 (1′) and GtVF2 (2′).

(Gunma, Japan) (Fig. 1A). The three venoms exhibited different spot patterns, showing that the venom compositions of *Gloydius* genus snakes in the areas separated by sea are different to one another. Since hypotension is a common sign observed at the occasion of Crotalinae snake bite and it has been reported that vascular permeability enhancing factor is responsible for this (Komori et al., 1999), vascular permeability enhancing activity was examined for *Gloydius* genus snake venoms by Miles assay. Strong vascular permeability enhancing activity was found only in *G. tsushimaensis* venom when examined together with the same amounts of the venoms of *G. blomhoffii* snakes from Hokkaido, Gunma and Gifu and of *G. ussuriensis* in South Korea as shown in Fig. 1B.

3.2. Purification of GtVFs and determination of their primary structures

Purification of GtVF to homogeneity was achieved by four chromatographic steps by monitoring vascular permeability enhancing activity with Miles assay. The crude *G. tsushimaensis* venom was gel-filtered on a Superdex 75 column (Fig. 2A). The active fractions were collected and chromatographed on a Mono Q column (Fig. 2B). The flow-through fractions were collected, concentrated and gel-filtered on a Superdex 75 column twice. Here, GtVF was almost purified to homogeneity as shown in Fig. 2C. SDS-PAGE analyses under nonreducing and reducing conditions gave molecular weights of about 26 kDa and 13 kDa, respectively (inset in Fig. 2C), suggesting that GtVF exists as homodimer in native condition.

Since it is assumed that the N-terminus of GtVF is blocked by the pyroglutamyl residue as observed for other known VEGFs (Yamazaki et al., 2003), GtVF was treated by pyroglutamyl aminopeptidase in order to remove the pyroglutamyl residue. Then, the remaining protein was analyzed by the Edman method for its amino acid sequence. The sequence obtained, VMPFMEVFKRSVXQTXE, can be considered as the residues 2-18 of GtVF. This was highly homologous to the corresponding segments of various snake venom VEGFs (Fig. 4). Then, we tried to determine the entire sequence of GtVF. Since the 5′ UTR of the snake venom VEGF cDNAs are well conserved, we conducted the 3′ RACE using the conserved sequence described above as the 5′ primer to obtain the full-length cDNA encoding GtVF. The two clones named *GtVF*1 and *GtVF*2 were obtained (Fig. 3). Only one nucleotide substitution was noted between them, showing that the deduced

