Role of activin receptor-like kinase 1 in vascular development and cerebrovascular diseases

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Abstract
Activin receptor-like kinase 1 (ALK1) is a transmembrane serine/threonine receptor kinase of the transforming growth factor beta (TGFβ) receptor superfamily. ALK1 is specifically expressed in vascular endothelial cells, and its dynamic changes are closely related to the proliferation of endothelial cells, the recruitment of pericytes to blood vessels, and functional differentiation during embryonic vascular development. The pathophysiology of many cerebrovascular diseases is today understood as a disorder of endothelial cell function and an imbalance in the proportion of vascular cells. Indeed, mutations in ALK1 and its co-receptor endoglin are major genetic risk factors for vascular arteriovenous malformation. Many studies have shown that ALK1 is closely related to the development of cerebral aneurysms, arteriovenous malformations, and cerebral atherosclerosis. In this review, we describe the various roles of ALK1 in the regulation of angiogenesis and in the maintenance of cerebral vascular homeostasis, and we discuss its relationship to functional dysregulation in cerebrovascular diseases. This review should provide new perspectives for basic research on cerebrovascular diseases and offer more effective targets and strategies for clinical diagnosis, treatment, and prevention.

Key Words: activin receptor-like kinase 1; aneurysm; atherosclerotic plaque; endoglin; extracellular matrix protein; intracranial arteriovenous malformation; matrix metalloproteinase; pericyte; transforming growth factor beta 1 pathway; vascular development

Introduction
During embryonic vascular development, the vascular endothelium senses cardiac pulsations and blood flow, and undergoes endothelial-mesenchymal transition (EndoMT) changes, including cellular budding, resulting in increased ability to hydrolyze extracellular matrix and decreased intercellular junctions. These cells recruit pericytes and smooth muscle cells, and also receive negative feedback, ultimately resulting in the formation of a mature cardiovascular system (Coultas et al., 2005). The transforming growth factor beta (TGFβ) pathway, and particularly activin receptor-like kinase 1 (ALK1), plays an important role in EndoMT changes and pericyte-endothelial cell interactions within blood vessels (Feige and Bailly, 2000; Tillet and Bailly, 2015). Bone morphogenetic protein-9 (BMP9) and BMP10 are endogenous ligands for ALK1 and activate the downstream SMAD1/5/8 signaling pathway to promote the migration of endothelial cells (David et al., 2007). Compared with BMP9 and BMP10, TGFβ isoforms have a lower affinity for ALK1 and ALK5. In contrast to SMAD1/5/8 activation, SMAD2/3 activation induces endothelial cell quiescence (Towner et al., 2012; Jonker, 2014). The biological effects of TGFβ may depend on the ALK1/ALK5 ratio. Overexpression of ALK5 simultaneously promotes the activation of SMAD1/5/8 and SMAD2/3; however, overexpression of ALK1 activates SMAD1/5/8, but inhibits the activation of SMAD2/3. This indicates that the regulation of SMAD1/5/8 is relatively independent of ALK1 expression compared with ALK5 (González-Núñez et al., 2013). The presence of the co-receptor endoglin significantly increases the affinity of BMP9/BMP10 for ALK1 and enhances the activation of SMAD1/5/8 (González-Núñez et al., 2013). To better understand the pathogenesis of cerebrovascular diseases, it is extremely important to elucidate the role of ALK1 in angiogenesis and the maintenance of physiological vascular homeostasis. Therefore, in this review, we focus on the role of ALK1 in angiogenesis, and discuss their relationship with cerebrovascular diseases.

