Arterial Endothelium-Specific Activin Receptor-Like Kinase 1 Expression Suggests Its Role in Arterialization and Vascular Remodeling

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Abstract—Hereditary hemorrhagic telangiectasia (HHT) is an autosomal dominant vascular disorder characterized by epistaxis, mucocutaneous telangiectases, and arteriovenous malformations (AVM). Two genes are linked to HHT: endoglin (ENG) in HHT1 and activin receptor-like kinase 1 (ACVRL1; ALK1) in HHT2. Although both genes are involved in the transforming growth factor β signaling pathways, the pathogenetic mechanisms for HHT remain elusive. It was shown that mutations in the Alk1 gene in mice and zebrafish resulted in an embryonic lethal phenotype due to severe dilation of blood vessels. We created a novel null mutant mouse line for Alk1 (Alk1<sup>bac2</sup>) by replacing its exons, including the one that encodes the transmembrane domain, with the β-galactosidase gene. Using Alk1<sup>bac2</sup> mice, we show that Alk1 is predominantly expressed in developing arterial endothelium. Alk1 expression is greatly diminished in adult arteries, but is induced in preexisting feeding arteries and newly forming arterial vessels during wound healing and tumor angiogenesis. We also show that hemodynamic changes, which require vascular remodeling, may regulate Alk1 expression. Our studies suggest the role of Alk1 signaling in arterialization and remodeling of arteries. Contrary to the current view of HHT as venous disease, our findings suggest that the arterioles rather than the venules are the primary vessels affected by the loss of an Alk1 allele, and that blood vessels with reduction in Alk1 expression may harbor defects in responding to demands for vascular remodeling. (Circ Res. 2003;93:682-689.)

Key Words: activin receptor-like kinase 1 ■ hereditary hemorrhagic ■ telangiectasia ■ angiogenesis ■ vascular remodeling

Hereditary hemorrhagic telangiectasia (HHT), also known as Osler-Weber-Rendu syndrome, is an autosomal-dominant vascular disorder that affects more than 1 in 10 000 individuals. HHT is characterized by recurrent epistaxis, localized mucocutaneous telangiectases in the nasal septum, oral mucosa, and gastrointestinal tract, as well as arteriovenous malformations (AVM) in the lungs, liver, gastrointestinal tract, and brain, which can cause severe ischemic injuries or stroke. Multiple mutations in endoglin (ENG) and activin receptor-like kinase 1 (ACVRL1; ALK1) have been identified for HHT1 and HHT2, respectively. Previous studies have shown that haploinsufficiency of ENG or ALK1 causes HHT. The earliest clinically detectable telangiectasia is focal dilation of postcapillary venules. However, the pathogenetic mechanisms by which reduced expression of Endoglin or ALK1 causes dilation of venules are not clearly understood. Furthermore, the questions of why only limited vascular beds in a HHT patient are affected and what determines the age of onset and the severity of disease manifestation among HHT patients remain to be answered.

Both endoglin and Alk1 are involved in TGF-β signaling. TGF-β family cytokines exert their effects by binding to heteromeric complexes of two types of transmembrane serine/threonine kinase receptors. The type II receptors function primarily as the binding receptors. On binding their ligand(s), type II receptors associate with and phosphorylate the type I receptors, which in turn activate downstream Smad proteins.

Activin receptor-like kinase1 (Alk1) is one of the seven type I receptors for TGF-β family proteins. Alk1 can interact with TGF-β1 and activins. We have previously shown that Alk1 in endothelial cells can bind TGF-β1. In contrast to the signaling pathway of Alk5 (the conventionally known TGF-β1 type I receptor) via Smad2/3, Alk1 transduces the TGF-β1 signal by phosphorylating Smad1 or Smad5.