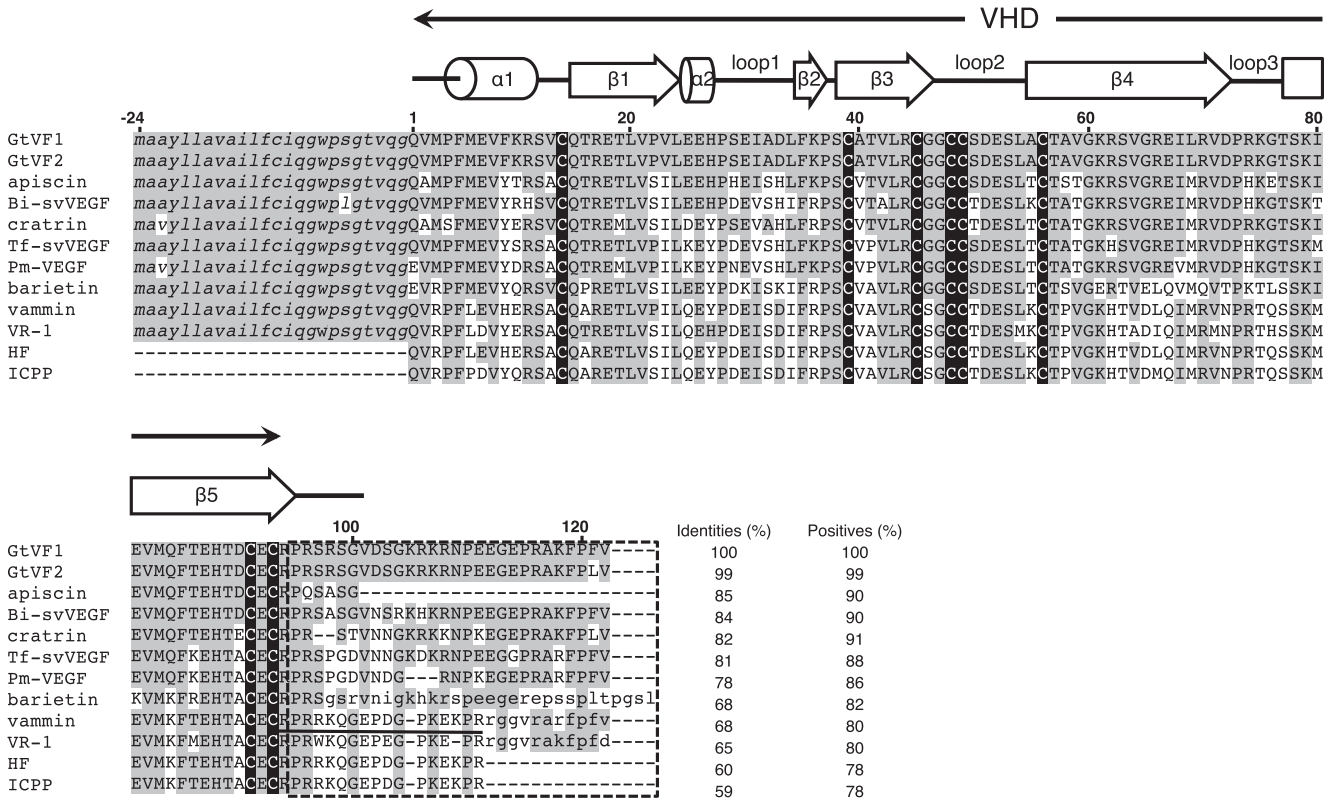

Fig. 4. The aligned amino acid sequences of snake venom VEGFs. The identical residues with those of GtVF1 are shaded and the conserved half-cystine residues are highlighted in black. Putative signal peptides are shown in italic lowercase letters. The numbers at the top refer to the residue numbers of GtVF1. The secondary structural elements are shown as arrows for β-strands and cylinders for α-helices, and loops are labeled. C-terminal putative coreceptor-binding regions are boxed with dotted lines, and the reported heparin binding sequences are marked with underlines. Note that the sequences of HF (Komori et al., 1999) and ICPP (Gasmi et al., 2000) are shown as mature proteins because cDNAs encoding HF and ICPP have not been reported. Accession numbers of the VEGFs in this figure are shown in the text.

amino acid sequences are identical except one amino acid substitution F to L in the C-terminal region (Fig. 3). Since four clones out of five obtained in the cloning coded for GtVF1, it could be assumed that GtVF1 is the major isozyme rather than GtVF2. Their amino acid sequence deduced from their cDNAs are well in accord with those of other snake venom VEGFs (Fig. 4). The signal peptides of GtVF1 and GtVF2 composed of 24 amino acids were judged from the alignment together with other snake venom VEGFs (Junqueira de Azevedo IL et al., 2001). Thus, the putative mature sequences of GtVF1 and GtVF2 are composed of 122 amino acids. The positions of the half-cystine residues of GtVFs completely matched with those of other snake venom VEGFs. Thus, it could be assumed that the second and fourth half-cystine residues of GtVFs form the inter-chain disulfide bond and the other half-cystine residues form the three intramolecular disulfide bonds similarly as reported (Junqueira-de-Azevedo Ide et al., 2004; Komori et al., 1999; Yamazaki and Morita, 2006).

