Isolation and Characterization of Vasohibin-2 as a Homologue of VEGF-Inducible Endothelium-Derived Angiogenesis Inhibitor Vasohibin

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Objective—We recently isolated vasohibin, a novel vascular endothelial growth factor (VEGF)-inducible endothelium-derived angiogenesis inhibitor. Our aim is to find DNA sequences homologous to vasohibin and determine their expression profile.

Methods and Results—By the search of DNA sequences in the database, we found one homologous gene and designated it vasohibin-2. Overall amino acid sequence homology between the prototype vasohibin (vasohibin-1) and vasohibin-2 was >50%. Vasohibin-2 exhibited antiangiogenic activity. Vasohibin-2 expression in cultured endothelial cells was low and not inducible by the stimulation that induced vasohibin-1. However, the immunohistochemical analysis revealed that vasohibin-1 and -2 were diffusely expressed in endothelial cells in embryonic organs during mid-gestation. After that time point, vasohibin-1 and -2 became faint, but persisted to a certain extent in arterial endothelial cells from late gestation to neonate. Expression of vasohibin-1 and -2 could be augmented in vivo by local transfection with the VEGF gene in the embryonic brain or by cutaneous wounding in adult mice.

Conclusion—These results suggest that vasohibin-2, in combination with vasohibin-1, forms a novel family of angiogenesis inhibitors. (Arterioscler Thromb Vasc Biol. 2006;26:1051-1057.)

Key Words: angiogenesis inhibitor ■ endothelial cells ■ vascular development ■ vasohibin

Angiogenesis, the formation of new blood vessels from pre-existing vessels, is essential for normal development but is also involved in various pathophysiological conditions. Although angiogenesis is thought to be controlled by the local balance between pro-angiogenic factors and angiogenesis inhibitors, little is known about the negative feedback regulation of angiogenesis. We recently isolated a gene for a candidate negative feedback angiogenesis regulator that was preferentially expressed in endothelial cells (ECs), and designated vasohibin. Vasohibin was isolated from a cDNA microarray analysis to search for vascular endothelial growth factor (VEGF)-inducible genes in human umbilical vein endothelial cells (HUVECs).

Generally, growth factors and cytokines belong to the families composed of homologous proteins. There are a number of examples of this among angiogenesis regulators, e.g., VEGF family members that play essential roles in angiogenesis or lymphangiogenesis; angiopoietin family members that regulate vascular stability as agonists or antagonists of the tyrosine kinase with Ig and endothelial growth factor (EGF) homology domains (TIE)-2 receptor; fibroblast growth factor (FGF) family members, a large group that plays important roles in angiogenesis as well as in other areas; and thrombospondin family members that participate in various functions including inhibition of angiogenesis.

Based on the sequence of the prototype vasohibin (vasohibin-1), we searched the NCBI database for homologous DNA sequences. Here we report one vasohibin-1 parologue in mammals with antiangiogenic activity, which we have designated vasohibin-2. A number of other angiogenesis inhibitors have already been reported, such as angiotatin, endostatin, 16 kDa prolactin, pigment epithelium-derived factor, thrombospondins, PF-4, and IP-10. In terms of origin, vasohibin-1 is intrinsic to ECs, whereas most of other angiogenesis inhibitors are extrinsic to ECs. Therefore, we examined whether vasohibin-2 shared this property with vasohibin-1. Our analysis revealed that...
both vasohibin-1 and -2 were expressed in ECs. Thus, we propose that vasohibin-2 forms part of an inhibitory angiogenesis regulation system in combination with vasohibin-1.

**Materials and Methods**

**Cell Culture**
HUVECs and human aortic endothelial cells were obtained from KURABO and were routinely cultured on type I collagen-coated dishes (IWAKI) in endothelial basal medium containing endothelial cell growth supplements and 10% fetal bovine serum.