Search Strategy and Selection Criteria
Searches were performed using PubMed, encompassing literature published from 1995 to December 31, 2019. The eligibility criteria were as follows: reviews, in vivo and in vitro studies, studies performed on humans and animals, and published in English. The key search words were as follows: ALK1, ENG, Cerebral arteriovenous malformation, hereditary hemorrhagic telangiectasia, HHT, cardiovascular disease, aneurysm, pericyte, hypertension, atherosclerosis, neural regeneration.
Relationship between Angiogenesis and Activin Receptor-Like Kinase 1 in Embryonic Development

The TGFβ signaling pathway, especially ALK1, plays an important regulatory role in angiogenesis. ALK1 is expressed specifically in endothelial cells. Knockout of the gene for ALK1 in endothelial cells causes vascular dilatation, the disappearance of pericytes (vascular smooth muscle cells and myofibroblasts), and results in abnormal vascular wall development (Lan et al., 2007; Tu et al., 2010; Corti et al., 2011; Chen et al., 2013b; Sweeney et al., 2016). Factors such as changes in shear force generated by blood flow and blood TGFβ1 concentration affect ALK1 activity, which in turn modulates vascular homeostasis and may be involved in the development of various vascular diseases (Winkler et al., 2017).

Cardiac tube pulsation is synchronized with activation of endothelial progenitor cell ALK1, and is involved in the initiation of angiogenesis

The expression of TGFβ receptor members (ALK1, endoglin) on endothelial cells and BMP10 concentration are temporally synchronized with blood island cell entry into the vasculature and vascular shaping (embryonic day 8.5) (Chen et al., 2013a). The increase in local blood flow stimulates endothelial cells at this site and leads to an increase in ALK1 expression (Seki et al., 2003). However, it is still unclear whether blood island cells or changes in luminal pressure induce activation of endothelial ALK1. Deletion of the cardiac excitation-contraction-related genes (mlc2a, NCX1, titin) leads to the weakening or disappearance of the original beating ability of the heart tube, which can perturb the angiogenic process (Koushik et al., 2001; May et al., 2004; Lucitti et al., 2007). It appears that endoglin, rather than ALK1, directly induces changes in blood flow shear force (Seghers et al., 2012). Therefore, ALK1 may induce changes in peripheral blood flow shear force with the help of endoglin, which increases the affinity for ligands and promotes angiogenesis. Studies in zebrafish show that during development, the intravascular pressure is highest at the blind end of the neovascularization duct and is accompanied by high expression levels of endothelial ALK1, which drives the outward migration of endothelial cells (Corti et al., 2011; Rochon et al., 2016; Figure 1).

Pericyte recruitment and differentiation

After progenitor cells in the blood islands enter the circulation, they are recruited to the outer edge of endothelial cells and differentiate into pericytes/vascular smooth muscle cells, which form the middle membrane of the vascular wall. This process depends on ALK1 activation, which regulates the expression of a series of genes in endothelial cells. The extracellular ligand-induced signal is transduced via phosphorylation of cytoplasmic SMAD proteins on C-terminal serines. Phosphorylated, dimerized SMADs bind to the common partner SMAD4, and this heterotrimeric complex translocates to the nucleus, binds DNA, and recruits coactivators or corepressors to upregulate or downregulate gene expression. Similar to ALK1, knockout of SMAD4 in endothelial cells also results in decreased recruitment of pericytes and abnormalities in the vascular wall (Crist et al., 2018). Transplantation of bone marrow from ALK1+/− transgenic mice, a model of cerebral arteriovenous malformation, into normal mice induces local expression of vascular endothelial growth factor (VEGF) and vascular malformations characterized by the loss of pericytes in the vascular wall. This may occur because although ALK1+/−-deficient endothelial progenitor cells recruited by local VEGF can differentiate into vascular cells, depletion of ALK1 cannot induce bone marrow-derived mesenchymal stem cells to accumulate, differentiate, and form normal vascular walls (Chen et al., 2013b). Endoglin also affects the recruitment of pericytes to endothelial cells, which in turn affects the formation of vascular walls (Rossi et al., 2016). It has been reported that blood flow and blood pressure affect the interaction between endoglin and ALK1 and significantly increase the sensitivity of ALK1 to its ligands. This, in turn, results in the activation of SMAD1/5/8 through phosphorylation and facilitates the recruitment of pericytes, which may be related to an impact of ALK1 on platelet-derived growth factor-B (PDGF-B; Stratman et al., 2010; Chen et al., 2013b; Baeyens et al., 2016). Platelet-deprived growth factor-B and its ligand platelet-derived growth factor receptor β are crucial for the formation of middle layer cells in vascular walls (Bjarnegård et al., 2004; Chen et al., 2013b). Genetic loss or mutation of the ligand (e.g., altering its extracellular matrix retention motif) or receptor leads to substantial loss of pericytes and vascular defects (Payne et al., 2019). Studies have shown that pericytes may be derived from CD44+ stem cells in the bone marrow. Therefore, reducing CD44+ cells in vivo may perturb neovascularization (Chan-Ling et al., 2011). TGFβ signaling also controls pericyte differentiation. Activation of ALK5, preferentially expressed in pericytes, leads to phosphorylation of the receptor-regulated SMAD2/3, which translocates to the nucleus after association with SMAD4 and regulates the transcription of specific target genes (e.g., SM22a, fibronectin, connexin 37, and plasminogen activator inhibitor-1) that inhibit cell migration, reduce proliferation, and promote vessel maturation and smooth muscle differentiation (Orlova et al., 2011) (Figures 2 and 3).

