Vascular Endothelial Growth Factor VEGF-like Heparin-Binding Protein from the Venom of *Vipera aspis aspis* (Aspic Viper)†

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ABSTRACT: The heparin-binding dimeric hypotensive factor (HF) was purified from *Vipera aspis aspis* (Aspic viper) venom [Komori, Y. and Sugihara, H. (1990) *Toxicon 28*, 359−369]. In this study, the amino acid sequence, and structure and function of HF, were elucidated. By electrospray ionization mass spectrometry (ESI-MS), the molecular weight of HF was determined to be 25 072.1. The complete amino acid sequence of HF was determined by Edman sequencing of the S-pyridylethylated HF and its peptides derived from enzymatic digestion. The theoretical molecular mass calculated from the primary structure agrees well with the molecular weight determined by ESI-MS. HF consists of two homogeneous monomers bound covalently. The monomer with an N-terminal blocked by pyroglutamic acid contains 110 amino acid residues, including eight cysteine residues, two of which are considered to be involved in intermolecular disulfide bonds. Sequential homology search revealed that the primary structure of HF is similar to that of vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) with a sequential homology of 45 and 22%, respectively. When injected intradermally into a rat, an increase in capillary permeability was observed with HF or VEGF. On the other hand, only HF exerted a strong hypotensive effect after intravenous injection of samples into a rat. Purified HF has a mitogenic effect on endothelial cells. Through the use of bovine aortic endothelial cells (BAEC), the half-maximal mitogenic concentration of HF was determined to be 5−5.5 nM (125−138 ng/mL). Similarly, VEGF had a mitogenic concentration at 0.5−1 nM. When incubated with HF and cycloheximide or HF and heparin, the cell growth was inhibited, suggesting that the mechanism of action of HF is similar to that of VEGF.

Hypotension is a common symptom observed following Viperidae and Crotalidae snake envenomation (*1*, *2*). Kallikrein-like enzymes and bradykinin potentiating factors are well-studied hypotensive factors in snake venoms (*3*−*9*); however, the mechanism of action of a basic hypotensive factor (HF)[1] purified from *Vipera a. aspis* venom (*10*) has not been clarified. HF is a heat-resistant dimeric protein. Intravenous injection of HF induces an immediate fall in the blood pressure of rats. The hypotensive effect was observed at a dose as low as 0.0125 μg/g, and the time required for the complete recovery of arterial blood pressure with 0.5 μg/g was approximately 180 min. The purity of HPLC-purified HF was proved by immunological, and by various electrophoretic procedures. The ESI-MS data also showed that HF preparation did not contain any trace of bradykinin-potentiating peptides or kallikrein-like enzyme. In addition, enzyme assay using fluorescence substrate indicated that this factor is devoid of kallikrein-like activity. None of venom proteins reported before possesses similar characteristics to HF (*10*).

During the course of determination of primary structure and homology search, HF was found to be homologous to growth factor VEGF, which belongs to the PDGF family. VEGF was first found by Senger et al. (*11*) as a vascular permeability factor (VPF) that is secreted from tumor cells. By a separate experiment, Ferrara and Henzel (*12*) identified the growth factor for vascular endothelial cells (VEGF) in the media conditioned by bovine pituitary follicular cells. VPF was subsequently found to stimulate endothelial cell growth and angiogenesis (*13*), and finally VPF and VEGF were identified as the same protein by cDNA cloning (*14*, *15*). VEGF/VPF is a disulfide-linked dimeric protein that causes capillary leakage in guinea pigs at a dose of 20 ng and promotes endothelial cell growth at concentrations as low as 50 pM (*16*). Analysis of cDNA clones revealed that

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[1] Abbreviations: HF, hypotensive factor; VEGF, vascular endothelial growth factor; ESI-MS, electrospray ionization mass spectrometry; PDGF, platelet-derived growth factor; BAEC, bovine aortic endothelial cell; VPF, vascular permeability factor; HAEC, human aortic endothelial cell; HUVEC, human umbilical vein endothelial cell; HPAEC, human pulmonary artery endothelial cell; HASMC, human aortic smooth muscle cell; NHEK, normal human epidermal keratinocyte (from neonatal foreskin); NRCE2, normal rabbit corneal epithelial cell; DMEM, Dulbecco's modified Eagle's medium; Pe-, S-pyridylethylated; Lys-N, lysylendopeptidase; EGF, epidermal growth factor; FGF, fibroblast growth factor.

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the heterogeneity of VEGF is apparently due to the alternative splicing of its RNA transcript. The existence of four different forms of human VEGF (121-, 165-, 189-, and 206-residue forms) has been identified (*17*, *18*). Of these isoforms, $VEGF_{121}$ and $VEGF_{165}$ was found to possess mitogenic activity when their clones were transiently expressed in human embryonic kidney 293 cells and then the conditioned medium from 293 cells were added to bovine capillary endothelial cells. The conditioned medium from 293 cells that expressed $VEGF_{189}$ and $VEGF_{206}$ had less effect on cell growth, indicating that $VEGF_{189}$ and $VEGF_{206}$ are predominantly cell-associated and are poorly secreted (18). Since $VEGF_{189}$ and $VEGF_{206}$ have a basic region in their COOH-terminals, they are expected to bind to negatively charged extracellular matrix. Houck et al. (*19*) compared chromatographic behavior of $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$ on S−Sepharose and heparin−Sepharose columns and showed that $VEGF_{189}$ strongly bound to both columns. On the other hand, $VEGF_{121}$ possessed very weak affinity for S−Sepharose and failed to bind to heparin−Sepharose. $VEGF_{165}$ displayed an intermediary behavior. Cell-associated VEGF isoforms were released by heparin, heparan sulfate, and heparinase, suggesting heparin-containing proteoglycans as candidate VEGF-binding site. Since plasmin-treatment also increased the dissociation of VEGF, it is expected that the bioavailability of VEGF is regulated by genetic proteolytic mechanisms (*19*). Flt-1 (VEGF receptor-1) and KDR/Flk-1 (VEGF receptor-2) were identified as VEGF receptor (*20*, *21*), and the role of VEGF and these receptor-type tyrosine kinase as a regulator of vasculogenesis and angiogenesis have been clarified (*22*−*25*).