**Reverse Transcriptase-Polymerase Chain Reaction**

**Analysis and Cloning of Human Vasohibin-2 cDNA**
Total RNA was extracted from HUVECs by an acid guanidinium-phenol-chloroform method using ISOGEN (Nippon Gene, Toyama, Japan). First-strand cDNA was generated from total RNA using a 1st Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany). Polymerase chain reaction (PCR) was performed in DNA thermal cycler (TAKARA, Tokyo, Japan) with 5 U TaqDNA polymerase per 100 µL reaction mixture. For cloning and quantification of human vasohibin-2, the primer pair 5'-GCAGCATGAAGGCCAAA-CGGT-3' (sense, Exon 1) and 5'-CTTCCTGGGCGATATGGG-3' (antisense, Exon 8) was synthesized. For quantification of human vasohibin-1, primer pairs for human vasohibin-1 (sense; 5'-AGATCCCCATACCGAGTGTG-3' and antisense; 5'-GGGCTTCTT-TGGTCATTTCC-3'), and human β-actin (sense; 5'-ACAATGAGCTGCGTGTGGCT-3' and antisense; 5'-TCTCCTTAAA-T GTCAAGCCAGA-3') were synthesized. PCR products were cloned into PCR2.1 TOPO vector (Invitrogen, Carlsbad, Calif) and directly sequencing using a DNA sequencing kit (Applied Biosystems, Foster City, Calif) and an ABI Prism 310 DNA sequencer (Applied Biosystems).

**Preparation of Recombinant Vasohibin-1 and Vasohibin-2 Proteins**
Recombinant vasohibin-1 protein was described previously. For the preparation of vasohibin-2, we cloned the cDNA of an N-terminal-truncated form of vasohibin-2 (66–355) into the expression vector p3xFLAG-CMV14 (Sigma, St. Louis, Mo). The FLAG-tagged vasohibin-2 protein was expressed using the Bac-To-Bac Baculovirus expression system (Invitrogen). We purified the protein using an A-agarose affinity chromatography (MAPS II kit, Bio-Rad Laboratories, Hercules, Calif).

**Mouse Corneal Micropocket Assay**
The mouse corneal micropocket assay was performed as described previously. Briefly, 0.3 µm hydroden pellets (IFN Sciences, New Brunswick, NJ) containing vehicle or 80 ng FGF-2 were implanted into the corneas of male BALB/c mice under pentobarbital anesthesia. A total of 5 ng vasohibin-1 or vasohibin-2 protein was added to the pellets. After 7 days, the corneal vessels were photographed, and neovascular area (mm²) was determined using the software package National Institutes of Health (NIH) Image.

**Network Formation by HUVECs**
Ice-cold growth factor reduced Matrigel was poured into a 35-mm dish and allowed to gel for 1 hour at 37°C. Thereafter, HUVECs (5×10⁴) were plated onto Matrigel and incubated with the vasohibin-2 (10 nM) or vehicle (20 mmol/L Tris/HCl, 0.1% NP40, 0.1 mol/L Glycine, pH 7.0) in medium199 (M-199) containing 5% fetal bovine serum. After 9-hour incubation, cells were photographed.

**Preparation of Antibodies**
Anti-human vasohibin-2 monoclonal antibody (mAb) was established using a human vasohibin-2 peptide (Ser206–Lys218) and keyhole limpet hemocyanin conjugate as described previously. The resultant mAb 5E3 (IgG1, subclass κ) was purified by protein A-agarose affinity chromatography (MAPS II kit, Bio-Rad Laboratories Inc, Hercules, Calif).

Anti-mouse vasohibin-1 and -2 polyclonal antibodies (Abs) were prepared using Cys-mouse vasohibin-1 peptide (Gly296–Arg309) or Cys-mouse vasohibin-2 peptide (Leu230–Arg242) conjugated with maleimide-activated keyhole limpet hemocyanin. Rabbits were immunized subcutaneously with mouse vasohibin-1 or mouse vasohibin-2 peptide conjugates. The first injection consisted of 200 µg of conjugate in an equal volume of Freund’s complete adjuvant. The rabbits were boosted with 100 µg conjugate in equal volumes of incomplete Freund’s adjuvant.