3.3. Comparison and classification of VEGF sequences

Multiple alignments of the amino acid sequences of the snake venom VEGFs demonstrated that GtVF1 and GtVF2 are well conserved in the VEGF homology domains (VHDs) (Fig. 4). They can be grouped nearly in accord with venomous snake taxonomy. Yamazaki et al. (2009) reported that modest accelerated evolution has occurred in the coding region of VHDs of snake venom VEGFs and that the functions of venom VEGFs may be extensively variegated especially in the functionally key regions such as the putative receptor binding loops 1 and 3 and the C-terminal putative coreceptor-binding region, whereas the sequences of mammalian VEGFs would be highly conserved. They also suggested that snake venom VEGFs were classified into three types based on their structures and receptor binding potentials: vammin type, that is, vammin (ACN22045), VR-1 (a VEGF homologous potent hypotensive factor from *Daboia russelli russelli* venom) (ACN22046), HF (P83942) (Komori et al., 1999) and ICPP (an increasing capillary permeability protein from *Macrovipera lebetina* venom) (P82475) (Gasmi et al., 2000), which selectively binds to KDR and heparin (Yamazaki et al., 2003), *Tf*-svVEGF type consisting of *Tf*-svVEGF (BAD38844) and *Pm*-VEGF (Q330K6) binds to Flt-1 in preference to KDR and heparin (Chen et al., 2005; Takahashi et al., 2004) and barietin type binds to KDR in equal affinity as vammin and Hs-VEGF-A do but binds to Flt-1 to a lesser degree and is tightly bound to heparin (Yamazaki et al., 2009). GtVF1 and GtVF2 branched with apiscin (ACN22039), being relatively close to *Bi*-svVEGF (AAK52102) and cratrin (ACN22040), in the phylogenetic tree, and are separately from other snake

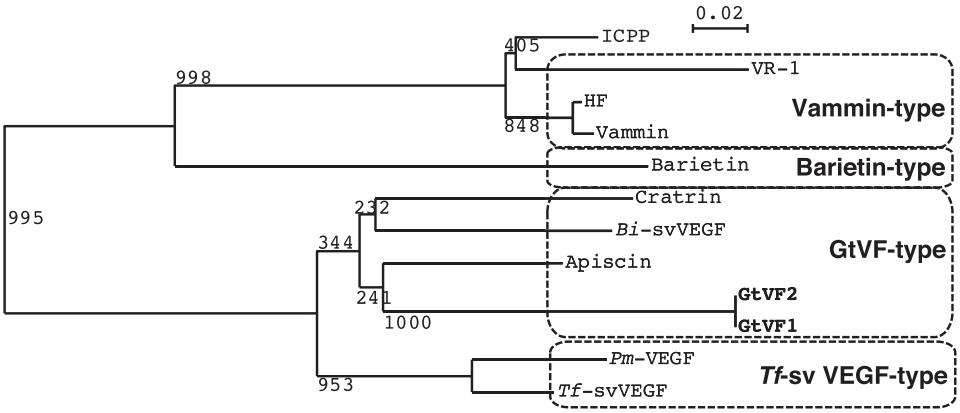

Fig. 5. Phylogenetic tree of the VHD regions of snake venom VEGF proteins generated with the Clustal W program and viewed using NJ plot. The unweighted pair group method with arithmetic phylogenetic tree was built by using the amino acid sequences of the VHDs of several snake venom VEGFs. Although snake venom VEGFs were classified into three types based on their structures and receptor binding potentials as reported by Yamazaki et al. (2009), that is, vammin type, *Tf*-svVEGF type and barietin type, we propose here the addition of *GtVF* type as the fourth type. The branch lengths are drawn to scale and represent the numbers of amino acid substitution per site.