In this study, the structure and chemical characteristics of HF purified from *V. a. aspis* venom were compared with those of VEGF. Effects of HF on vascular permeability, blood pressure of rats, and endothelial cell growth were also tested and discussed in comparison with VEGF.

EXPERIMENTAL PROCEDURES

Materials. Lyophilized crude venom of *V. a. aspis* was purchased from Latoxan (Rosans, France). Heparin−Sepharose CL-6B was the products of Amersham Pharmacia Biotech. 4-Vinylpyridine and pyroglutamyl-peptidase from *Bacillus amyloliquefaciens* were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Lysyl-endopeptidase from *Achromobactor lyticus* (Lys-N), heparin sodium salt, and cycloheximide were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) Endoproteinase Glu-C from *Staphyloccoccus aureus* V8 was the product of Boehringer Mannheim Gmbh (Germany), and clostripain was from Sigma (St. Louis, MO). The reagents used for protein sequencing were supplied by Perkin-Elmer Applied Biosystems (Tokyo, Japan). Recombinant human $VEGF_{165}$ and rabbit anti-human VEGF (polyclonal) were purchased from Immugenex Corp. (Los Angeles, CA), recombinant human PDGF-AA, PDGF−BB, PDGF-AB, and rabbit anti-human PDGF-AA and rabbit anti-human PDGF−BB (polyclonal) were from Pepro Tech EC Ltd. (London, England). Cryopreserved human and rabbit cells (HAEC, HPAEC, HUVEC, HASMC, NHEK, and NRCE2), their respective cell culture media (HuMedia EB-2 for human endothelial cells, HuMedia SG2 for HASMC, HuMedia KG2 for NHEK, and RCGM2 for NRCE2), and other cell culture supplements and reagents were obtained from Kurabo (Osaka, Japan). Dulbecco's modified Eagle medium was the product of Nissui Seiyaku, Co. (Tokyo, Japan), antibiotic antimycotic lyophilized powder was from Sigma, and cell counting kit was from Dojindo (Kumamoto, Japan). Other chemicals used were of analytical grade from commercial sources.

Purification Procedure of HF. Purification of HF was performed as previously described (*10*). Briefly, crude venom of *Vipera a. aspis* (500 mg) was fractionated on Sephadex G-75 gel filtration and S−Sepharose columns. The fraction possessing hypotensive activity was further purified by reversed-phase HPLC to eliminate the peptide contamination and used for all bioassays.

Heparin−Sepharose CL-6B Column Chromatography. Partially purified HF fraction from Sephadex G-75 and S−Sepharose columns was dialyzed against 0.01 M Tris-HCl buffer (pH 7.2) and applied to a column of heparin−Sepharose CL-6B (1 × 15 cm) equilibrated with the same buffer. After the column was extensively washed with an equilibration buffer, the column was eluted with a linear gradient from 0.1 to 0.3 M NaCl in a total volume of 200 mL at a flow rate of 15 mL/hr and then washed with the buffer containing 0.5 and 1 M NaCl.

S−Pyridylethylation of HF. HF (6.0 mg) was dissolved in 2.0 mL of 5 mM Tris-HCl buffer (pH 7.5) containing 8 M urea. 2-Mercaptoethanol (200 μL) was added to the sample and incubated at 37 °C for 60 min under nitrogen. 4-Vinylpyridine (200 μL) was then added to the reduced sample and stirred at room temperature for 2 h. The excess reagents were removed by dialysis against distilled water. S−Pyridylethylated (Pe-) HF was used as the starting material for the primary structural analysis.

Chemical Cleavage of Pe-HF. Pe-HF (1.5 mg) was cleaved with a 200-fold molar excess of CNBr over methionine residues of HF in 70% formic acid (1 mL) under nitrogen for 72 h at room temperature. The reaction mixture was diluted with 20 mL of water and lyophilized. The digests were separated by reversed-phase HPLC using Gilson Model 302 chromatograph with a Develosil 300 ODS-7 column (4.6 × 250 mm).

Enzymatic Cleavage of Pe-HF. Pe-HF (1.5 mg) was digested with lysyl endopeptidase Lys-N (2 wt %) for 15 h at 37 °C in 0.01 M ammonium acetate buffer (pH 9.0) containing 4M urea or with endoproteinase Glu-C (V8 protease-2 wt %) for 20 h at 37 °C in 0.01 M ammonium carbonate buffer (pH 7.8) containing 4 M urea. Pe-HF (1.5 mg) was also digested with clostripain (2 wt %) for 15 h at 37 °C in 0.05 M Tris-HCl buffer (pH 7.2) containing 1% 2-mercaptoethanol and 10 mM $CaCl_2$. All digests were separated by reversed-phase HPLC using the same system described above. The N-terminal-blocked peptide obtained from the clostripain digest was further incubated with pyroglutamylpeptidase (0.25 mU) in 250 μL of 0.1 M phosphate buffer (pH 8.0) containing 10 mM EDTA and 10 mM 2-mercaptoethanol at 37 °C for 24 h and purified by reversed-phase HPLC.

Amino Acid Analysis and Sequence Analysis. Enzymatically digested peptides were hydrolyzed in boiling HCl at 110 °C for 24 h. The amino acid composition was determined with a JEOL Model JLC-300 high-speed automatic analyzer. The sequence analysis of chemically and enzymatically digested fragments were performed with an Applied Bio-

systems model 477A protein sequencer. The phenythiohydantoin (PTH) derivatives of the amino acids were identified with an Applied Biosystems model 120A PTH-analyzer.

