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A Negative Regulatory System of Angiogenesis Genetically Programmed in Endothelial Cells

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Abstract—Biological phenomena are under the precise control by the genome. For the regulation of angiogenesis, proangiogenic genes such as VEGFs and angiopoietins are highly conserved, act specifically on endothelial cells, and play a fundamental role. In this sense, nature should prepare specific antiangiogenic genes as well. However, this counterpart of genomic regulation of angiogenesis remains to be established. We recently isolated a novel endothelium-derived angiogenesis inhibitor and named it vasohibin. Vasohibin is dominantly expressed in endothelial cells, induced by the stimulation with VEGF or FGF-2, and selectively affects on endothelial cells and inhibits angiogenesis. Although the mechanism of how vasohibin inhibits angiogenesis remains to be elucidated, our discovery of vasohibin as an endothelium-derived VEGF-inducible angiogenesis inhibitor should shed light on the genomic basis of the negative regulation of angiogenesis. (*Arterioscler Thromb Vasc Biol*. 2007;27:37-41.)

Key Words: endothelial cell ■ angiogenesis inhibitor ■ VEGF ■ negative feedback

B lood vessels are one of the most quiescent tissues in the body, but have the capacity to form neovessels under certain conditions. Angiogenesis, ie, the formation of neovessels from existing ones, is a key event in various processes that takes place under physiological and pathologic conditions. Physiological conditions include embryonic development, reproduction, and wound healing; whereas pathologic conditions include cancers, proliferative retinopathy, and rheumatoid arthritis. Angiogenesis consist of multiple sequential steps: detachment of mural pericytes for vascular destabilization, extracellular matrix degradation by endothelial proteases, migration of ECs, proliferation of ECs, tube formation by ECs, and reattachment of pericytes for vascular stabilization.[1]

The local balance between angiogenesis stimulators and inhibitors regulates angiogenesis. Understanding of the mechanism of angiogenesis regulation has advanced significantly since the discovery of endothelium-specific proangiogenic factors, namely vascular endothelial growth factor (VEGF) and angiopoietins (Ang) family proteins. VEGFs bind to specific VEGF receptors (VEGFRs), while Angs bind to a tyrosine kinase receptor having Ig and EGF homology domains (TIE) receptor expressed exclusively in the endothelium. Among the VEGF family members, VEGF-A is the most important factor for angiogenesis, stimulating protease synthesis, migration, and proliferation of endothelial cells (ECs), and most of the VEGF-A–mediated signals are transduced via VEGFR-2.[2] TIE-2–mediated signals determine vascular mat- uration by the pericyte attachment. Amid Ang family members (Ang 1–4), Ang-1 and Ang-3/4 are agonistic ligands, whereas Ang-2 is a very weak ligand and acts as an antagonist of TIE-2 receptor.[3] Ang3 (mouse) and Ang4 (human) are interspecies orthologs.[4]

Various molecules are listed as angiogenesis inhibitors.[5] Most of them, such as pigment epithelium derived factor (PEDF), platelet factor 4, angiostatin, and endostatin, are extrinsic to ECs. In addition, ECs themselves have the capacity to express some angiogenesis inhibitors, eg, soluble VEGFR-1 (sVEGFR-1), vascular endothelial growth inhibitor (VEGI), Down syndrome critical region gene 1 (DSCR1), and vasohibin.

The VEGFR-1 gene encodes for both the full-length receptor and a soluble form. sVEGFR-1 carries 6 Ig-like domains as well as a 31-amino-acid stretch derived from intron 13.[6] sVEGFR-1 can be distinguished from the other angiogenesis inhibitors because of its specific activity. It is able to bind specifically VEGF-A as well as VEGF-B and PlGF with high affinities, and functions as a decoy receptor by sequestering them.[6] sVEGFR1 cannot inhibit angiogenesis stimulated by other angiogenic factors such as fibroblast growth factor 2 (FGF-2) or hepatocyte growth factor (HGF) because of its binding specificity. The regulation of the expression of sVEGFR-1 is yet to be characterized.