**Northern Blot Analysis**
Human embryonic message HUNTER was obtained from Geno-Technology (St. Louis, Mo). Northern blotting was performed as described previously. Briefly, the membranes were hybridized with 3P-labeled human vasohibin-2 cDNA probe in hybridization solution at 42°C for 24 hours. After hybridization, membranes were washed once with 0.1% SDS in 2× standard saline citrate (SSC) at room temperature and then 3 times with 0.1% SDS in 0.2× SSC at 65°C. Autoradiography was performed using an imaging plate and the results analyzed by a FLA2000 Image Analyzer (Fuji Film, Tokyo, Japan).

**Immunohistochemical Analysis**
Human tissue specimens were obtained after obtaining informed consent and ethical committee approval from Tohoku University. Specimens were fixed in 10% formalin, embedded in paraffin, and cut into 3-µm-thick sections. Sections were dewaxed in xylene, rehydrated in a graded ethanol series (100%, 90%, 80%, and 70%), and bleached in 0.3% H₂O₂/ethanol. Sections were then incubated in 0.1% Trypsin/0.05 mol/L Tris buffer (pH 7.6) for 30 minutes at 37°C for PECAM-1 staining or incubated in citrate buffer (pH 6.0) for 5 minutes at 120°C for vasohibin-1 and vasohibin-2 staining. Sections were blocked with 1% bovine serum albumin (BSA)/PBS for 30 minutes at room temperature. Primary antibody reactions were performed overnight at 4°C at a dilution of 1:20 for anti-human PECAM-1 mAb (Dako Cytomation, Kyoto, Japan), and 1:400 for anti-human vasohibin-1 mAb and anti-human vasohibin-2 mAb. For all specimens, secondary antibody reactions were performed using biotinylated goat anti-mouse Ab (Vector Laboratories Inc., Burlingame, Calif) at a dilution of 1:500 for 30 minutes at room temperature. Vectastain ABC kit (Vector Laboratories Inc) was used according to the manufacturer’s instructions. Sections were visualized using 3,3'-Diaminobenzidine (DAB) (Sigma)/0.03% H₂O₂ in 0.05 mol/L Tris buffer (pH 7.6) and counterstained with hematoxylin.

All the animal studies were approved by the committee for animal study in our institute. Mouse tissues were fixed in 4% paraformaldehyde/PBS overnight at 4°C, dehydrated in a graded ethanol series (70%, 80%, 90%, and 100%) and xylene, and embedded in paraffin. Sections were dewaxed, rehydrated, and bleached as described. For PECAM-1 and vasohibin-2 staining, sections were incubated in Proteinase K (Dako Cytomation) at room temperature for 5 minutes. For vasohibin-1 and von Willebrand factor (vWF) staining, sections were incubated in citrate buffer (pH 6.0) for 5 minutes at 120°C. Primary antibody reactions were performed at a dilution of 1:20 for rat anti-mouse PECAM-1 mAb (Research Diagnosis Inc.), 1:400 for rabbit anti-mouse vasohibin-1 Ab and rabbit anti-mouse vasohibin-2 Ab, or 1:100 for rabbit anti-human vWF Ab (Dako Cytomation) overnight at 4°C. Secondary antibody reactions were performed at a dilution of 1:500 for PECAM-1 (biotinylated goat anti-rat Ab, Vector Laboratories Inc.), or 1:500 for vasohibin-1, vasohibin-2, and vWF (biotinylated goat anti-rabbit Ab, Vector Laboratories Inc) at room temperature for 30 minutes.