ESI-MS. The ESI mass spectra were measured with a double-focusing mass spectrometer JMS HX-110 (JEOL, Japan) equipped with an ESI ion source (Analytica of Branford, U.S.A.). The mass spectra were acquired with a mass resolution of 1000 for ESI. The instrument was calibrated in the first atom bombardment ionization mode using CsI as the calibrant. The acceleration voltage was 5 kV. About 300 pmol of purified samples were dissolved into 30 μL of methanol/water (3:1, vol/vol) containing 1% acetic acid, and the solution was infused into the ESI ion source at a flow rate of 1 μL/min by a Harvard syringe pump (Harvard Apparatus). Dry nitrogen was heated to 200 °C and introduced into the capillary region at a flow rate of 8 L/min. Mass spectra were processed with JEOL DA7000 data system.

Hypotensive Activity. Male Wister rats weighing 200−250 g were anesthetized with ketamine hydrochloride by im injection. Arterial blood pressure was measured by means of a polyethylene tubing inserted in the carotid artery and connected to a pressure transducer. The systolic and diastolic pressures were recorded on a Graphtec Linearcorder F, WR 3701 attached to a carrier Amplifier AP-621G (Nihon-Koden, Japan). The samples dissolved in saline were injected through another polyethylene tube connected to the jugular vein.

Vascular Permeability Assay (Miles Assay). Vascular permeability assay described by Miles and Wilhelm (*26*) was modified. Briefly, the dorsal hair on Wister rats weighing 300−350 g was depilated, and 0.2 mL of 1% Evans blue solution in saline was injected intravascularly. The samples dissolved in 50 μL of PBS were then injected intradermally at various concentrations in the depilated area. An increase in vascular permeability allows the Evans blue dye to move out of the capillaries into the surrounding tissues, causing a blue spot to appear.

Cell Culture and Bioassays. Bovine aortic endothelial cells (BAEC) were obtained by the method of Jaffe et al. (*27*) and maintained in Dulbecco's modified Eagle's medium (DMEM) containing fetal calf serum (10%, v/v), penicillin (100 units/mL), streptomycin (0.1 mg/mL) and amphotericin B (0.25 μg/mL). Frozen human aortic endothelial cells (HAEC), human umbilical vein endothelial cells (HUVEC), and human pulmonary artery endothelial cells (HPAEC) were cultured and maintained in commercially available media, HuMedia-EB2 supplemented with fetal calf serum (2%, v/v), hEGF (10 ng/mL), hFGF−B (5 ng/mL), hydrocortisone (1 μg/mL), heparin (10 μg/mL), gentamicin (50 μg/mL), and amphotericin B (50 ng/mL). Frozen human aortic smooth muscle cells (HASMC) were cultured and maintained in HuMedia-SG2 supplemented with fetal calf serum (5%, v/v), hEGF (0.5 ng/mL), hFGF−B (2 ng/mL), insulin (5 μg/mL), gentamicin (50 μg/mL), and amphotericin B (50 ng/mL). Frozen normal human epidermal keratinocyte (NHEK) were cultured and maintained in HuMedia-KG2 supplemented with hEGF (0.1 ng/mL), insulin (10 μg/mL), hydrocortisone (0.5 μg/mL), brain pituitary extract (4%, v/v), gentamicin (50 μg/mL), and amphotericin B (50 ng/mL). Frozen normal rabbit corneal epithelial cell (NRCE2) were cultured and maintained in RCGM2 supplemented with mEGF (10 ng/mL), insulin (5 μg/mL), hydrocortisone (0.5 μg/mL), brain pituitary extract (0.4%, v/v), gentamicin (50 μg/mL), and amphotericin B (50 ng/mL). At confluency, cells were trypsinized, washed with serum/supplements-free medium, and then resuspended in their respective growth media containing 1% fetal calf serum and antibiotics/antimycotics. For bioassays, these cells were seeded at a density of 5×10^3 cells/well in 0.2 mL of medium in 96-multiwell plates. Aliquots of fractions to be assayed were diluted in PBS and then added to the cells. After 5 days, cell densities were determined by the colorimetric method using a cell counting kit (product of DOJINDO, Japan) that was based on the tetrazolium salt/formazan system (*28*).

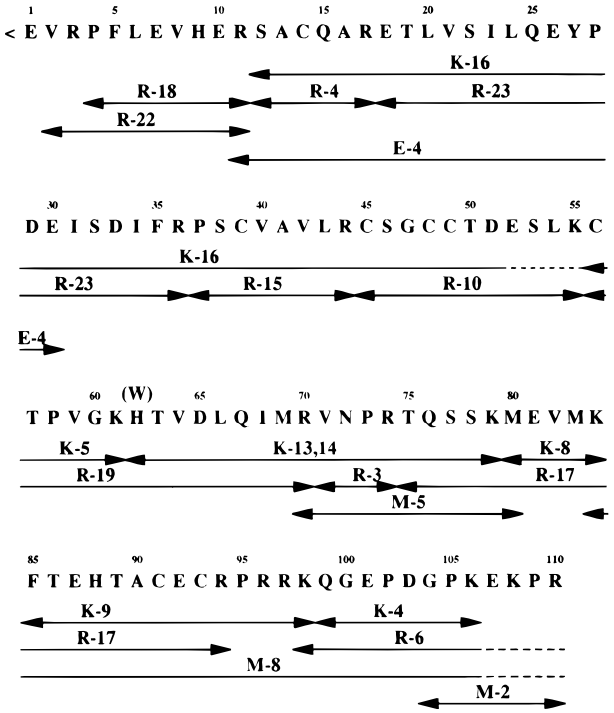

FIGURE 1: Primary structure of HF. Amino acid sequence of HF by Edman degradation. Horizontal arrows indicate residues determined by sequence analysis. The following abbreviations are used for the various peptides: K; lysylendopeptidase, R; clostripain, E; V8 protease, M; CNBr.