Maintenance of vascular homeostasis

During blood vessel formation and maturation, endothelial cells must transition from an activated to a stable state, which depends on feedback from pericytes in the vascular wall. The vascular basement membrane participates in the maintenance of endothelial cell and pericyte functions. Endothelial cells secrete most of the proteins that form the basal membrane, but can also secrete matrix metalloproteinases, whereas pericytes secrete tissue inhibitors of metalloproteinases to inhibit basement membrane hydrolysis (Stratman and Davis, 2012). In tumor neovascularization, pericytes form gap junctions with endothelial cells via the gap junc-
tion protein connexin 43, which promotes endothelial stability (van Dijk et al., 2015).

At the end of vascular remodeling, the levels of BMP10 secreted by the fetal heart are significantly reduced (only small amounts are secreted by the mature heart, liver, and lungs), resulting in decreased ALK1 activity, and the endothelium tends to be static (Chen et al., 2004). TGFβ1 also has different effects on endothelial cells and pericytes. Because TGFβ1 has a greater affinity for ALK5 than ALK1, high concentrations of TGFβ1 activate endothelial ALK1 and ALK5, thereby in turn simultaneously activating SMAD1/5/8 and SMAD2/3. The overall effect is to induce EndoMT-like changes in endothelial cells. However, low concentrations of TGFβ1 tend to stimulate ALK5 in endothelial and smooth muscle cells, thereby activating downstream plasminogen activator inhibitor 1 and SMAD7, which in turn promote the secretion of cellulose and elastic fibers, and ultimately make endothelial cells more static (Van Geest et al., 2010). Lack of ALK5 expression in cerebrovascular pericytes can lead to decreased expression of tissue inhibitors of metalloproteinase 3 and destruction of the blood-brain barrier, leading to diffuse malignant intraventricular hemorrhages (Dave et al., 2018a, b). Currently, the interaction between pericytes and endothelial cells is considered critical to the maintenance of vascular homeostasis; however, the molecular feedback mechanisms that regulate the interaction between these cell types remain unclear and need further study for clarification (Figure 3).

In general, angiogenesis can promote ALK1 expression and the activation of endothelial cells via hemodynamic changes in pressure or shear force and lead to EMT-like changes in endothelial cells. However, as the fetal heart matures, the secretion of BMP10 decreases, and the relatively low TGFβ1 concentrations in the circulation promote the activation of ALK5 in recruited endothelial cells and pericytes, which weakens the ALK1 signal in endothelial cells. The endothelial cells then transition from an activated state to a quiescent state, which helps in the maintenance of vascular homeostasis (Capasso et al., 2019; Otterbein et al., 2019).