VEGI is a novel member of the tumor necrosis factor (TNF) family identified from the human umbilical vein endothelial cell (HUVECs) cDNA library.[7] VEGI is a type II

transmembrane protein composed of 174 amino acid residues. Unlike other members of the TNF family, VEGI is expressed predominantly in ECs,[7] but importantly, the effect of VEGI is not selective to ECs, and inhibits proliferation of various caner cells as well.[8] The expression of VEGI is regulated mainly by transcription factor NF-κB in parallel with other inflammatory cytokines.[9]

DSCR1 and vasohibin are VEGF-inducible molecules in ECs.[10] DSCR1 is a cytoplasmic protein and is shown to act as an endogenous calcineurin inhibitor,[11] and because of this property, DSCR1 is thought to inhibit angiogenesis.[12] Indeed, overexpression of DSCR1 in ECs inhibited angiogenesis.[13] However, our analysis revealed that specific knockdown of DSCR1 in ECs inhibited angiogenesis.[14] Moreover, our subsequent analysis showed that DSCR1 bind not calcineurin but also Raf-1.[15] Thus, the role of DSCR1 in angiogenesis may not be simple.

Vasohibin and its homologue vasohibin-2 are the most recently identified angiogenesis inhibitors.[16,17] This review will focus on the vasohibin family, a negative regulatory system of angiogenesis genetically programmed in endothelial cells.

Vasohibin

Isolation
DNA microarray analysis was used to identify VEGF-inducible genes in ECs.[10] Among 7267 human sequences, 97 were induced more than 2-fold by VEGF stimulation in HUVECs at the 24 hour time point. Of these 97 sequences, 11 were uncharacterized in terms of their biological function, and we could isolate 1 of these11 genes that had antiangiogenic activity, and named it vasohibin.[16] Human vasohibin protein is composed of 365 amino acid residues, without any detectable glycosilation. A cluster of basic amino acids was present in the C-terminal region, but neither a classical secretion signal sequence nor any other functional motif was found among these amino acid sequences by the database search. The lack of classical signal sequence suggests that vasohibin is an unconventional secretory protein.

Initially the antiangiogenic activity of vasohibin was shown using the in vitro Matrigel assay.[16] Recombinant vasohibin protein inhibited the spontaneously formed network-like structures of HUVECs when plated on Matrigel. The antiangiogenic activity of vasohibin was then further determined by 3 independent in vivo assays When matrigel mixed with VEGF with or without vasohibin protein was inoculated subcutaneously to mice, vasohibin inhibited the VEGF-stimulated angiogenesis. However, vasohibin did not inhibit phosphorylation of VEGFRs in HUVECs.[16] Moreover, the "Miles assay" revealed that vasohibin exhibited no inhibitory effect on VEGF-stimulated acute vascular permeability (unpublished observation, 2005). Indicating that vasohibin is not merely an antagonist of VEGF.

When vasohibin was applied to pellets containing fibroblast growth factor (FGF)-2 in a mouse corneal micropocket assay, vasohibin inhibited FGF-2–stimulated angiogenesis.[16] Introduction of the vasohibin gene into a replication-defective adenovirus vector, and applied it to chicken chorioallantoic

membrane (CAM) assay, the adenovirus vector encoding vasohibin abrogated the vessel formation whereas the control adenovirus vector encoding β-galactosidase (AdLacZ) did not.[16] All these data implicate that the antiangiogenic effect of vasohibin is not restricted to VEGF-stimulated angiogenesis. The mechanism as to how vasohibin inhibits angiogenesis remains to be elucidated.

Expression Profile
The expression profile of vasohibin was examined in both in vitro and in vivo. In vitro, vasohibin was predominantly expressed in ECs. The expression in ECs was induced not only by VEGF but also by FGF-2. However, human aortic smooth muscle cells (HASMCs) expressed vasohibin weakly, and platelet derived growth factor (PDGF) modestly increased its expression. In addition, fibroblasts did express very low levels of vasohibin, but was unresponsive to the FGF-2 stimulation. Vasohibin expression was not observed in keratinocytes under either basal or EGF-stimulated conditions.[16]