RESULTS

Amino Acid Sequence Analysis of HF. Previous report on the biochemical nature of HF showed that HF is a dimeric protein with an isoelectric point of ~7.95 and a molecular weight of 25 000 (*10*). The N-terminal amino acid of Pe-HF was not detected by Edman degradation. To determine the complete amino acid sequence, lysylendopeptidase digestion of Pe-HF was performed. The digest was fractionated by reversed-phase HPLC (data not shown), and the sequences of residues 12−51, 56−61, 62−79, 80−84, 85−98, and 99−106 were determined by direct sequencing of the peptides K-16, K-5, K-13,14, K-8, K-9, and K-4, respectively (Figure 1). The clostripain digestion produced peptides R-18, R-4, R-23, R-15, R-10, R-19, R-3, R-17, and R-6, which consisted of residues 4−11, 12−17, 18−36, 37−44, 45−55, 56−70, 71−74, 75−94, and 98−106, respectively. The N-terminal-blocked peptide R-22 obtained from clostripain digest was further incubated with pyroglutamylpeptidase. The amino acid sequence of deblocked peptide was determined as VRPFLEVHER, which was considered as residues 2−11.

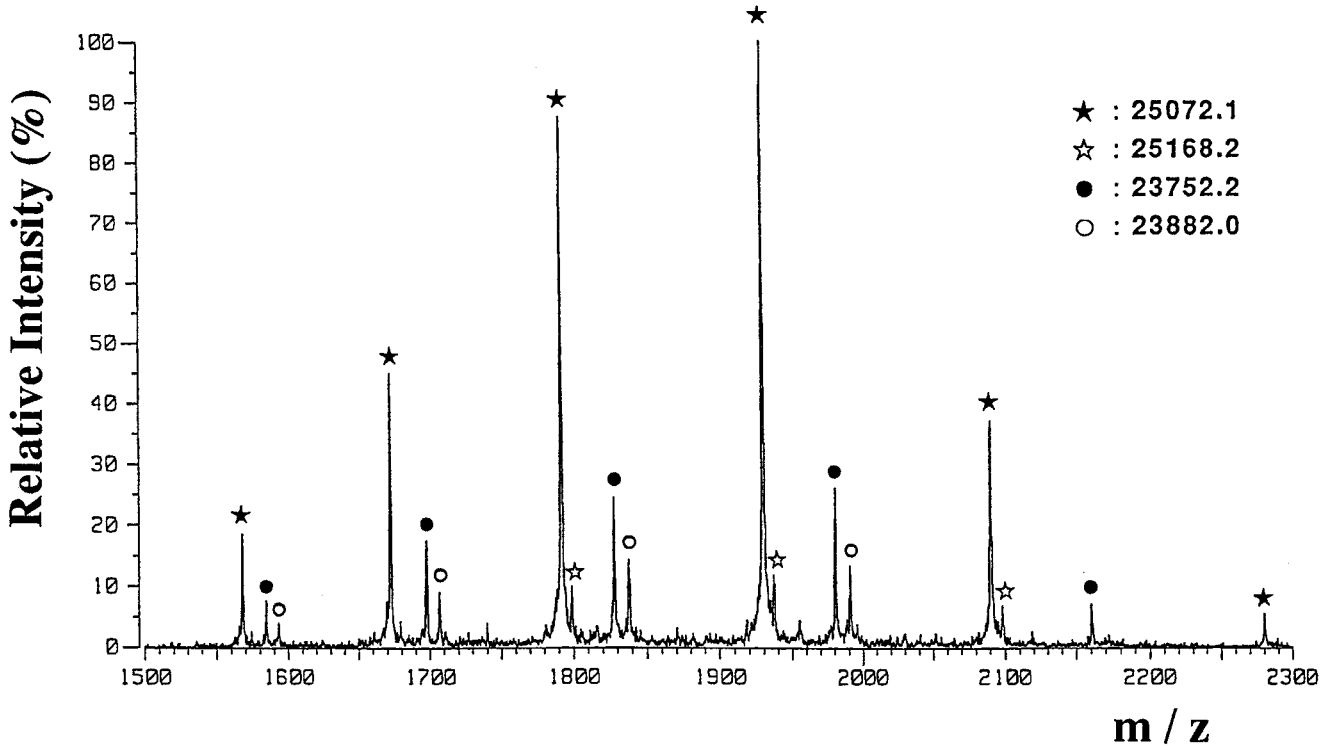

FIGURE 2: ESI-mass spectrometry of HF. The multiple charged ion spectra were deconvoluted on a real mass scale. ★: mol.wt. of the intact HF-dimer composed of 220 amino acids, ☆: mol. wt. of Trp-62 HF-dimer, ● and ○: mol. wts. of heterodimers composed of an intact HF-monomer and a digested HF-monomer. The digestion occurred at the position of Lys(98)-Gln(99) or Gln(99)-Gly(100), and each dimer consists of 208 and 209 amino acids, respectively.

The V8 protease digestion of Pe-HF resulted in Glu-C cleavage, producing peptides E−4 (residues 11−30). Chemical cleavage by CNBr resulted in peptides M-5, M-8, and M-2, which were determined to be residues 70−80, 84−106, and 104−110, respectively. On the process of sequence analysis of peptides K-13, K-14, and R-19, tryptophan residue was detected with histidine at the position 62. This indicates the possibility of existence of mutant protein Trp-62-HF.