ALK1 plays an extremely important role in vascular homeostasis during angiogenesis. Most cerebrovascular diseases are caused by disruption of vascular homeostasis, which in turn impairs normal cellular functions (Winkler et al., 2011). For example, if the balance between lumen pressure and vessel wall stiffness is perturbed, the blood vessel wall grows and the number of pericytes increases gradually, leading to vascular plaques, stenosis, fibrosis, and a series of clinical symptoms. Decreased recruitment of pericytes or their ability to inhibit endothelial cell feedback leads to arterial dysplasia or decreased blood vessel wall stiffness, which is the main pathophysiological change in the formation of aneurysms and arteriovenous malformations, ultimately leading to arterial rupture and bleeding (Winkler et al., 2017, 2018). Therefore, clarifying the relationship between ALK1 expression and functions and the occurrence and progression of cerebrovascular diseases is of great significance for future basic and clinical research in cerebrovascular diseases.

**Relationship between Activin Receptor-Like Kinase 1 in Vascular Endothelial Cells and Cerebrovascular Diseases**

**ALK1 and cerebral arteriovenous malformation**

Cerebral arteriovenous malformation is the leading cause of spontaneous intracranial hemorrhage in young people (Cheng et al., 2019; Shaligram et al., 2019; Winkler et al., 2019). In addition to heterozygous mutations in the ACVR1L gene, which encodes ALK1, that cause type II hereditary telangiectasia, a single nucleotide polymorphism in this gene is also closely associated with sporadic cerebral arteriovenous malformations (Sturiale et al., 2013; Kremer et al., 2016; Weinsheimer et al., 2016). The pathological features of arteriovenous malformation include lack of normal development of capillaries between arteries and veins, significant reduction in cellular (e.g., smooth muscle cells and pericytes) and extracellular matrix components of the arterial vascular membrane, lowered recruitment capacity caused by the reduction of pericytes, and impaired communication between pericytes and endothelial cells. This results in arterial dysplasia or decreased wall toughness, leading to aneurysms and arteriovenous malformations (Winkler et al., 2010, 2011, 2017, 2018).

The formation of the vascular network depends on the budding growth of blood vessels, which requires the migratory ability of endothelial cells and the ability to hydrolyze the basement membrane. In pathological conditions, mutation or decreased expression of ALK1 causes an imbalance in cerebral vascular homeostasis. Under the influence of blood flow, the blood vessels form abnormal vascular clusters and undergo various degrees of expansion, causing cerebral arteriovenous malformations. In this process, deficiency of ALK1 will lead to a decrease in the ability of endothelial cells to germinate, which ultimately leads to a decreased density of the vascular network. ALK1 deletion also affects the recruitment of pericytes. In a mouse model of cerebral arteriovenous malformation, dysplastic blood vessels have no layer of smooth muscle cells. ALK1 knockout reduces the expression of the chemokine PDGF-B in human microvascular endothelial cells after VEGF stimulation and reduces the ability of endothelial cells to recruit pericytes (Chen et al., 2013b, 2014; Zhu et al., 2018). Additionally, ALK1 has a strong impact on endothelial lineage differentiation. The tip cell interacts with stalk cells via Notch signaling to induce stalk cell differentiation. ALK1 upregulates hairy/enhancer-of-split related with YRPW motif proteins 1 and 2 (Itoh et al., 2004), downstream of Notch, by activating SMAD1/5/8 and antagonizing the effect of VEGF (Larrivée et al., 2012). Furthermore, abnormalities in ALK1 affect local inflammation, which is another feature of the formation of cerebral arteriovenous malformations. More macrophages are present in arteriovenous malformation lesions in mice with ALK1 knockout endothelial cells than in endoglin knockout mice (Zhang et al., 2016). Macrophage-secreted matrix metalloproteinase 9 can degrade key components of the cerebral vascular matrix, including laminin, collagen, and cellular tight junction
proteins, leading to leakage and bleeding of the blood-brain barrier (Hashimoto et al., 2003; Chen et al., 2009). Although it is currently unclear how ALK1 abnormalities cause local inflammation in arteriovenous malformations, hypoxia may play a key role. Although blood flow is increased in cerebral arteriovenous malformations, the rarefaction of the capillary network reduces oxygen exchange, leading to hypoxia in the local tissue (Chen et al., 2008; Neyazi et al., 2017). However, whether this is related to increased secretion of inflammatory chemokines and increased recruitment of inflammatory cells remains unclear and is in need of further study.