venom VEGFs such as vammin type, barietin type and *Tf*-svVEGF type (Fig. 5). Interestingly, it was observed that GtVF was not trapped on a Hitrap-Heparin column under the same condition as conducted for vammin and barietin (Yamazaki et al., 2009) (Fig. 2D). *Bi*-svVEGF was reported to be a relatively weak affinity to heparin-Sepharose (Junqueira de Azevedo IL et al., 2001). Although there is no information about apiscin or cratrin as regards the binding affinity to heparin, we propose here that snake venom VEGFs such as *Bi*-svVEGF, apiscin, cratrin, and GtVFs constitute a new type of VEGFs, that is, the GtVF type, which is characterized with no or weak affinity to heparin. By contrast, other three types of snake venom VEGFs, that is, vammin-type, barietin-type and *Tf*-svVEGF-type VEGFs show relatively high affinity to heparin. The C-terminal regions of some VEGF isoforms have been shown to interact with heparin/heparan sulfate (Makinen et al., 1999; Mamluk et al., 2002; Tokunaga et al., 2006). Although the C-termini of GtVF and *Tf*-svVEGF seem to have similar property that is positively charged, they exhibited different binding behaviors to heparin. On the other hand, it was reported that although the C-terminal region of barietin is less similar to those of other VEGFs, barietin was able to bind to a heparin affinity column even more tightly than others (Yamazaki et al., 2009). Therefore, it could be assumed that the heparin binding region may not be limited to positively charged C-terminal region. Thus, the presence of two types of snake venom VEGF molecules as regards heparin binding, that is, strong binding type and no (or weak) binding type, provide a clue for considering a biological meaning of heparin binding of snake venom VEGFs.

3.4. Relationship of GtVF and KDR as to vascular permeability enhancing activity

For determining whether vascular permeability enhancement is caused by GtVF through the aid of KDR, Miles assay was conducted in the presence of anti-KDR antibody. The bar graph shows that vascular permeability

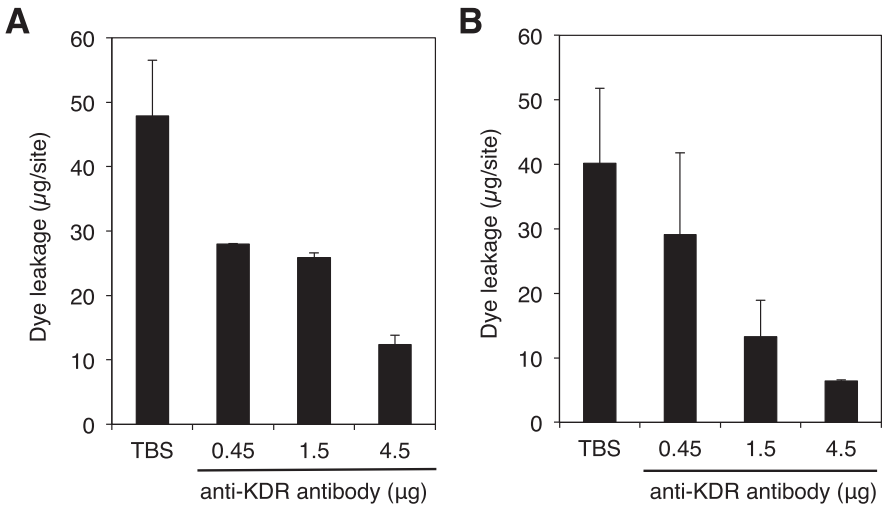

Fig. 6. Effects of anti-KDR antibody on vascular permeability enhancing activity of GtVF. A hundred microliter of anti-KDR antibody or TBS was injected intradermally into each guinea pig. After 30 min, 50 μl of GtVF (30 ng/ml) (A) or mouse recombinant VEGF-A (25 ng/ml) (B) was injected intradermally into the pre-injected sites. Animals were sacrificed 10 min after sample injection and the injected sites were harvested. Leaked Evans blue dye was quantified as indicated in "Materials and methods." Data represent mean plus or minus SEM ($n = 2$).

enhancing activity of GtVF is inhibited by anti-KDR antibody in a dose dependent manner (Fig. 6), suggesting that GtVF gives rise to vascular permeability enhancing activity through the aid of KDR. Since GtVF does not bind to a heparin column while other sv-VEGFs are known to bind to heparin, GtVF might have a peculiar physiological property being not annexed to other snake venom VEGFs.

Here, we discovered that the strong vascular permeability enhancing activity induced by *G. tsushimaensis* venom is due to a novel VEGF acting through the aid of KDR but being independent of heparin binding. The venomous VEGFs distribute in Viperidae venoms and their structures and functions are extensively variegated among species. It could be assumed that snake venom VEGFs possibly variegated their receptor binding potentials during the evolutionary process and acquired the biological functions specifically to aid envenomation.

Ethical statement

The contents of this article do not contain any thing against an ethic.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

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