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An endothelium-derived angiogenesis inhibitor vasohibin and its significance in tumor angiogenesis

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Abstract Angiogenesis is regulated by a local balance between the levels of endogenous stimulators and inhibitors of angiogenesis. Understanding of the mechanism of angiogenesis has advanced significantly since the discovery of two members of the family of angiogenesis stimulators—vascular endothelial growth factor (VEGF) family proteins and angiopoietins. These factors act on endothelial cells to stimulate angiogenesis. In contrast, few genes encoding molecules that selectively inhibit angiogenesis have been discovered. We recently isolated a novel angiogenesis inhibitor, whose expression was augmented in endothelial cells by stimulation with VEGF. We named it vasohibin. Vasohibin is thought to be the first negative feedback regulator of angiogenesis, which is selectively expressed in endothelial cells.

Keywords Angiogenesis · VEGF · Negative feedback · Endothelial cell

Introduction

Blood vessels are one of the most quiescent tissues in adult humans, but have the capacity to form neo-vessels under certain conditions. Angiogenesis, i.e., the formation of neo-vessels, is a key event in various developmental or remodeling processes that take place under physiological and pathologic conditions. Physiological conditions include organ growth and development, wound healing, and reproduction; whereas, pathologic conditions include tumor growth and metastasis.

It has been shown that carcinogenesis is a consequence of multiple steps of oncogene and tumor suppressor gene mutation. However, the growth of tumors beyond 2 mm^3 requires the additional step of angiogenesis. The original hypothesis for the dependence of solid tumors on angiogenesis was presented by Folkman in 1971 [7]. Since then, numerous studies have been performed to prove this hypothesis. Animal studies clearly showed the dependence of solid tumors on angiogenesis. Moreover, multiple clinical studies for the assessment of tumor microvascular density in human cancers have supported the view that tumors are angiogenesis dependent.

Angiogenesis inhibitors

Angiogenesis is thought to be regulated by a local balance between the levels of endogenous stimulators and inhibitors of this process. Various endogenous molecules have been reported to inhibit angiogenesis. These endogenous angiogenesis inhibitors can be classified into two major categories, proteolytic fragments/metabolites and gene products.

Proteolytic fragments/metabolites

Proteolytic fragments and metabolites of hormones are derived from parental molecules with no obvious

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anti-angiogenic activity. The parental molecules of proteolytic fragments can be divided into two subgroups: extracellular matrix (ECM) proteins and non-ECM proteins. Molecules derived from ECM proteins include arrestin [3], canstatin [11], endostatin [16], and tumstatin [12]. Molecules derived from non-ECM proteins include angiostatin [15] and anti-angiogenic antithrombin [17]. Metabolites of hormones include 2-methoxyestradiol [4, 8] and 16-kDa prolactin fragment [6].

Most of the proteolytic fragments in this category are reported to inhibit angiogenesis selectively, and most of the targets are either integrins or heparan sulfate proteoglycans (HSPGs). However, if the only targets are integrins and/or HSPGs, it is difficult to explain the selective effect of these molecules on angiogenesis inhibition. Although a number of reports have suggested that proteolytic fragments have a function in pathological angiogenesis, the physiological significance of these molecules in the regulation of angiogenesis remains to be established.

Gene products

Molecules in this category include chondromodulin-I [10], interferon-α/β [19], interferon-γ-inducible protein 10 [14], maspin [22], pigment epithelium-derived factor (PEDF) [5], platelet factor-4 [13], soluble vascular endothelial growth factor receptor 1 (sVEGFR1) [21], thrombospondins [2], and vasohibin [20]. Most members of this category have pleiotropic effects that include angiogenesis inhibition.

Vasohibin and sVEGFR1 are distinguished because of their selective expression in endothelial cells and their selective activities. Soluble VEGF receptor 1 blocks VEGF signaling, and thus inhibits VEGF-mediated effects including angiogenesis and vascular permeability. Naturally, sVEGFR1 cannot inhibit angiogenesis stimulated by other angiogenic factors. Vasohibin is proposed to be the first negative feedback regulator of angiogenesis.