Molecular Weight Determination of HF by ESI Mass Spectra. HF isolated by reversed-phase HPLC was lyophilized and the molecular mass was examined by ESI mass spectrometry. The spectrum of HF revealed the protonated molecular ion $[M+H]^+$ at *m*/*z* 25 072.1 (average data) giving the molecular weight of 25 071.1 (Figure 2). Since the sequence data (Figure 1) indicated the existence of eight cysteine residues in HF monomer, theoretical molecular weight of HF dimer was calculated in various cases by using the molecular weights of C (12.011), H (1.00794), N (14.0067), O (15.9994), and S (32.06). The model containing eight cystine bridges ($C_{1064}H_{1724}N_{324}O_{332}S_{22}$: mol. wt. 25 072.2) possessed the closest molecular weight to the ESI-MS data. On the spectrum, some minor ion peaks were also observed at *m*/*z* 25 168.2, 23 752.2 and 23 882.0 (Figure 2). Since the ion peak observed at *m*/*z* 25 168.2 matches with the theoretical molecular weight of mutant protein Trp-62-HF at 25 170.3, this ion peak may be derived from this mutant. Trypsin digestion of native HF for 24 h followed by the separation of digest on reversed-phase HPLC showed only two detectable peaks (data not shown) and these were identified as residues 99−106 (Figure 1, K-4) and 107−110. These data indicated that native HF is resistant against the protease digestion, except for the C-terminal of the protein. Theoretical molecular weight of heterodimers consisting of an intact HF-monomer and a C-terminal-deficient HF-monomer produced by the digestion at the position of Lys(98)-Gln(99) was calculated to be 23 880.6 ($C_{1013}H_{1642}N_{308}O_{315}S_{22}$) and that of an intact HF-monomer and C-terminal-deficient HF-monomer produced by the digestion at the position of Gln(99)-Gly(100) to be 23 752.6 ($C_{1008}H_{1634}N_{306}O_{313}S_{22}$). This suggests the possibility that the ion peaks detected at *m*/*z* 23 882.0 and 23 752.2 were derived from these heterodimers described above. Since the crude venom of *V. a. aspis* contains various serine-proteases (*29*−*31*), these phenomena might occur on the venom components during the purification.

Comparison of Primary Structure of HF with VEGF and PDGF. Homology search showed that the primary structure of HF has similarity in sequence with the vascular endothelial growth factor (VEGF) from human and bovine (*14*, *15*) (Figure 3). Platelet-derived growth factor (PDGF) (*32*−*34*) was also found to be a homologous protein to HF. The percentage of amino acid residues matched with HF were 44.5% for human $VEGF_{165}$, 45.5% for bovine VEGF, 20.0% for PDGF−B, and 23.6% for PDGF-A. It is remarkable that the positions of HF cysteine residues completely matched with these growth factors. Assignment of intramolecular disulfide bonds and interchain disulfide bonds between monomers of PDGF or VEGF has been performed by using a mutant protein and X-ray analysis (*35*−*37*). The data suggests that the second and fourth cysteine residues of PDGF or VEGF monomers are involved in interchain disulfide bonds, and the other cysteine residues form three intramolecular disulfide bonds between Cys(1)-Cys(6), Cys(3)-Cys(7), and Cys(5)-Cys(8) (Figure 3). These data indicate

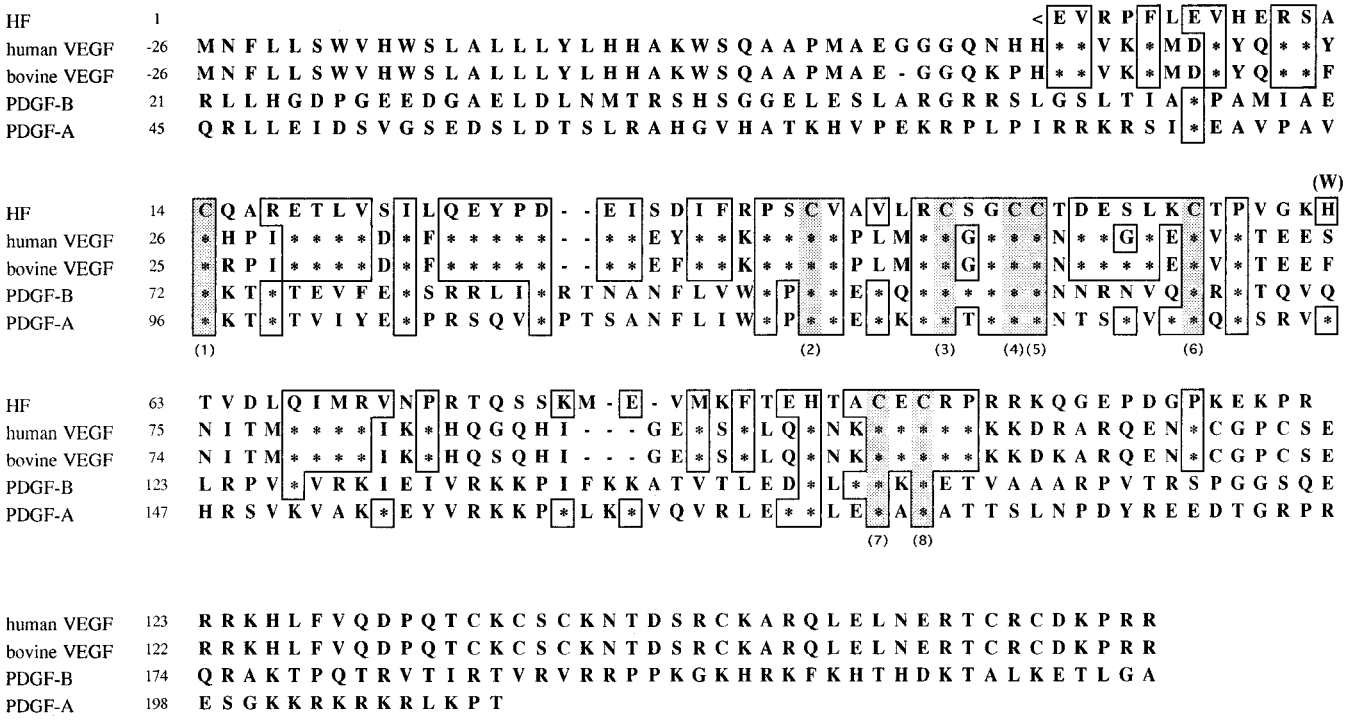

FIGURE 3: Comparison of primary structure of HF with growth factors. The sequences are aligned according to the position of Cys (numbered from one to eight). Only part of the sequences of PDGF-A and -B chains are shown, and identical residues with HF (*) were boxed.

that HF molecule forms disulfide bridges in a manner similar to VEGF and PDGF.