**ALK1 and cerebral aneurysms**

At present, no study has shown that ALK1 is directly related to the occurrence of clinical cerebral aneurysms. This may be because the treatment of cerebral aneurysms mainly involves craniotomy clipping and intracranial embolization, and consequently, corresponding tissue specimens are lacking. Notably, ALK1 is closely associated with hypertension, one of the major risk factors for cerebral aneurysms. Studies
have shown that TGFβ1, an ALK1 ligand, is closely related to hypertension, and elevated TGFβ1 levels are significantly correlated with target organ damage (Lin et al., 2015; Chen et al., 2019). Serum TGFβ1 is significantly elevated in hypertensive patients with atrial fibrillation. The steady-state levels of TGFβ1 mRNA are also associated with the degree of hypertension (Lin et al., 2015), and hypertensive patients with heart necrosis have higher serum TGFβ1 concentrations than patients without cardiac damage. Furthermore, polymorphisms in the TGFβ1 gene, serum TGFβ1 levels, the severity of hypertension, and hypertension-induced organ damage are correlated (Xi et al., 2012; Ferrario et al., 2013; Ge et al., 2014). Genetically, the 915C single nucleotide polymorphism in the TGFβ type I receptor is associated with increased hypertension risk in European and American populations (Cambien et al., 1996; Lu et al., 2012), and the 869C polymorphism is associated with hypertension risk in Asian populations (Niu, 2011). In animal studies, hypertension is also closely related to elevated concentrations of TGFβ1 in the serum (Tipton et al., 2017). Small molecule drugs against TGFβ1 or anti-TGFβ1 antibodies can antagonize the TGFβ1 signaling pathway to alleviate hypertensive disease in rats (Dahly et al., 2002; Lavoie et al., 2005; Murphy et al., 2012; Liang et al., 2017; Fu et al., 2018). The maturation of elastin microfibril interactor 1 (EMILIN1) can be inhibited by binding to pre-TGFβ, and deletion of EMILIN1 promotes TGFβ1 maturation and stimulation of the TGFβ1 signaling pathway (Zaccigna et al., 2006; Shen et al., 2009). EMILIN1 knockout mice showed increased TGFβ1 signaling in blood vessel walls, as well as hypertension. These EMILIN1 knockout mice have reduced blood vessel diameters, resulting in increased peripheral vascular resistance and elevated blood pressure. In contrast, the combined knockdown of EMILIN1 and TGFβ1 prevents hypertension (Zaccigna et al., 2006; Shen et al., 2009). This indicates that increased activity of the TGFβ pathway is closely related to vascular remodeling. Moreover, ALK1 transgenic mice also show activation of the renin-angiotensin system, reduction of cholinergic neurons, and phenotypically significant hypertension (González-Núñez et al., 2015). Endoglin, the ALK1 co-receptor, has been shown to be associated with neurovascular abnormalities. Endoglin mRNA level changes (Cooke et al., 2018), mutation of the ENG gene (p.A60E) (Santiago-Sim et al., 2009; Ruigrok et al., 2012) and a single-nucleotide polymorphism (rs1800956) (Zhohdybayeva et al., 2018) are significantly correlated with the occurrence of cerebral aneurysms. However, direct empirical evidence of a link between ALK1 and aneurysms is lacking. Therefore, the role of ALK1 in the pathogenesis of cerebral aneurysms requires further study. Whether changes in endoglin affect the susceptibility for cerebral aneurysms through ALK1 also remains unclear.

