REVIEW ARTICLE

Delta-like 4 and vasohibin 1: two endothelium-produced negative regulators of angiogenesis with distinctive roles

Yasufumi Sato

Department of Vascular Biology, Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan

Correspondence: Y. Sato, Department of Vascular Biology, Institute of Development, Aging and Cancer, Tohoku University, 4-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan
<y-sato@idac.tohoku.ac.jp>

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ABSTRACT. Angiogenesis is regulated by the local balance between angiogenesis stimulators and inhibitors. A number of endogenous angiogenesis inhibitors have been found in the body. The origin of these inhibitors is mostly extrinsic to the vasculature. Recently however, endothelial cells themselves have been found to produce angiogenesis inhibitors including delta-like 4 and vasohibin 1. These intrinsic factors are thought to regulate angiogenesis by an autoregulatory or negative-feedback mechanism. This review focuses on such negative regulators of angiogenesis produced by endothelial cells.

Keywords: angiogenesis, endothelium, autoregulation, negative feedback

The vascular system, a hierarchical network of arteries, capillaries and veins, is one of the most quiescent organs in the body, but it has the capacity to form neo-vessels under certain conditions. Angiogenesis or neovascularization, i.e., the formation of neovessels, is a fundamental process that occurs in the vascular system, and it occurs under both physiological and pathological conditions. Angiogenesis can be classified into sprouting angiogenesis and intussusceptions. Although the process resulting in intussusceptions has been poorly investigated, that of sprouting angiogenesis is better characterized at present.

The vascular system is primarily composed of luminal endothelial cells (ECs) and surrounding mural cells (smooth muscle cells or pericytes). The presence of mural cells causes blood vessels to become mature and stabilized. The initial step of sprouting angiogenesis is the detachment of mural cells for vascular destabilization. Thereafter, specialized ECs, so-called tip cells, start to migrate by extending numerous filopodia, whereas following ECs, so-called stalk cells, proliferate, cause elongation of sprouts, and form immature, tube-like structures. Finally, redistributed mural cells attach to newly formed vessels for vascular restabilization. By this final process, ECs stop their proliferation, thus terminating angiogenesis.

Angiogenesis is thought to be regulated by the local balance between stimulators and inhibitors of this process. A number of endogenous angiogenesis stimulators and inhibitors have been found in the body. Angiogenesis stimulators include certain growth factors and cytokines, whereas angiogenesis inhibitors are varied and include hormones, chemokines, proteolytic fragments of various proteins, proteins accumulated in the extracellular matrix, and so forth [1]. The origin of these angiogenesis inhibitors is mostly extrinsic to the vasculature. Recently however, ECs have been found to produce angiogenesis inhibitors by themselves, including delta-like 4 and vasohibin 1 (see below). Such intrinsic factors may regulate angiogenesis in an autoregulatory or negative-feedback fashion.

DELTA-LIKE 4 (DLL4)

The Notch-signaling system is evolutionarily conserved from Drosophila to humans, regulating cell fate specification, growth, differentiation, and patterning of neighboring cells through lateral inhibition. The Notch-signaling system in mammals consists of four type I transmembrane receptors (Notch1, Notch2, Notch3, and Notch4) and five type I transmembrane ligands (Jagged1, Jagged2, Dll1, Dll3, and Dll4) collectively referred to as the DSL (Delta/Serrate/Lag-2) family. The Notch receptor consists of an extracellular domain and an intracellular domain. The Notch extracellular domain (NECD) is composed of epidermal growth factor (EGF)-like repeats, followed by Lin12-Notch (LN) repeats. These EGF-like repeats contain the ligand-binding sites, whereas the LN repeats are involved in preventing ligand-independent signaling. On the other hand, the Notch intracellular domain (NIC) contains recombination signal binding protein for the immunoglobulin kappa J region (RBPJκ)-associated molecular region in the juxtamembrane region, followed by ankyrin repeats, a putative transactivating domain, and a C-terminal PEST motif. PEST is defined by a cluster