We have previously demonstrated that Alk1-knockout mice die at midgestation, and exhibit severe vascular abnormalities, which are characterized by excessive fusion of capillary plexus into cavernous vessels and hyperdilation of large vessels. Zebrafish Alk1 mutant, <i>violet beauregarde</i> (vbg), also exhibits blood vessel dilation reminiscent of that of <i>alk1<sup>bac2</sup></i> mice, indicating that the vessel dilation phenotype of the Alk1 mutant mice is not due to an indirect effect of the
hypoxic condition. In addition, Roman et al also showed an increased number of endothelial cells within the affected vessels of vbg mutants. Furthermore, Alk1-knockout embryos show defects in the differentiation and recruitment of vascular smooth muscle cells, suggesting the critical role of Alk1 in the development of vascular smooth muscle cells. These data suggest the role of Alk1 in the inhibition of endothelial cell proliferation and in the stabilization of vessel structure integrity.

In this article, using a novel Alk1-null mutant mouse line in which the bacterial β-galactosidase gene is inserted into the Alk1 locus, we show dynamic regulation of Alk1 expression during embryonic and postnatal development as well as various pathophysiological conditions. These data suggest that Alk1 plays an important role in the arteriolarization, maturation, and stabilization of developing arteries and in the remodeling of the arterial vessels to accommodate physiological demands.

Materials and Methods
Alk1lacZ strain was generated at the University of Florida. C57BL/6j and ICR wild-type mice were purchased from Harlan (Indianapolis, Ind), and Flk+/− mice were purchased from Jackson Laboratory (Bar Harbor, Maine). All animal procedures performed were reviewed and approved by the University of Florida Animal Care and Use Committee.

Immunohistochemistry and X-Gal Staining
Adult mice and embryos were stained with X-gal as described. For immunohistochemical staining, whole-mount X-gal–stained samples were fixed with zinc fixative followed by paraffin embedding. Anti-PECAM (clone: Mec13.3; PharMingen) and anti-smooth muscle α-actin (clone: 1A4; Sigma) antibodies were used for the identification of endothelial and smooth muscle cells, respectively. All other adult tissue samples were cryosectioned at 8 μm thickness and stained with X-gal followed by immunostaining, using the Vectastain staining kit (Vector Laboratory).

Wound Healing Study
Four-month-old mice were anesthetized by intraperitoneal injection with 0.015 mL of 2.5% Avertin per gram of body weight. The back of the mouse was shaved and swabbed with Betadine. Three 4-mm-diameter full-thickness excisional wounds were placed on the mid-dorsum using a biopsy punch. The wounds were left unsutured and were treated with Betadine. The wound areas had completely recovered after 14 days in most cases. The mice were euthanized at six different time points (3, 5, 8, 10, 12, and 14 days after wounding), and full dorsal-side skins were removed and stained with X-gal, followed by postfixation with 10% formalin. Photographs were taken before and after clearing with organic solvent (benzyl benzoate: benzyl alcohol = 1:1).

Formation of Teratoma
To induce teratoma, 0.1 mL of 1×10⁸ wild-type J1 embryonic stem cells in HEPES solution were subcutaneously injected into both the left and right flanks of the Alk1lacZ or Flk−/− mice on 129S4/SvJae or 129/B6 hybrid background. In most cases, the tumor became visible within 2 weeks. At around 3 weeks when the teratomas become 1 cm in diameter, the tumor-bearing mice were euthanized. The dorsal side of skin containing the teratomas was stained with X-gal and then cleared with organic solvent.

Mesenteric Artery Ligation
Adult mice (at least 2 months old) were anesthetized by intraperitoneal injection of 0.015 to 0.017 mL of 2.5% Avertin per gram of body weight. An incision was made in the abdominal skin and muscles to exteriorize the ileum and cecum on sterile surgical drape. The branch of the mesenteric artery supplying terminal ileum was identified, and arterial ligation was made using a sterile 6-0 polyester suture. The abdominal wall was sutured with sterile 5-0 Ethicon Chromic Gut, and the skin was closed with surgical clips. Two days after surgery, the small intestine with mesenteric vessels was stained with X-gal and then cleared with the organic solvent.

An expanded Materials and Methods section is available in the online data supplement at http://www.circresaha.org.