**Quantitative Real-Time Reverse-Transcriptase PCR**
Total RNA was extracted using ISOGEN. First-strand cDNA was generated using a first-strand cDNA synthesis kit for reverse-transcriptase (RT)-PCR. Quantitative real-time RT-PCR was per-
formed using a Light Cycler System (Roche Diagnostics Corp). PCR conditions consisted of an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of 15 seconds at 95°C, 5 seconds at an annealing temperature (as noted), and 15 seconds at 72°C. The primer pairs used were: mouse β-actin forward 5’-TGGAGTGAGGAGGACAGCAT and reverse 5’-ACGGGTCAGCCAGCTTGG; mouse vasohibin-1 forward 5’-AGGAGGATGAAAGTAG (annealing temperature 68°C); mouse vasohibin-2 forward 5’-GGGAGCAGTCCTCCTTGAG (annealing temperature 68°C); and mouse VEGF forward 5’-AGCACAGAGAGATGAGGAAC and reverse 5’-CGTCGTCGGCTGCTGGAAG (annealing temperature 68°C); mouse vasohibin-2 forward 5’-GCCCGCTGAGCCTCGCCAC and reverse 5’-CATGGACAACCTGTAGTITG (annealing temperature 68°C); and mouse VEGF forward 5’-TCGTCTCTCTTTGTTGCACT and reverse 5’-TCCGGTGAAGAGGTGTG (annealing temperature 66°C). Each mRNA level was measured as a fluorescent signal corrected according to the signal for β-actin.

**VEGF Plasmid Construction**

Total RNA was extracted from OP9, a murine bone marrow-derived stromal cell line, using ISOGEN. First-strand cDNA was derived from total RNA using a 1st Strand cDNA Synthesis Kit for RT-PCR. Primers were designed to amplify full-length mouse VEGF164 (sense ATGAACTTTCTGCTCTCTCTTG, antisense: 5’-AGCACAGAGAGATGAGGAAC and reverse 5’-CGTCGTCGGCTGCTGGAAG; mouse vasohibin-1 forward 5’-GGGAGCAGTCCTCCTTGAG (annealing temperature 68°C); mouse vasohibin-2 forward 5’-GGGAGCAGTCCTCCTTGAG (annealing temperature 68°C); and mouse VEGF forward 5’-TCGTCTCTCTTTGTTGCACT and reverse 5’-TCCGGTGAAGAGGTGTG (annealing temperature 66°C). Each mRNA level was measured as a fluorescent signal corrected according to the signal for β-actin.

**Mouse Whole Embryo Culture**

Mouse whole embryo culture and electroporation were performed according to the method described by Osumi and Inoue. Briefly, time-pregnant Institute of Cancer Research mice at embryonic day 10.5 (E10.5) were euthanized. Embryos were freed from the uterine wall, deciduas, and Reichert’s membrane. The yolk sac and chorionicallantoic placenta were left intact. Embryos were then transferred into culture bottles that contained 2.5 mL 100% rat serum (Charles River, Yokohama, Japan), 2 mg/mL glucose, and penicillin/streptomycin solution (Invitrogen, Carlsbad, Calif), and then inserted into the pCAX expression vector containing the chicken β-actin promoter and CMV-IE enhancer (a generous gift from Dr N. Osumi, Tohoku University Postgraduate School of Medicine).

**Mouse Wound Healing Model**

The back of the mouse was shaved and disinfected with 70% ethanol. A 6-mm-diameter circle was drawn on the skin of the mid-dorsal region and a full-thickness wound created by excision of the area with curved scissors. After 5 days, mice were euthanized and the skin obtained to determine vasohibin-1 and -2 expression.

**Calculations and Statistical Analysis**

The statistical significance of differences was evaluated by unpaired analysis of variance (ANOVA), and probability values were calculated by Student t test. P<0.05 was considered statistically significant.