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of proline (P), glutamic acid (E), serine (S) and threonine (T) residues. Upon Notch receptor-ligand binding at the cell surface, a series of sequential cleavages of the Notch receptor occurs. The final cleavage is mediated by the γ-secretase complex, which results in the release of the NICD, which is then translocated to the nucleus, where it interacts with members of the CSL (CBF-1, Suppressor of Hairless, Lag-1) family of transcription factors. The best-characterized CSL family members of Notch targets are the Hairy and enhancer-of-split (HES), and Hairy and enhancer-of-split-related (HEY, HESR, HRT, or CHF) gene families. These basic, helix-loop-helix (bHLH) proteins act mostly as transcriptional repressors, either by direct binding to an E-box and N-box for the recruitment of corepressors such as groucho (TLE in mammals) or by mechanisms independent of direct DNA binding. Thus, the interaction of the NICD with CSL family members results in the derepression/activation of CSL targets [2].

Multiple Notch receptors and ligands are expressed in the vascular system during both embryonic development and postnatal remodeling. Among them, Notch2, Notch4, Jagged1, and Jagged2 expression are restricted mainly to arterial endothelium, whereas Notch1 and Dll4 are expressed in both capillary and arterial endothelium [3, 4]. Consistent with their restricted expression patterns, the Notch-mediated signaling has been shown to play a critical role in arterial specification. This activity was initially highlighted in studies on zebrafish. The blockade of the Notch-mediated signaling in zebrafish embryos resulted in the loss of arterial markers accompanied by ectopic expansion of venous markers into arteries [5, 6]. In contrast, the activation of Notch-mediated signaling exhibited the opposite effects, suppressing expression of venous markers and promoting ectopic expansion of arterial markers into veins [5, 6]. Similar results were also obtained in mice by targeted disruption of *Notch1/Notch4*, *Rbpsuh* encoding RBP-Jκ protein, *Hey1/Hey2* or *Dll4* [7-11]. Thus, consistent with its restricted expression pattern in the vasculature, the Notch signaling system plays a critical role in arterial specification.

Dll4 gene-targeted mice showed an increased number of vessel branches and vascular sprouts associated with the leading edge of certain growing vascular beds, such as in the yolk sac [11]. This phenotype of the *Dll4*-knockout mice resembled that of the *Notch1*-knockout mice [7, 11]. These observations indicate that Dll4-Notch1-mediated signaling is involved not only in arterial specification, but also in angiogenesis. Importantly, heterozygous *Dll4*-knockout mice show embryonic lethality. Selective disruption of *Notch1* in the endothelium results in embryonic lethality at a similar time in the development [12], indicating that embryonic lethality of heterozygous *Dll4*-knockout mice is closely related to loss of *Notch1* in the endothelium. The precise role of Dll4-mediated signaling in angiogenesis was characterized further by studies using zebrafish or newborn mouse retina. These studies have demonstrate that the expression of Dll4 and Notch1 are detected mainly in tip cells and stalk cells respectively, and that Dll4 and Notch1 contribute to the regulation of tip cells versus stalk cells during sprouting angiogenesis [13-15]. Notably, the expression of Dll4 and Notch1 are induced by VEGF [16, 17]. Dll4-mediated signaling then limits the number of sprouts *via* Notch1 [14, 18, 19], which inhibition is attributable to the reduced expression of VEGFR2, neuropilin-1, and CXCR4 as a negative-feedback regulator [17, 20] *(figure 1)*. In contrast, the Dll4-Notch1 signal induced Notch-regulated ankyrin repeat protein (Nrarp) in stalk cells and promoted Wnt signaling through interactions with lymphoid enhancer factor 1 (Lef1). This Lef1-dependent Wnt signaling in stalk cells is further involved in the stabilization of newly formed vessels [21].