Vasohibin

Negative feedback regulation is one of the most important physiological mechanisms, and has been demonstrated to control a wide range of phenomena. However, no negative feedback regulators had been recognized for the regulation of angiogenesis until the discovery of vasohibin, which is proposed to be the first endothelium-derived negative feedback regulator of angiogenesis.

Isolation

Vasohibin is the most recently identified angiogenesis inhibitor with endothelium-specific expression [20]. We searched for VEGF-inducible genes in endothelial cells by cDNA microarray analysis [1]. Among 7,267 human sequences, 97 were induced more than twofold by VEGF stimulation in human umbilical vein endothelial cells (HUVECs) at the 24 h time point. Of these 97 sequences, 11 were novel or uncharacterized in terms of their biological function. We focused on one such sequence, namely AF055021. An expressed sequence tag (EST)-clustered database, AssEST, indicated that AF055021 encodes the 3′-untranslated region (UTR) of KIAA1036 cDNA (GenBank accession number AB028959). We named KIAA1036 as vasohibin. Human vasohibin gene localizes in chromosome 14, and includes a 5′-UTR of 385 base pairs (bp), an open reading frame of 1,098 bp, and a 3′-UTR of 3,998 bp. Based on the sequence of the human vasohibin gene, we cloned a mouse counterpart of vasohibin cDNA. We then prepared recombinant vasohibin protein and examined whether vasohibin protein exhibited any biological effects. HUVECs spontaneously form network-like structures when plated on matrigel (Collaborative Research, Bedford, MA, USA). When the recombinant vasohibin protein was added to the medium, it inhibited this network formation. This is the first evidence to suggest an anti-angiogenic activity of KIAA1036 protein. Three independent assays were then performed to determine whether vasohibin exhibited anti-angiogenic activities in vivo. Matrigel was mixed with VEGF and/or vasohibin protein and inoculated subcutaneously in mice. Seven days after the inoculation, the matrigel was removed and a histological analysis was performed. It was shown that vasohibin had inhibited VEGF-stimulated neo-vessel formation in matrigel. Mouse corneal micropocket assay was then performed. Vasohibin protein apparently inhibited fibroblast growth factor (FGF-2)-stimulated angiogenesis in mouse cornea. We introduced vasohibin gene into a replication-defective adenovirus vector, and evaluated it in a 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. These three independent assays confirmed the anti-angiogenic activity of vasohibin protein [20].

Signals for induction in endothelial cells

We characterized a signal for the up-regulation of vasohibin in HUVECs by VEGF [18]. VEGF transduces

signals through two receptor tyrosine kinases, namely VEGFR1/Flt-1 and VEGFR2/Flk-1. We employed blocking anti-VEGFR1 or VEGFR2 monoclonal antibodies (mAbs) to test which receptor was responsible for the up-regulation of vasohibin. The anti-VEGFR2 mAb, but not the anti-VEGFR1 mAb, inhibited VEGF-stimulated up-regulation of vasohibin. The downstream intracellular signaling pathways of VEGFR2 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. Phorbol myristate acetate, an activator of PKC, enhanced the expression of vasohibin in HUVECs. PD98059, a specific inhibitor of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK 1), partially inhibited the up-regulation of vasohibin mRNA and protein by VEGF, whereas inhibitors of p38, c-Jun NH_2-terminal protein kinase and phosphatidylinositol 3-kinase did not influence this. We further used selective PKC isoform inhibitors to clarify which PKC isoforms were involved in the up-regulation of vasohibin. Rottlerin, a specific inhibitor of PKCδ, completely blocked the up-regulation of vasohibin, whereas Gö6976, a specific inhibitor of PKCα, and 2,2′,3,3′,4,4′-hexahydroxy-1,1′-biphenyl-6,6′-dimethanol dimethyl ether (HBDDE), an inhibitor of PKCα and PKCγ, partially inhibited it. Hispidin, a specific inhibitor of PKCβ, did not affect the up-regulation of vasohibin. VEGF activated PKCδ in HUVECs. From these results, we concluded that PKCδ transduced a principal signal for the up-regulation of vasohibin through VEGF. FGF-2 increased the expression of vasohibin in endothelial cells to a level comparable to that obtained with VEGF, and rottlerin again completely blocked FGF-2-stimulated up-regulation of vasohibin. Accordingly, the principal signaling pathways for the up-regulation of vasohibin by two representative angiogenic growth factors considerably overlap [18].