Heparin-Binding Ability of HF. The homologous primary structure of HF and VEGF indicated the possibility that the function of HF is also similar to VEGF. To compare the heparin-binding ability of HF with VEGF isoforms, a partially purified sample from Sephadex G-75 gel filtration and S−Sepharose ion-exchange columns purification was applied to a Heparin-Sepharose CL-6B column equilibrated with 5 mM Tris-HCl buffer, pH 7.2 containing 0.1 M NaCl. HF bound to the column, but was completely eluted with 0.3 M NaCl (Figure 4). Houck et al. (*19*) observed that $VEGF_{165}$ was eluted in the presence of 0.9 M NaCl, but $VEGF_{121}$ failed to bind the column. These experiments indicated that HF has a relatively weak affinity for heparin-Sepharose.

Effect of HF, $VEGF_{165}$, and PDGF on Rat Blood Pressure. Since HF has been reported as a potent hypotensive factor (*10*), the effect of growth factors on rat arterial blood pressure was tested. Injection of HF (0.25 μg/g) into the vein induced acute and continuous effect, persisting for ~100 min (Figure 5A). Administration of $VEGF_{165}$ at the same dose affected the blood pressure, but the effect was not comparable to HF (Figure 5B). Injections of PDGF-AA, -BB, and -AB caused little changes in blood pressure (Figure 5C-E). As previously reported (*10*), readministration of HF after the recovery of blood pressure did not cause hypotension on animals (Figure 5A, second arrow), while injections of HF into the VEGF/PDGF-treated animals lowered blood pressure (Figure 5B-E, second arrow). Although HF and VEGF/PDGF are similar in structure, the hypotensive effect is shown only by HF.

Effect of HF and $VEGF_{165}$ on Vascular Permeability. Since VEGF was first reported as a vascular permeability factor (*11*) and the lowest dose of VPF producing a positive response on guinea pig was 20 ng (*16*), the effect of HF on

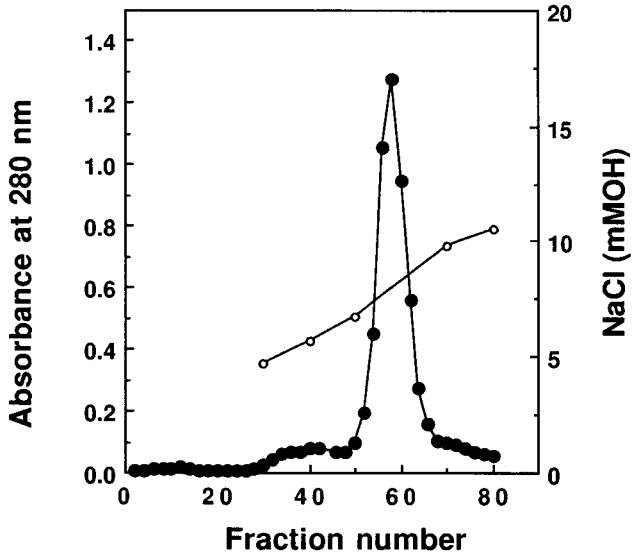

FIGURE 4: The affinity of HF for heparin−Sepharose CL-6B column. Partially purified hypotensive factor from Sephadex G-75 and S−Sepharose columns was applied to a heparin−Sepharose CL-6B column (1 × 20 cm) equilibrated with 5 mM Tris-HCl buffer (pH 7.2), containing 0.1 M NaCl. Elution was performed with a linear gradient from 0.1 to 0.3 M NaCl in a total volume of 200 mL at a flow rate of 15.0 mL/hr.

vascular permeability was further examined. As shown in Figure 6, 5 ng of HF clearly increased permeability in a rat. Spot size caused with the same amount of HF and $VEGF_{165}$ were similar.

Effect of HF on the Growth of Endothelial Cells. The bioactivity of HF was also tested with different cell types. As shown in Figure 7A, apparent effects on the cell growth were observed only in cells of vascular endothelial origin with 10 nM of HF. The percentage of increase in cell growth were approximately 150 to 210% against the control. In contrast, smooth muscle cells, epidermal keratinocytes and