Results
Alk1 Is Predominantly Expressed in Arteries Over Veins
It has been shown by RNA in situ hybridization on histological sections that Alk1 transcripts were detected primarily in blood vessels. By exploiting the β-galactosidase (β-gal) reporter gene under the control of the endogenous Alk1 promoter, we analyzed Alk1 gene expression in embryos and adult organs of Alk1lacZ mice at various stages (see details in the online data supplement, available at http://www.circresaha.org). Consistent with the previous section in situ data, the β-gal activity was detected primarily in the blood vessels of Alk1lacZ embryos throughout development. In E8.5 embryos, the β-gal activity was detected in vitelline veins, the endocardium, the dorsal aorta, and allantoids (Figure 1a). In E9.5 embryos, the β-gal activity was detected in the dorsal aorta and intersomitic arteries as well as in some capillaries (Figure 1b). The β-gal expression pattern of Alk1lacZ was comparable to whole-mount embryos hybridized with Alk1-antisense probe (Figure 1c).

When compared with the evenly distributed β-gal expression in Flk−/− mice (Figure 1d), in which the lacZ gene is inserted into the vascular endothelial growth factor receptor 2 (Kdr; Vegf-r; Flk1) gene locus, the β-gal activity (the Alk1 expression hereafter) in Alk1lacZ mice was detected only in a subset of blood vessels. In E11.5 embryos, Alk1 expression was detected in all major arteries, such as the dorsal aorta, carotid arteries and intersomitic arteries, and capillaries surrounding the neural tube (Figure 1f), but was undetectable in veins. The cross section of whole-mount X-gal staining of E11.5 embryos shows X-gal–positive cells in the endothelium of dorsal aorta, but not in the cardinal veins (Figure 1e).

As the development proceeded, Alk1 expression was observed in small arteries and some capillaries in addition to the major arteries and the arterial-specific expression became more apparent. The best representative example of arterial-specific expression of Alk1 is the blood vessels on the thoracic wall. Figure 1g shows blood-filled internal thoracic veins and arteries running parallel on the thoracic wall. X-gal staining detected the Alk1 expression only in arteries and their connecting small arteries and arterioles, but not in veins or venules (Figure 1h). It is also interesting to note that Alk1 expression in capillaries was selective; Alk1 expression in alveolar (Figure 1i) and glomerular capillaries (data not shown) and in the capillaries surrounding the neural tube (Figure 1f) was strong during development, whereas that in sinusoidal capillaries of the liver (Figure 1i) was undetectable. Because endoglin is expressed uniformly in almost all blood vessels including the liver sinusoids, we tested our expression data using the Northern blot analysis. Total RNA
was isolated from the lung and liver at 1-day-old, 2-week-old, and adult (4 months or older) wild-type mice. Consistent with the X-gal staining results, around 4.5 kb Alk1 transcript was detected in the lung but not in the liver at all three stages (Figure 1l). It is worth mentioning that some hepatic arteries were X-gal positive. To determine which cell type in the blood vessels expresses Alk1, we stained cryosections of E15.5 embryos with X-gal followed by immunostaining with anti-PECAM antibodies for endothelium or anti-smooth muscle \(\alpha\)-actin antibodies for vascular smooth muscle layers. X-gal–positive cells were mostly in the endothelium, indicating that endothelial cells express Alk1 during embryonic development (Figures 1j and 1k).

**Alk1 Expression Is Diminished in Adult Vessels**

Throughout development, Alk1 expression was the highest in the lung (Figure 1i). To determine whether cells expressing Alk1 in the lung are endothelial cells, we stained cryosections of E15.5 embryos with X-gal followed by immunostaining with anti-PECAM antibodies for endothelial cells (j) or anti-smooth muscle actin antibodies for smooth muscle cells (k). Note that X-gal–positive cells (arrowheads) in both aorta (j) and the right carotid artery (rca, k) are in endothelium. l, Northern blot showing Alk1 transcripts in the lungs but not in the liver from wild-type newborn (nb), 2 weeks (2w), and adult (ad) mice. Scale bars in (d, j, and k) are equal to 100 \(\mu\)m.