PKCδ is known to be a transducer of anti-angiogenic signals in endothelial cells [9]. Thus, vasohibin could be a downstream effector of PKCδ in endothelial cells for angiogenesis inhibition.

Expression profile

The expression profile of vasohibin was examined in more detail. Human aortic smooth muscle cells (HASMCs) expressed vasohibin weakly, and PDGF modestly increased its expression. Although fibroblasts did express vasohibin, the level of expression was low and unresponsive to FGF-2 stimulation. Vasohibin expression was not observed in keratinocytes under either basal or EGF-stimulated conditions. The expression of vasohibin in various organs in vivo was examined. Northern blotting revealed that vasohibin was predominantly expressed in the brain and placenta, and, to a lesser extent, in the heart and kidney. The placenta is a site at which active vascular remodeling takes place. Immunohistochemical analysis revealed that vasohibin was present selectively in the endothelial layer of the human placenta. The strong expression of vasohibin was demonstrated in the developing organs of the human embryo, suggesting a role for this molecule in development [20].

Hypoxia is known to act as a trigger of both physiological and pathological angiogenesis by inducing VEGF. Hypoxia did not affect the expression of vasohibin. However, hypoxia did inhibit VEGF-stimulated vasohibin mRNA expression, as well as vasohibin protein synthesis in endothelial cells. Pathological angiogenesis is often associated with inflammation. The inflammatory cytokine tumor necrosis factor-α alone did not significantly affect the expression of vasohibin, but did inhibit VEGF-stimulated vasohibin mRNA expression and protein synthesis in endothelial cells [20].

Anti-tumor activity

Tumors are generally associated with hypoxia and inflammation. It was therefore assumed that exogenous vasohibin would affect tumor growth and tumor angiogenesis, and we undertook experiments to determine if this were the case. We transfected human vasohibin cDNA into Lewis lung carcinoma (LLC) cells, established permanent transfectants, and further isolated two vasohibin-producing clones, namely clone16 and clone19. Vasohibin cDNA transfection did not alter the proliferation of LLC cells in vitro. To show the effect of vasohibin produced by LLC cells, a modified Boyden chamber was used. Mock- or vasohibin-transfected LLC cells were plated on the lower chambers, and the migration of HUVECs toward the LLC cells was analyzed. The number of migrated HUVECs was significantly reduced when vasohibin-transfected LLC cells were plated on the lower chamber. We then inoculated LLC cells intradermally in mice, and tumor growth was observed. The growth of vasohibin-producing clones, clone16 and clone19, in mice was significantly retarded. Immunohistological analysis of CD31 was performed to evaluate the extent of tumor angiogenesis. It showed that tumors of mock-transfectants contained large luminal vessels, whereas those of clone16 and clone19 contained small ones, even when the size of tumors did not differ greatly on day 8.

Quantitative analysis of vascular luminal space revealed that the vascular area within tumors was significantly decreased in clone16 and clone19.

Conclusion

In general, biological phenomena are precisely organized by the genome. As angiogenesis is an important event that can have drastic consequences if dysfunctional, it should be under strict genomic control. Indeed, specific pro-angiogenic genes such as VEGFs and angiopoietins are highly conserved and play a fundamental role in the regulation of angiogenesis. In this sense, nature should prepare specific anti-angiogenic genes to regulate angiogenesis as well. However, this counterpart of angiogenesis regulation is ill-defined, since most of the anti-angiogenic genes are pleiotropic and not specific for angiogenesis inhibition. Furthermore, their expression is not specifically related to angiogenesis. The recent discovery of vasohibin as an endothelium-derived VEGF-inducible angiogenesis inhibitor may shed light on the genomic basis of inhibition of angiogenesis.

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