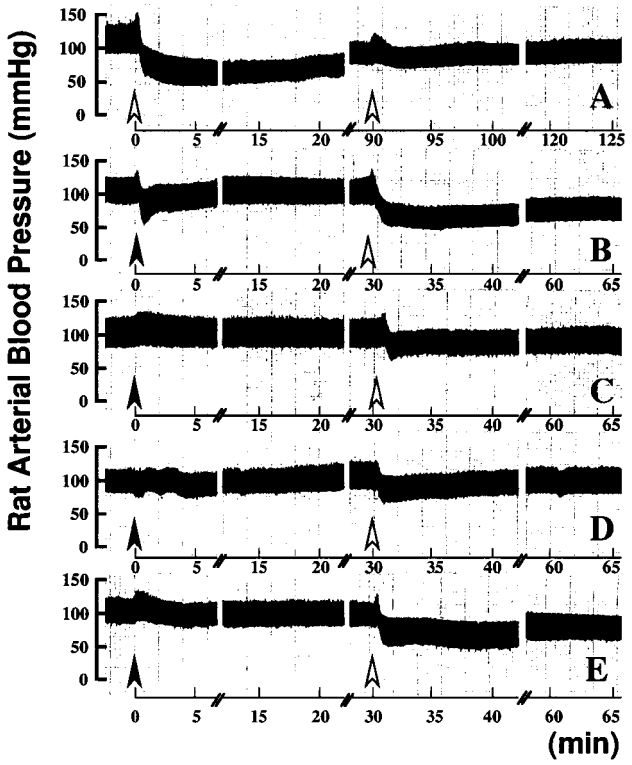

FIGURE 5: Effect of intravenous injections of HF and growth factors on rat arterial blood pressure. Each factor was injected into the jugular vein at a dose of 0.25 μg/g at indicated time points. The figures under the physiograph tracing denote the time after the administration of HF (white arrow) or growth factors (black arrow). (A) A typical hypotension observed after an iv administration of HF. Effect of growth factors on rat blood pressure is shown in (B) $VEGF_{165}$, (C) PDGF-AA, (D) PDGF−BB, and (E) PDGF-AB.

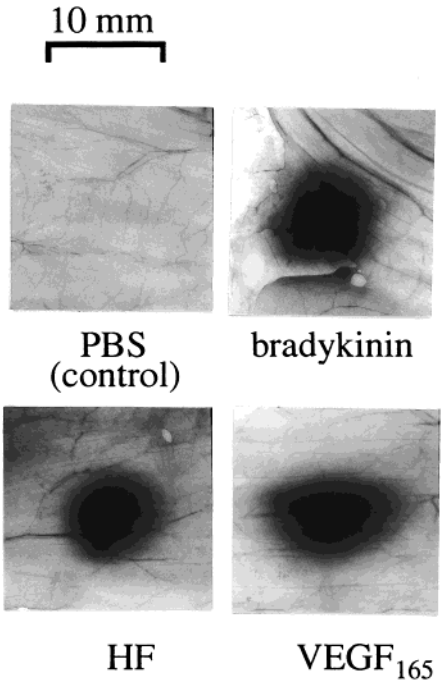

FIGURE 6: Miles assay of purified HF and $VEGF_{165}$. Samples (50 μL) dissolved in PBS (0.1 μg/mL) were injected intradermally into rat. Blue spots of Evans blue were observed 3 to 5 min after the injections, indicating the leakage of dye from the plasma into the skin. Bradykinin (5 ng) was injected as a control.

epithelial cells failed to show any significant mitogenic response with ~500 nM of HF. The mitogenic effect of HF was further examined with BAEC. The dose−response curve for HF is compared with $VEGF_{165}$ in Figure 7B. The half-maximal effect was observed with 5−5.5 nM (125−138 ng/mL) of HF, while human $VEGF_{165}$ possessed similar effect on BAEC with 0.5−1 nM.

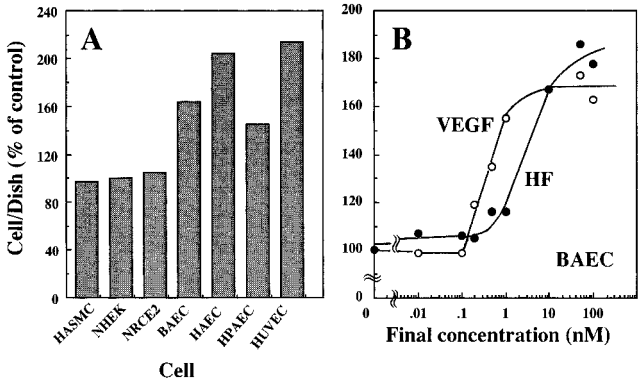

FIGURE 7: Mitogenic effect of HF. Cells were seeded at the density of 1.5×10^3/well in their respective growth media in 96 well plates as described in Experimental Procedures. (A) Effect of HF on the growth of various cell types. Cells were incubated with HF (10 nM at the final concentration) for 5 days. (B) Dose-dependent growth of bovine aortic endothelial cell in the presence of HF or $VEGF_{165}$. The indicated amounts of HF or $VEGF_{165}$ were added 1 h after plating in a 10 μL medium. After 5 days, cells were counted by the colorimetric method. The results shown represent the average of 6 experiments and the percentage of control.

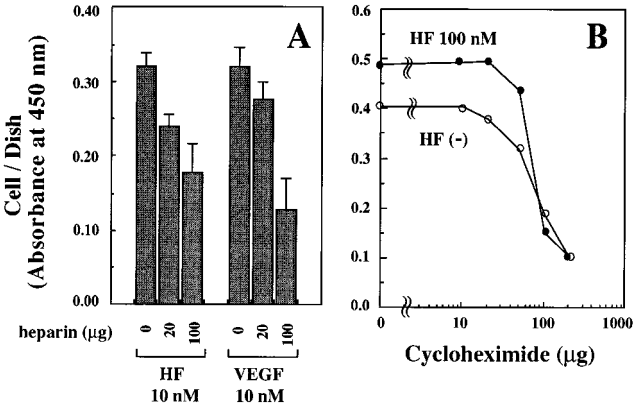

FIGURE 8: Inhibitory effect of heparin and cycloheximide on HF-mediated endothelial cell growth. BAEC were seeded at the density of 5×10^3/well in 96 well plates. The indicated amounts of (A) heparin or (B) cycloheximide were added with HF or $VEGF_{165}$ simultaneously into the culture medium (total 0.22 mL). The results shown represent the average of 3 experiments.

Inhibitory Effect of Heparin and Cycloheximide on Cell Growth. The effects of heparin and cycloheximide, a protein synthesis inhibitor, on the HF-mediated BAEC growth were tested. As shown in Figure 8A, cell growth mediated by 10 nM of HF and $VEGF_{165}$ was inhibited in the presence of heparin with the similar manner. The cell number was approximately 40−60% with 100 μg (450 μg/mL) of heparin. The effect of cycloheximide on BAEC growth was examined in the presence or absence of 100 nM HF, and the IC_{50} was determined to be 60 and 80 μg, respectively (Figure 8B).