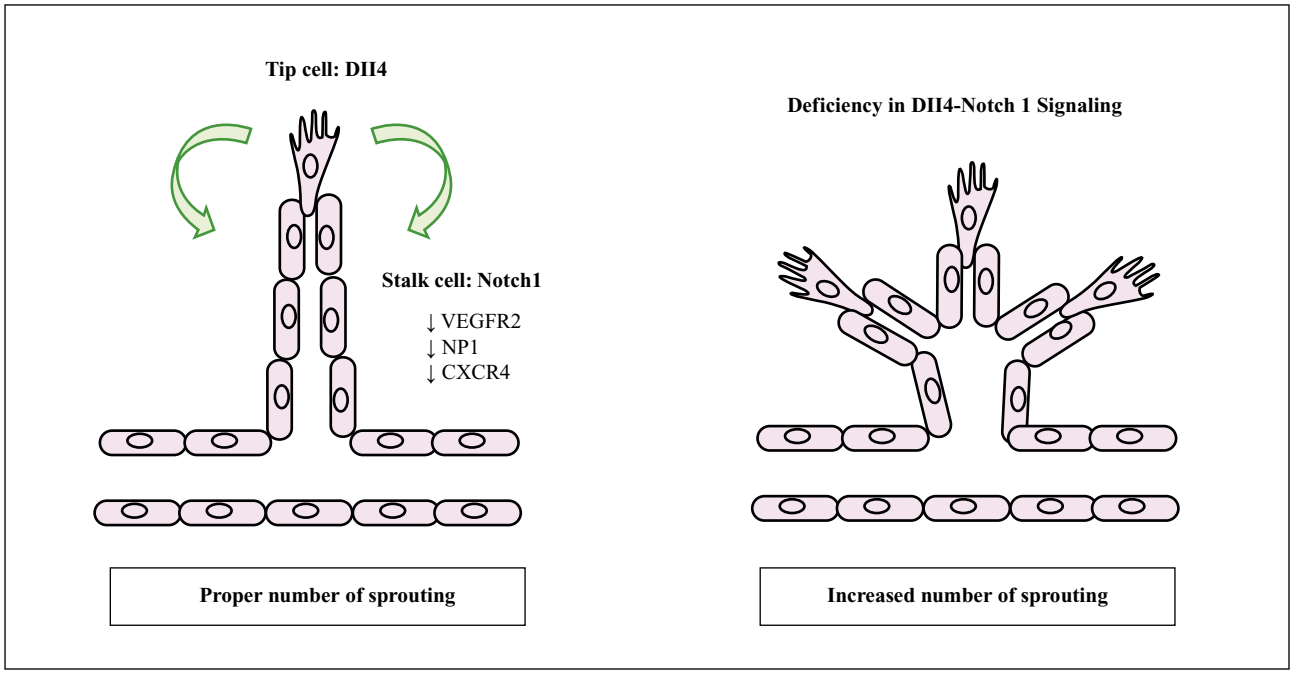

Figure 1
Role of Dll4-Notch1 in angiogenesis. Dll4 on tip cell activates Notch1 on stalk cell, and this determines the number of sprouts by downregulating VEGFR2, neuropilin1 (NP1) and CXCR4.

The inhibitory role of Dll4 in angiogenesis has also been documented in tumors. It has been revealed that Dll4 is up-regulated in the tumor vasculature [22, 23]. When this Dll4-mediated signaling was blocked, the tumors developed numerous microvessels. Interestingly, these vessels were non-functioning and devoid of blood flow, and this unrestrained angiogenesis paradoxically decreased tumor growth even in certain tumors resistant to anti-VEGF therapies [24-26]. Alternatively, as Dll4 is defined as a negative regulator of angiogenesis, activation of Dll4-mediated signaling can inhibit tumor angiogenesis and tumor growth in distinct tumor models [27]. So far, the growth of carcinomas, gliomas, and melanomas has been reported to have been inhibited by the Dll4/Notch blockade, whereas that of lymphomas, plasmacytomas, and myelomonocytic tumors has been reported to be inhibited by Dll4/Notch activation [24, 25, 27]. Future studies will be required to identify the determinants of responsiveness to Dll4/Notch blockade or activation in various tumors.