Figure 1. Arterial blood vessel–specific Alk1 expression during embryonic development. a, Dark-field image of E8.5 embryo stained with X-gal. Dorsal aorta (da), endocardium (ec) and allantois (al), and vitelline veins (vv) are shown X-gal positive. b, Whole-mount X-gal staining of E9.5 Alk1\(^{lacZ}\) embryo followed by clearing. c, Whole-mount in situ hybridization of E9.5 embryo with anti-Alk1 RNA probe. d, Whole-mount X-gal staining of E9.5 Flk1\(^{lacZ}\) embryos. e, Histological section of X-gal–stained E11.5 embryo, demonstrating that dorsal aortas are X-gal positive, but cardinal veins (cv) are not. n indicates notochord. f, Whole-mount X-gal staining of E11.5 Alk1\(^{lacZ}\) embryo, showing X-gal–positive staining in the dorsal aorta, intersomatic arteries (isa), and capillaries surrounding the neural tube (*). g and h, Blood vessels on the thoracic wall of newborn pups before (g) and after (h) X-gal staining. Similar sizes of internal thoracic arteries (ita) and veins (itv) were running side-by-side in unstained sample (g), whereas only arteries and connecting small arteries were X-gal positive (h). X-gal–positive staining in the sternal skeleton (*) is from the endogenous \(\beta\)-gal activity. i, Whole-mount X-gal staining of E18.5 Alk1\(^{lacZ}\) embryo, showing intensive expression in the lung and major arteries, but not in the liver. ica indicates internal carotid artery; eca, external carotid artery; va, vertebral artery; and sa, spinal artery. j and k, Endothelial cell–specific Alk1 expression. Cryosections of E15.5 Alk1\(^{lacZ}\) embryos were stained with X-gal followed by anti-PECAM antibodies for endothelial cells (j) or anti-smooth muscle actin antibodies for smooth muscle cells (k). Note that X-gal–positive cells (arrowheads) in both aorta (j) and the right carotid artery (rca, k) are in endothelium. l, Northern blot showing Alk1 transcripts in the lungs but not in the liver from wild-type newborn (nb), 2 weeks (2w), and adult (ad) mice. Scale bars in (d, j, and k) are equal to 100 \(\mu\)m.
Alk1 Is Induced in Adult Mice During Angiogenesis

To investigate whether Alk1 is more specific for nascent vessels, we studied Alk1 expression in blood vessels during skin wound healing and tumor growth in adult mice. Three 4-mm-diameter excisional wounds were made on the backside of adult Alk1$^{+/+}$ or Flk1$^{+/+}$ mice. The wound areas were completely closed by day 14 in most cases. The mice were euthanized at six time points (3, 5, 8, 10, 12, and 14 days after wounding), and full dorsal skins were removed and stained with X-gal, followed by clearing with organic solvents. The healing processes can be roughly divided into two stages by 1-week intervals: early and late. In the early stage, a tremendous number of newly formed capillary-like vessels developed in the rim of skin surrounding the wound. In the late stage, small arteries and veins were established and converged at the center of the incision as the wound closed.

On day 3, sparse Alk1 expression was found in small blood vessels at the rim of wound (Figure 3a). On day 5, Alk1 was found to have been induced in the preexisting arteries supplying the wound area and in the newly branching small arteries (Figure 3b), but its expression in the capillary-like vessels of the wound rim area was minimal compared with Flk1 expression (Figure 3c). As the wounds closed in the late stages, newly forming blood vessels converging at the center of the wound expressed Alk1. Lower magnification images show that these neovascular vessels were connected to feeding arteries (online Figure 2). The number of these Alk1-expressing arterial vessels began to increase on day 8 (Figure 3d) and culminated on day 10 (Figure 3e). Alk1-expressing vessels on days 8 and 10 were fewer in number but comparable to Flk-expressing vessels at the same stages (Figure 3f). Alk1-expressing vessels were slightly decreased on day 12 (Figure 3g), and on day 14, when the wound was completely closed, Alk1 expression was almost completely diminished (Figure 3h). Figure 3i shows that neovascular vessels converging to the center of the wound were still present at this stage. We also investigated Alk1 expression in the blood vessels during tumor angiogenesis. We injected J1 wild-type ES cells subcutaneously into Alk1$^{+/+}$ or Flk1$^{+/+}$ mice. When the tumor reached a size of about 1 cm in diameter after 2 weeks, the tumor and surrounding skin were collected and stained with X-gal. Similar to the wound model, Alk1 expression was detected in the arteries feeding the tumors (Figure 4a). Alk1 expression was detected only in large arteries (Figure 4b), whereas Flk1 expression was detected in large and small arteries as well as in capillary-like vessels in the tumor (Figure 4c).