DISCUSSION

In this study, we investigated the primary structure of HF purified from *Viperidae* snake venom gland and compared it with VEGF. Although VEGF is secreted from mammalian

cells, both proteins are homologous with the percentage of matched amino acid residues of approximately 45%. The position of eight cysteine residues in HF-monomer also matched with that of VEGF (Figure 3). The molecular weight of HF obtained by ESI-MS supports the existence of eight cystine bridges in HF-dimer. Since intact HF-dimer was resistant against trypsin digestion, identification of disulfide bridges could not be performed. However, the data described above indicates that the folding manner of HF-monomer might be similar to that of VEGF (*37*), and HF-dimer is presumably formed by two intermolecule disulfide bridges.

The ESI mass spectra indicated the existence of three minor molecular species in purified HF fraction (Figure 2). From the sequence data, one of them is predicted to be a mutant protein Trp-62-HF. Other two molecules were speculated to be heterodimers of HF-monomer and C-terminal-deficient HF-monomer. Unlike VEGF, which heterogeneity arised from an alternative splicing of RNA transcript, C-terminal-deficient HF-monomers might be proteolytic products of venom proteases during the process of purification or storage. DNA sequencing would be necessary to clarify the heterogeneity of HF.

Since various growth factors possess heparin-binding ability, chromatographic behavior of HF on heparin-Sepharose column was tested. Houck et al. (*19*) reported that the longer forms of VEGF possess higher affinity for heparin, and $VEGF_{121}$ does not bind to heparin−Sepharose. $VEGF_{165}$ and $VEGF_{189}$ strongly bound to the column and were eluted in the presence of 0.9 and 2.0 M NaCl, respectively. Plasmin digestion of $VEGF_{165}$ and $VEGF_{189}$ released the C-terminal basic peptides and, therefore, decreased the heparin-binding ability of these isoforms. However, this step did not diminish their mitogenic activity. When compared with $VEGF_{165}$ and $VEGF_{189}$, HF possesses a weak affinity for heparin−Sepharose and was eluted with 0.2 M NaCl (Figure 4). HF also weakly bound to S−Sepharose and its isoelectric point was determined to be 7.95 (*10*). The smaller protein HF (110 amino acids for a monomer), which possesses sequence homology to N-terminal region of $VEGF_{165}$ only (Figure 3), might account for the weak basic nature and low heparin-binding ability. As shown in Figure 8A, mitogenic activity of HF on BAEC was inhibited in the presence of high concentrations (100∼500 μg/mL) of heparin. This result coincides with the previous report (*38*), showing that the binding of VEGF to cell surface receptors was affected by excess amount of heparin. Two receptor-type tyrosine kinases (Flt-1 and KDR/Flk-1) had been identified as VEGF receptors (*20*, *21*), and the role of heparin (or heparan sulfate proteoglycan) in the binding of VEGF to its receptor has been discussed (*39*−*41*). However, HF receptor on endothelial cells has not been identified, the homologous structure and similar binding effects of heparin suggest the possibility that HF shares the same receptor with VEGF. Preliminary studies performed with HF and VEGF antibodies, which demonstrate partial cross reactivity (unpublished data), may support the speculation. Detailed study for HF receptors would be necessary.

It is obvious that HF possesses mitogenic effect on various endothelial cells, but the effective concentrations are 5 to 10 times higher than that of VEGF (Figure 7B). The mechanisms of VEGF to generate mitotic signals are not clearly understood. However, recent report by Takahashi and Shibuya (*42*) indicated that an activation of PLCγ-PKC and MAP kinase is involved in the processing and signal transduction of KDR/Flk-1. Since the mitogenic activity of HF was inhibited by cycloheximide, it is supeculated that HF also generates some signals and increases protein synthesis in endothelial cells.

The angiogenesis cascade occurred by growth factors such as bFGF and VEGF involves induction of tissue-type and urokinase-type plasminogen activator (tPA and uPA) and generation of plasmin (*43*, *44*). Plasmin plays an important role in the regulation of VEGF bioavailability by releasing $VEGF_{189}$ and $VEGF_{206}$ from stably incorporated extracellular matrix (*19*), and activation of procollagenase (*22*). Plasmin and collagenase are able to degrade laminin and collagen (type I and III), which are important components of extracellular matrix and basement membrane. Although the mechanism of increasing vascular permeability by VEGF has not been clarified, these proteolytic effect may cause hyperpermeability of the capillaries. Increase in vascular permeability and hypotension by snake envenomation are usually results from kallikrein-like enzyme action that releases kinin(s) from circulatory kininogen (*3*, *4*); however, HF did not possess any enzyme activities. Bradykinin-potentiating peptide (ACEI; angiotensin converting enzyme inhibitor) is another venom component known to cause hyperpermeability of blood vessels and a hypotensive response by inhibition of the conversion of angiotensin I to hypertensive angiotensin II (*6*−*9*). Although HF possessed depressive effect on rat blood pressure, its structure was entirely different from ACEI. These data indicate that HF is a new venom component and its mechanism of action has not been reported. On Miles assay, 5 ng each of HF, VEGF, and PDGF (data not shown) caused similar effect, while continuous hypotensive effect on rat arterial blood pressure was a characteristic of HF (Figure 5). Although HF and VEGF/PDGF have similar structures and behave as mitogenic agents on vascular endothelial cells, some biological activity might be different.

Angiogenesis is an important process for cardiovascular biology and medicine, and various growth factors have been reported as potential regulators. VEGF, a member of PDGF family, is a well-studied growth factor that acts on various steps of angiogenesis (*22*). In this report, we showed various similarities between HF and VEGF. More detailed study of HF receptor system will prove that HF is a new member of PDGF family.

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