**ALK1 and atherosclerosis**

Cerebral atherosclerosis is a major senile cerebrovascular disease (Li et al., 2018; Yuan et al., 2019). Studies have shown that ALK1 is expressed in the middle layer of the endothelium, the neointima, and in human coronary atherosclerotic lesions (Yao et al., 2007). ALK1 is highly expressed in blood vessel bifurcations, where increased shear force is present, and these sites are also prone to atherosclerosis. ALK1 expressed by endothelial cells can co-bind low-density lipoprotein-containing ApoB100 in a low-affinity manner and mediate lipid deposition in endothelial cells through endocytosis (Kraehling et al., 2016). This process does not rely on the activation of BMP, endoglin or the ALK1 signaling pathway. However, transgenic mice overexpressing apolipoprotein A1 exhibit upregulation of BMP4 through high-density lipoprotein, and activation of the downstream ALK2/ALK1 and SMAD pathways, and show reduced formation and severity of atherosclerotic plaques (Yao et al., 2008). Therefore, during the process of atherosclerotic plaque formation, ALK1 may have two distinct roles in low-density lipoprotein endocytosis and BMP signaling. The role of ALK1 defects in vascular endothelial cell lipid deposition and atheromatous plaque formation remains to be clarified. The infiltration of vascular smooth muscle cells and macrophages in atherosclerotic plaques may be similar to processes during vascular development. During plaque formation, the expression of chemokines is promoted by ALK1 activation in endothelial cells, which in turn results in the recruitment of circulating macrophages and mesenchymal stem cells. However, this concept needs experimental confirmation.

In addition to endothelial cell lipid deposition, increased proliferation and migration of vascular smooth muscle cells is also involved in the formation of atheromatous plaques (Tong and Qi, 2018; Novikova et al., 2019). Under pathological conditions, vascular smooth muscle cells also express ALK1 to a certain extent. ALK1 can induce the expression of matrix-Gla-protein in vascular mesenchymal cells and promote their proliferation and phenotypic differentiation into smooth muscle cells (i.e., positive for alpha-smooth muscle actin and calponin) (Yao et al., 2007). In patients with chronic kidney disease, high levels of BMP9 induce vascular smooth muscle calcification via the ALK1-SMAD pathway and alkaline phosphatase-dependent mechanisms (Zhu et al., 2015). Because of a lack of studies on ALK1 in smooth muscle cells, further study is required to elucidate how ALK1 signaling pathways in vascular smooth muscle cells affect atherosclerosis. Conditional knockout of the ACVR1 gene in smooth muscle cells in the course of atherosclerosis may help clarify its biological functions.

**Prospects**

ALK1 plays a critical role in angiogenesis and in the maintenance of vascular homeostasis. The occurrence of vascular diseases, especially cerebrovascular diseases, is often closely related to an imbalance in vascular homeostasis. ALK1 also plays an extremely important role in the development of various cerebrovascular diseases. However, there are many unknowns in the study of the relationship between ALK1 and cerebrovascular diseases. For example, the elevated TGFβ1 pathway activity in the vascular wall may be the cause or consequence of hypertension. Furthermore, the endothelial cell-expressed protein that directly senses blood flow chang-
Aortic regenerative therapy is a promising approach for the treatment of cerebrovascular diseases, aiming to improve the functional outcome after stroke. The use of autologous stem cells derived from bone marrow, adipose tissue, or umbilical cord blood has been explored in preclinical and clinical studies. These studies have demonstrated the potential of stem cells to differentiate into vascular cells, promote vessel repair, and improve blood flow by reducing infarct size and improving neurological outcomes.

Recent advances have focused on the development of new cell sources, delivery methods, and therapeutic strategies to enhance the efficacy of aortic regenerative therapy. For instance, the use of mesenchymal stem cells (MSCs) labeled with magnetic nanoparticles has shown promise in improving the delivery and retention of MSCs in the ischemic brain. Additionally, the use of bioresorbable vascular scaffolds loaded with stem cells has been investigated as a means to maintain therapeutic levels of cells in the target area for an extended period.

However, several challenges remain to be addressed before aortic regenerative therapy can be translated into clinical practice. These include the identification of optimal cell sources, the development of efficient delivery systems, the modulation of inflammatory responses, and the establishment of standardized protocols for patient selection and treatment regimens. Continued research is needed to overcome these challenges and to enhance the potential of aortic regenerative therapy for the treatment of cerebrovascular diseases.
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