VASOHIBIN 1 (VASH1)

We hypothesized that ECs might produce novel or uncharacterized regulators of angiogenesis. To test our hypothesis, we performed DNA microarray analysis to examine VEGF-inducible genes in ECs [28]. Among a number of VEGF-inducible genes, we focused our attention on genes whose functions were previously undefined. We then performed a functional assay, isolating a protein that showed antiangiogenic activity, and named it vasohibin (VASH) [29]. Through the subsequent DNA sequence search of genomic databases, we found one gene homologous to VASH and named it VASH2 [30], and thus the prototype VASH is now called VASH1. The gene for human VASH is located on chromosome 14q24.3, and consists of eight exons and seven introns, which encodes a protein of 365 amino acid residues. Mouse VASH1 is more than 90% identical to its human counterpart in amino acid sequence, indicating that VASH1 is highly conserved at least between humans and mice [29]. A cluster of basic amino acids is present in the C-terminus region of VASH1 protein, but neither a classical secretion signal sequence nor any other functional motifs are found in its amino acid sequence. The lack of a classical signal sequence suggests that VASH1 is an unconventional secretory protein [29]. One minor alternative splicing form of VASH1 lacking exons 5 to 8 is present in humans [30-32]. In addition, there are multiple different molecular forms that are processed post-translationally [33].

Immunohistological analysis has revealed that VASH1 is shown in ECs in the developing embryo and placenta, but is down-regulated in the postnatal period, and detected in ECs preferentially at the site of angiogenesis [29, 30, 34]. We further defined the spacio-temporal expression pattern and function of VASH1 during angiogenesis. Our analysis, using the mouse subcutaneous angiogenesis model, has revealed that VASH1 is expressed not in ECs at the sprouting front (tip and stalk cells), but in newly formed blood vessels behind the sprouting front where angiogenesis ceases (termination zone) [35]. Thus, although Dll4 and VASH1 are expressed in ECs during angiogenesis, their expression patterns are totally distinctive. We further demonstrated, in a subcutaneous angiogenesis model, that *VASH1 (-/-)* mice contained immature microvessels in the area where angiogenesis should be terminated [35]. These results indicate that the central function of endogenous VASH1 is to terminate angiogenesis *(figure 2)*. Importantly, newly formed immature microvessels in *VASH1 (-/-)* mice function with blood flow [35].

We investigated the expression of VASH1 under various conditions accompanying pathological angiogenesis. The presence of VASH1 in ECs was evident in cancers,