**Results**

**Isolation of Vasohibin Homologue**

Searching the NCBI database for DNA sequences homologous to human vasohibin-1 revealed the gene FLJ112505 and related cDNAs. FLJ112505 was composed of 8 exons and 7 introns located on human chromosome 1q32.3 (Figure 1A). We designated this gene as vasohibin-2. The related cDNAs appeared to be alternative splicing forms of FLJ112505 (transcript 1: AK022567, transcript 2: BC051856) (Figure 1A). Transcripts 1 and 2 differed by the lack of ∼200 bp in the mid region of exon 2 in transcript 1, and the lack of exon 4 and exon 5 in transcript 2. For transcript 1, the missing 200 bp of exon 2 resulted in an altered translation start codon, such that its open reading frame started ∼200 bp downstream from that of transcript 2 (Figure 1A). Because vasohibin-1 was isolated as a VEGF-inducible gene in HUVECs, we investigated whether vasohibin-2 transcripts were also present in HUVECs. We designed PCR primer pairs that amplify both transcripts 1 and 2 (Figure 1A). Theoretically, transcripts 1 and 2 would amplify 1196 and 1189 bp RT-PCR products, respectively. However, RT-PCR revealed only a single transcript of 1300 bp in HUVECs (Figure 1B). Direct sequencing showed that this RT-PCR product was composed of 8 exons including a complete exon 2 (Figure 1A). We entered this cDNA sequence into the NCBI database as AY834202. Of the 3 possible alternative splicing forms of vasohibin-2, transcript 3 exhibited the highest similarity to vasohibin-1. The deduced amino acid sequence of transcript 3 compared with that of vasohibin-1 with asterisks indicating identical amino acids (Figure I, available online at http://atvb.ahajournals.org). Based on the human vasohibin-2 cDNA sequence, we identified a mouse orthologue of vasohibin-2 located on chromosome 1H6. The human and mouse vasohibin family members and the percent identity between the sequences are summarized (Table I, available online at http://atvb.ahajournals.org).
Antiangiogenic Activity of Vasohibin-2

We performed a mouse corneal micropocket assay to examine whether vasohibin-2 exhibited antiangiogenic activity. Vasohibin-2 protein inhibited angiogenesis in the cornea (Figure 2A). Like vasohibin-1, vasohibin-2 protein alone had no effect on the cornea (data not shown). Quantitative analysis revealed that the antiangiogenic activity of vasohibin-2 was significant and almost equivalent to that of vasohibin-1 (Figure 2B). To examine whether vasohibin-2 acts directly on ECs, we plated HUVECs on Matrigel and treated them with vasohibin-2. Vasohibin-2 apparently inhibited network formation by HUVECs (Figure 2C).

Expression of Vasohibin-1 and Vasohibin-2

We analyzed vasohibin-2 expression. RT-PCR analysis showed that basal expression of vasohibin-2 was very low in both HUVECs and human aortic endothelial cells, and was not inducible by stimulators that induced vasohibin-1 (Figure 3A). We then characterized the mRNA expression in vivo. We have previously shown that vasohibin-1 expression is abundant in human embryonic tissues. Northern blotting revealed vasohibin-2 mRNA expression in various human embryonic organs at 6 to 12 embryonic weeks (Figure 3B). Thus, the expression of vasohibin-2 mRNA was evident in human embryonic tissues.

We compared mRNA levels of vasohibin-1 and -2 in mice embryos at various gestational periods by real-time RT-PCR. When standardized with respect to β-actin, expression of vasohibin-2 was twice that of vasohibin-1 (Figure 3C), with both showing peak levels at E14.5. In contrast to vasohibin-1 and -2, VEGF expression reached maximum levels by E14.5 but showed sustained expression to E18.5 (data not shown).

Vasohibin-1 and Vasohibin-2 Protein Localization In Vivo

We next investigated the localization of vasohibin-1 and -2 proteins in vivo. We confirmed the specificity of our mAbs and revealed that both proteins were selectively expressed in PECAM-1–positive ECs (Figure II, available online at http://atvb.ahajournals.org). We then analyzed various human em-
Development of vasohibin-2

Vasohibin-2 was expressed in developing embryonic organs. We performed immunohistochemical analysis of mouse embryonic tissues. We confirmed the specificity of our anti-mouse vasohibin-1 and -2 polyclonal Abs and found that mouse vasohibin-1 and -2 proteins were present in ECs (Figure IV, available online at http://atvb.ahajournals.org). Vasohibin-1 and -2 proteins were diffusely present in ECs at mid-gestation (E10.5) (Figure 5A to 5C) but became faint thereafter (E16.5) except in arterial ECs (Figure V, available online at http://atvb.ahajournals.org).