Inflammation often associates pathological angiogenesis. Our analysis revealed that inflammatory cytokines such as TNFα, interleukin (IL)-1β, and interferon (IFN)γ reduced the VEGF-induced expression of vasohibin in ECs.[16,18] The effect of IL-1β was comparable to that of TNFα, whereas the effect of IFNγ was less pronounced. Hypoxia is known to act as a trigger of both physiological and pathological angiogenesis by inducing VEGF. Hypoxia did not affect the basal expression of vasohibin in ECs. However, hypoxia did inhibit the VEGF-stimulated vasohibin mRNA expression, as well as vasohibin protein synthesis in ECs.[16]

Northern blot analysis of the samples from various tissues revealed that vasohibin was expressed in the brain, and to a lesser extent, in the heart and kidney in the adult.[16] Moreover, a robust expression of vasohibin was demonstrated in the placenta and various developing organs of the human embryo.[16] Furthermore, immunohistochemical analysis revealed that vasohibin was present only in ECs of the human placenta and developing organs in embryo.[16,17] Thus vasohibin is thought to be a molecule selectively expressed in ECs during angiogenesis.

Signals for the Induction of Vasohibin in ECs
The intracellular signaling for the induction of vasohibin in HUVECs by VEGF was characterized using blocking anti-VEGFRs mAbs to test which receptor was involved in the induction of vasohibin.[18] Anti–VEGFR-2 antibodies but not anti–VEGFR-1 antibodies inhibited the VEGF-stimulated induction of vasohibin. The downstream intracellular signaling pathways of VEGFR-2 for the induction of vasohibin were further investigated. GF109203X, a broad-spectrum inhibitor of protein kinase C (PKC), strongly inhibited the increase of vasohibin mRNA and protein in response to VEGF, which was in line with the observation that Phorbol 12-myristate 13-acetate (PMA), an activator of PKC, enhanced the expression of vasohibin in HUVECs. Selective PKC isoform inhibitors were used to clarify which PKC isoforms were involved in the upregulation of vasohibin. Rottlerin, a specific inhibitor of PKCδ, completely blocked
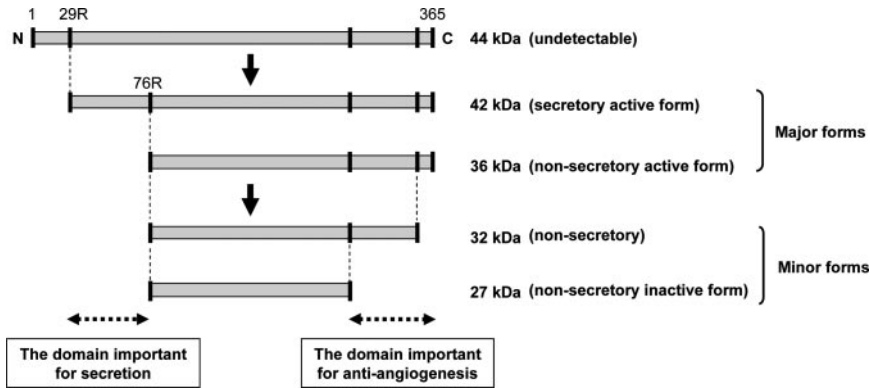
Figure 1. Posttranslational processing, secretion, and biological activity of vasohibin-1. Forty-four–kDa full-length vasohibin-1 cannot be detected. Four different forms of vasohibin-1 are generated after the translation. Forty-two–kDa vasohibin-1 is the major secretory form with antiangiogenic activity. N terminus region is important for secretion, whereas C terminus region is important for antiangiogenesis. The antiangiogenic activity of 32-kDa form is not determined.

the upregulation of vasohibin, whereas Gö6976, a specific inhibitor of PKCα, and HBDDE, an inhibitor of PKCα and PKCγ, partially inhibited it. Hispidin, a specific inhibitor of PKCβ, did not affect the upregulation of vasohibin.[18] From these results it is concluded that PKCδ transduced a principal signal for the upregulation of vasohibin through VEGF. FGF-2 increased the expression of vasohibin in ECs to a level comparable to that obtained with VEGF, and rottlerin again completely blocked FGF-2–stimulated upregulation of vasohibin.[18] Accordingly, the principal signaling pathways for the induction of vasohibin by 2 representative angiogenic growth factors considerably overlap. PKC-δ is known to be a transducer of antiangiogenic signals in ECs.[19] Thus, vasohibin can be a downstream effecter of PKC-δ in ECs for angiogenesis inhibition.