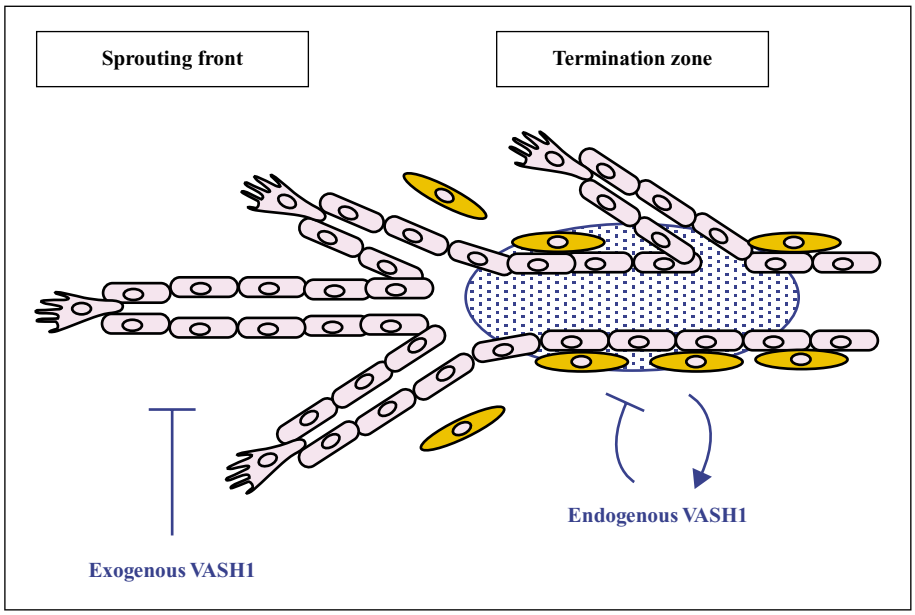

Figure 2
Role of VASH1 in angiogenesis. Endogenous VASH1 is expressed in ECs at the termination zone and stops angiogenesis, whereas exogenous VASH1 preferentially inhibits sprouting.

adventitia of atherosclerotic lesion, age-dependent macular degeneration (AMD), and diabetic retinopathy [36-41]. As cancers contain complex lesions where angiogenesis continues asynchronously and sprouting occurs randomly, it is difficult to dissect the expression profile of VASH1. Nevertheless, we showed that VASH1 was prevalent in tumor vessels of non-small cell lung cancers when they were associated with mural cells [41]. This observation suggests that the spacio-temporal expression pattern of VASH1 is maintained even in tumor angiogenesis. Indeed, tumors inoculated into *VASH1 (-/-)* mice contained numerous immature vessels, and this resulted in increased growth of tumor [41]. In the case of AMD, angiogenesis may subside during its natural course. Interestingly, active AMD tended to have a lower vasohibin-to-VEGF ratio, whereas inactive AMD had a higher vasohibin-to-VEGF ratio [38]. These observations suggest that the expression level of VASH1 may determine certain pathological condition.

When added exogenously, VASH1 inhibits migration and proliferation of ECs, and inhibits angiogenesis. The receptor for vasohibin and its intracellular signaling pathways are now under investigation. Even so, one may ask how exogenous VASH1 can exhibit its effect on angiogenesis in the presence of endogenous VASH1. Our recent analysis clarified that exogenous VASH1 exhibited little effect in the termination zone where endogenous vasohibin was present, but effectively inhibited angiogenesis in the sprouting zone where endogenous VASH1 was not present [35] *(figure 2)*. Since exogenous VASH1 can efficiently inhibit angiogenesis, one may anticipate the application of VASH1 in antiangiogenic therapy. So far, we have been able to show the effect of VASH1 on at least three different states of pathological angiogenesis; tumor angiogenesis, arterial adventitial angiogenesis related to atherosclerosis and ocular angiogenesis [29, 36, 41, 42].

CONCLUSION

The present mini-present review focuses on two angiogenesis inhibitors, Dll4 and VASH1, produced by ECs. Accumulating evidence indicates that the spacio-temporal expression patterns and roles of these two angiogenesis inhibitors are distinct. Dll4 is expressed in tip and some stalk cells and determines the number of sprouts *(figure 1)*, whereas VASH1 is expressed in ECs in the termination zone and determines the termination of angiogenesis *(figure 2)*.

Recently, several other angiogenesis inhibitors have also been reported to be expressed in ECs. Netrin family members were originally identified as a regulator of axon guidance. Among them, netrin-4 was recently shown to be induced in ECs by VEGF stimulation, and to inhibit angiogenesis *via* binding to neogenin and recruitment of Unc5B [43]. However, since the spacio-temporal expression pattern of netrin-4 is not known, the precise role of netrin-4 in the regulation of angiogenesis remains to be elucidated. Nevertheless, we propose that angiogenesis inhibitors produced by ECs orchestrate and regulate angiogenesis in a complementary manner.

Another issue is the balance of angiogenesis stimulators and inhibitors. The original idea of this balance theory is based on the scenario that angiogenesis is initiated when stimulators are up-regulated and inhibitors are down-regulated [44]. This theory is derived from the idea that angiogenesis inhibitors act as barriers of angiogenesis. However, the scenario is not so simple, as some angiogenesis inhibitors are up-regulated in ECs during angiogenesis, and finely tune this process. Clearly, this theory needs to be re-evaluated depending on the individual inhibitors.

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