Inducible Expression of Vasohibin-1 and Vasohibin-2 In Vivo

Vasohibin-2 expression was very low and not inducible in vitro, vasohibin-2 was clearly expressed in ECs of developing embryonic organs. We therefore examined the inducibility of vasohibin-2 in mice using a whole mouse embryo culture system. Genes for VEGF or GFP were transfected into mouse embryonic brains. Twenty hours after the transfection, neovessels were evident in brains transfected with VEGF but not with GFP (Figure 6A and 6B). We determined mRNA levels of vasohibin-1 and -2 in the brain by real-time RT-PCR. Results showed the increase of vasohibin-1 and -2 mRNA in VEGF-transfected brain (Figure 6C). We also applied a postnatal mouse skin wound healing model. Granulation became evident at 5 days after injury, at which point the mRNA expression of both vasohibin-1 and -2 was augmented (Figure VI, available online at http://atvb.ahajournals.org). Immunohistochemical analyses revealed that vasohibin-1 and -2 proteins were present in ECs of the granulation tissue (Figure VI).

Discussion

Here we identified a gene that showed significant homology to the vasohibin-1 gene and termed it vasohibin-2. Whereas alternative splicing variants of this gene have already been entered into the database, our present study identified a novel transcript with 8 complete exons. This transcript, termed transcript 3, was the only vasohibin-2 transcript detected in normal tissues by our hands (data not shown), and was also
the transcript that showed the highest homology to vasohibin-1. Thus, although the significance of other transcripts remains unclear, we concluded that transcript 3 represented the principal isoform of vasohibin-2.

Similar to vasohibin-1, we could not identify any classical signal sequences or known functional motifs in the primary structure of vasohibin-2. Nevertheless, vasohibin-2 protein was present in the culture medium when COS7 cells were transfected with this gene (data not shown). Similar to vasohibin-1, vasohibin-2 protein inhibited network formation of HUVECs (Figure 2C), but it did not exhibit any effect on the migration of human smooth muscle cells (data not shown). Moreover, vasohibin-2 protein inhibited angiogenesis with a potency equivalent to that of vasohibin-1 (Figure 2B). We therefore propose that vasohibin-1 and -2 form a novel family of endogenous angiogenesis inhibitors. It is not known whether or not vasohibin-1 and -2 share the common signaling pathway for angiogenesis inhibition. Identification of the receptors for vasohibin-1 and -2 in the future should answer this question.

Although vasohibin-2 expression was very low and was not inducible in vitro, vasohibin-2 was clearly expressed in vivo. The reason for this discrepancy remains unclear. It is possible that as yet unknown factor(s) present in vivo are required to induce vasohibin-2 in ECs. Nonetheless, the spatial and temporal expression pattern of vasohibin-2 in vivo was akin to that of vasohibin-1. Indeed, vasohibin-2 expression increased when angiogenesis was induced in embryonic brain or postnatal skin in mice. These results suggest the involvement of vasohibin-1 and -2 in the control of angiogenesis.

Immunohistochemical analysis confirmed that both vasohibin-1 and -2 proteins were localized to ECs. Interestingly, immunostaining of vasohibin-2 was more intense than that of vasohibin-1. Although the expression levels of different proteins cannot be directly compared by immunostaining, results from real-time RT-PCR in mice suggested that vasohibin-2 expression was higher than that of vasohibin-1. The precise quantification of the two proteins awaits future analysis.

Whereas high levels of VEGF persisted from E14.5 to E18.5 in mouse embryos, vasohibin-1 and -2 levels peaked at E14.5 and declined thereafter. However, vasohibin-1 and -2 proteins persisted to some extent in arterial ECs in late gestation and through to the neonate. It will be interesting to determine whether the vasohibin proteins in arterial ECs exhibit any functions beyond antiangiogenesis.

Acknowledgments

This work was supported by the 21st Century COE Program Special Research Grant “The Center for Innovative Therapeutic Development for Common Diseases” and by a grant-in-aid for Scientific Research on Priority Areas of the Japanese Ministry of Education, Science, Sports, and Culture from the Ministry of Education, Science, Sports, and Culture of Japan.

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*Arterioscler Thromb Vasc Biol.* 2006;26:1051-1057; originally published online March 9, 2006; doi: 10.1161/01.ATV.0000216747.66660.26

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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