Actinnomycin D treatment did not change the decay of VEGF-induced vasohibin mRNA.[18] Thus, the increase of vasohibin mRNA by VEGF is not determined by mRNA stability. However, when cycloheximide was added, the expression of vasohibin mRNA was completely abolished in both basal and VEGF-stimulated condition.[18] Thus, de novo protein synthesis is indispensable for the induction of vasohibin mRNA.

Posttranslational Processing, Secretion, and Biological Activity

To understand the posttranslational modification of vasohibin protein, vasohibin cDNA was overexpressed in ECs.[20] The calculated vasohibin protein is 44 kDa. When the retroviral vector encoding human vasohibin cDNA was transfected to the HUVEC-derived HUV-SV8 cells, 2 major (42, 36 kDa) bands and 2 minor (32, 27 kDa) bands were detected in their cellular extract, whereas 42 kDa product was detected in the conditioned medium. Because the 44 kDa complete from was not seen, amino terminal region is thought to be processed simultaneously or immediately after the translation. To characterize the structures of these multiple forms of vasohibin proteins, various vasohibin cDNA mutants were generated to substitute some basic amino acids. This analysis revealed that there were 2 cleaving sites in the amino terminal region; arginine 29 and arginine 76. The 42 kDa form is generated by the cleavage at arginine 29, whereas the 36 kDa form is generated by the cleavage at arginine 76. Because only 42 kDa vasohibin was shown in the conditioned medium, the domain from arginine 29 to arginine 76 is thought to be

important for the secretion. The mechanism of its secretion is not known at present. Cleaving sites in the carboxyl terminal region are not determined yet. However, because the calculated molecular weight of the vasohibin protein from methionine 77 to carboxyl terminal end is 33 kDa, the carboxyl terminal of the 32 kDa form should be very close to the end. From the calculation of the molecular weight, the 27 kDa form may lack about 47 amino acids from the carboxyl terminal, and this lacked region contains the cluster of basic amino acids (Figure 1).

To determine the biological function of these processed forms of vasohibin, mouse corneal miropocket assay was used to check for antiangiogenic activities using purified recombinant proteins for Vh(77–365) and Vh(77–318).[20] Vh(77–365) inhibited FGF-2–induced angiogenesis, suggesting that truncation of the 76 amino terminal residues does not influence antiangiogenic activity of vasohibin. On the other hand, Vh(77–318) could not exert antiangiogenic activity, suggesting that the carboxyl terminal is essential for antiangiogenic activity (Figure 1).

Application to Antiangiogenic Therapy

Because vasohibin is identified as a novel angiogenesis inhibitor, one may anticipate the application of vasohibin to antiangiogenic therapy. We have examined the effect of vasohibin on 3 different states of pathological angiogenesis: tumor angiogenesis, arterial adventitial angiogenesis, and retinal angiogenesis.

For tumor angiogenesis, we transfected human vasohibin cDNA into Lewis lung carcinoma (LLC) cells, establishing two permanent human vasohibin-producing clones.[16] Vasohibin cDNA transfection did not alter the proliferation of LLC cells in vitro. To show the effect of vasohibin produced by LLC cells on ECs, mock or vasohibin-transfected LLC cells were plated on the lower compartment of modified Boyden chambers, and the migration of HUVECs toward LLC cells was analyzed. The number of migrated HUVECs was significantly reduced when vasohibin-transfected LLC cells were plated on the lower chamber. Then LLC cells were inoculated intradermally in mice, and the growth of tumor was observed. The growth of vasohibin producing LLC cells in mice was significantly retarded, and immunohistological analysis of CD31 revealed that tumors of mock-transfectants contained large luminal vessels whereas those of vasohibin

producing LLC cells contained very small ones, even when the size of tumors did not differ dramatically.[16]

It has been documented that the extent of adventitial angiogenesis from vasa vasora correlates with atherosclerosis.[21] Arterial neointimal formation was investigated using the mouse cuff model.[22] In this model, cuff placement around the femoral artery does not denudate luminal endothelium, but induces adventitial angiogenesis, and that causes neointimal formation. To apply vasohibin protein to mice, we injected replication-defective adenovirus vectors encoding human vasohibin gene (AdVh) to mice via the tail vein. In this way, vasohibin was synthesized in the liver, secreted in the plasma, and was able to exhibit antiangiogenic activity in the remote sites after the delivery through systemic circulation.[22] We observed that adventitial angiogenesis and neointimal formation were significantly inhibited in AdVh-injected mice in this model. Thus, vasohibin is thought to play a preventive role in angiogenesis-dependent neointimal formation.

Retinal angiogenesis is the major cause of acquired blindness.[23] The mouse model of retinopathy of premature (ROP) is a useful model to study the hypoxia-induced regulation of VEGF expression.[24] In this model, placement of neonatal mice into a high oxygen environment results in decreased expression of VEGF and regression of newly developed retinal blood vessels. When mice are returned to room air, the poorly vascularized retina becomes hypoxic and VEGF is induced, which causes retinal angiogenesis. Interestingly, when endogenous vasohibin expression in the retinal vessels was knocked down by siRNA, retinal angiogenesis was augmented.[25] This result indicates that endogenous vasohibin plays a role in the inhibition of angiogenesis. However, it is assumed that the extent of endogenous vasohibin expression is not enough to control retinal angiogenesis. To determine the effect of exogenous vasohibin, we used AdVh or recombinant vasohibin protein. Intraocular injection of recombinant vasohibin or AdVh strongly suppressed retinal angiogenesis.[25]

A Homologue of Vasohibin and Splicing Variants

By the search of DNA sequences in the database, a homologous gene was found. This gene was named vasohibin-2, and the prototype vasohibin as renamed vasohibin-1.[17] Human vasohibin-2 is composed of 355 amino acid residues, and also exhibits antiangiogenic activity. The overall homology between human vasohibin-1 and vasohibin-2 is 52.5% at the amino acid level. The genes for human vasohibin-1 and vasohibin-2 are located on chromosome 14q24.3 and 1q32.3, respectively.

So far 8 exons for the vasohibin-1 gene and 11 exons for the vasohibin-2 gene have been shown in Ensembl human genome database to form multiple transcripts for these paralogous genes owing to alternative splicing (Figure 2). Prototype full-length vasohibin-1 is composed with 5589 bp consisting of 8 exons, and will receive posttranslational processing as described previously. In addition, two splicing variants of 522 bp and 1459 bp have been registered in the database. Although open reading frames encoding 42 and 204 amino acids, respectively, exists in these transcripts and the expression of all three splicing variants are confirmed by real-time polymerase thain reaction using unique primers (unpublished observation, 2006), the biological significance
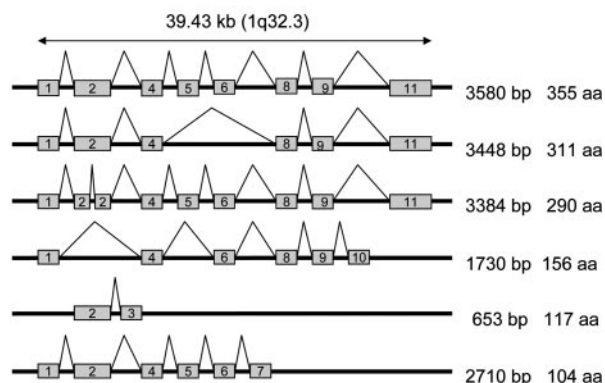
Human vasohibin-1 gene
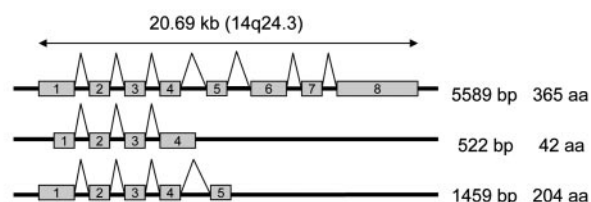
Human vasohibin-2 gene

Figure 2. Schematic representation of human vasohibin-1 and -2 genomic structures and splicing patterns. Exons of human vasohibin-1 and vasohibin-2 genes are numbered in their orders on the chromosomes. Length of each transcript (bp) and polypeptides encoded in each transcript (aa) are shown in the right side of each splicing pattern.

of the 2 shorter variants have not been clarified yet. No alternative splicing for the mouse vasohibin-1 gene has been reported.

We have recently described the existence of 3 splicing variants for human vasohibin-2 transcripts, which encode polypeptides of 290, 311, and 355 amino acids.[17] Eight exons are joined to generate the variant of 355 amino acids, and this isoform is predominantly expressed in HUVECs. The isoform consisting of 290 amino acids has been confirmed to have antiangiogenic activity. In addition to those 8 exons, 3 additional exons are now found in Ensembl database generating 3 small different splicing variants encoding polypeptides of 104, 117, and 156 amino acids. The biological significance of these shorter variants have not been clarified yet. In the mouse genome, vasohibin-1 gene is located at 12D2 spanning 13.39 kb and consisting of 7 exons. Mouse vasohibin-2 gene is located at chromosome 1H6 spanning 31.48 kb and single splicing pattern with 8 exons is reported in Ensembl database.

Whereas the expression of vasohibin-2 was compared with that of vasohibin-1, vasohibin-2 expression in cultured endothelial cells was low and not inducible by the stimulation that induced vasohibin-1. However, the expression pattern of vasohibin-2 in vivo resembled to that of vasohibin-1.[17] Immunohistochemical analysis revealed that vasohibin-1 and vasohibin-2 were diffusely expressed in ECs in embryonic organs during midgestation. After that time point,
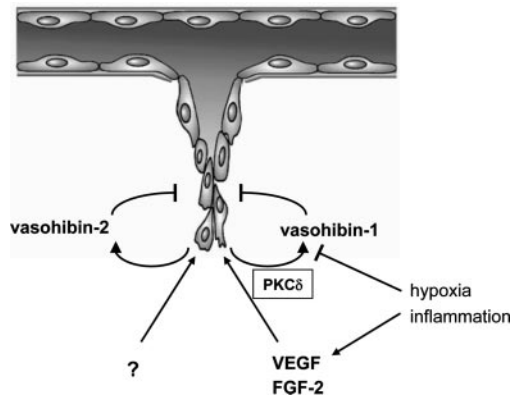
Figure 3. The role of vasohibin family in the regulation of angio-genesis. Vasohibin-1 and vasohibin-2 form a novel family of angiogenesis inhibitors genetically programmed in ECs. Vasohibin-1 is induced in ECs by VEGF and FGF-2. Hypoxia and inflammatory cytokines, which induce VEGF, may abort the expression of vasohibin-1 in ECs. The mechanism for the regulation of vasohibin-2 expression is not known.

vasohibin-1 and vasohibin-2 became faint, but persisted to a certain extent in arterial ECs from late-gestation to neonate. Interestingly, expression of vasohibin-1 and vasohibin-2 could be augmented in vivo by the local expression with the VEGF gene in the embryonic brain, or by cutaneous wound-ing in adult mice.[17]

Concluding Remarks

A summary of the vasohibin family is shown in Figure 3. Negative feedback regulation is one of the most important physiological mechanisms, and has been demonstrated to control a wide range of phenomena. However, very few endothelium-derived negative feedback regulators have been established for the regulation of angiogenesis. Vasohibin-1 is the first secretory antiangiogenic factor induced by VEGF in ECs. We would like to propose that vasohibin-1 has the property of negative feedback regulator of angiogenesis. Thus far Vasohibin-2 is a sole homologue of vasohibin-1, which exhibits antiangiogenic activity as well. Although vasohibin-2 lacks the property of VEGF or FGF-2 inducibility in vitro, its expression pattern is resemble to that of vasohibin-1. Thus, vasohibin-1 and vasohibin-2 form a novel family of angiogen-esis inhibitors genetically programmed in ECs. The discovery of vasohibin family should shed light on the novel genomic basis of the negative regulation of angiogenesis.

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