Figure 3. Induced Alk1 expression in the blood vessels during wound healing. Full-thickness wounds were generated by surgical biopsy punch on back skin of Alk1$^{+/+}$ (a, b, d, e, g, and h) and Flk1$^{+/+}$ (c, f, and i) adult mice. Skins were stained with X-gal at the indicated days after injury, followed by clearing. All photographs were taken in the same magnification. Arrowheads in panel b indicate sprouting arterial vessels. w indicates wound.
We demonstrate that Alk1 is differentially expressed in the wound healing, tumor growth, and hemodynamic challenges of postnatal life, and pathophysiological conditions, including physiological factors such as blood flow (shear stress) or pressure (cyclic strain) during remodeling of the feeding arteries, because the blood flow is increased in the arteries supplying the wounds.

To test this, we used a mesenteric artery ligation model, which displays arterial wall remodeling associated with shear-mediated luminal expansion. In this rat model, blood flow, but not pressure, was significantly increased in the counter region of the second-order arterial branch. Ligation was made at a second-order branch of the mesenteric artery supplying ileum (Figure 5b), and we examined the Alk1 expression in whole mesenteric vessels by X-gal staining 2 days after ligation. X-gal–positive vessels were found in the marginal arteries running from the cecum area, away from the affected hypoxic area. Close examination of arterial connections to the ileum indicated that the X-gal–positive artery was the only vessel supplying blood to the hypoxic area on ligation (Figures 5b and 5c). Whereas the mechanism involved in the induction of Alk1 in preexisting arteries during wound healing and mesenteric artery ligation model remains uncertain, these findings collectively suggest that Alk1 may play an important role in the remodeling and stabilization of arteries in response to blood flow increase.

**Discussion**

We created a novel lacZ knock-in mouse line in the Alk1 gene locus and studied the expression of Alk1 during development, postnatal life, and pathophysiological conditions, including wound healing, tumor growth, and hemodynamic challenges. We demonstrate that Alk1 is differentially expressed in the endothelium of arteries over veins. We also show that Alk1 is expressed in the subset of blood vessels. For example, Alk1 expression is high in the capillaries of the lung, but undetectable in the sinusoidal endothelium of the liver. In addition, Alk1 expression is diminished in blood vessels of adult mice as their growth slows, but is induced in the neovascular endothelium during wound healing and tumor growth and in the arteries corresponding to increased blood flow.

Genetically engineered mouse strains in which a reporter gene is inserted into a locus of interest have been invaluable resources in the study of detail expression patterns of the genes of interest. Although this genetic approach is powerful and widely used, it is possible that genetic manipulations may create some alterations in regulatory sequences, and thus the reporter gene expression may not faithfully reflect the endogenous gene expression. Therefore, it is important to verify findings from genetic approaches with those from other conventional methods. The Alk1 expression pattern in E9.5 embryos detected by whole-mount in situ hybridization using antisense RNA grossly overlaps with the β-galactosidase expression pattern (Figures 1b and 1c). The lacZ expression patterns are also consistent with other expression studies published. Roelen et al have shown that Alk1 is mostly expressed in the developing vasculature. The RNA probe detected Alk1 transcripts mostly in arteries but not in veins. Antibody staining also identified the Alk1 protein predominantly in arterial, as opposed to venous, endothelium. Studies consistently showed that Alk1 transcript or protein was highest in the lung, but undetectable in the liver.

Considering the variations in sensitivity, species, and the stability of mRNA or proteins, we anticipate some minor differences between genetic approaches and conventional staining methods. Nonetheless, our findings are consistent with the previous results, suggesting that the gene replacement in the Alk1 locus in the Alk1lacZ strain did not alter or delete major regulatory sequences.

**Role of Alk1 in Angiogenesis**

Angiogenesis refers to the process of neovascularization by sprouting from preexisting blood vessels. Angiogenesis consists of two phases: activation and resolution. In the activation phase, the perivascular basement membrane is degraded, and endothelial cells proliferate, migrate, and form capillary lumen. In the resolution phase, endothelial cells cease to proliferate and migrate, and reconstitute basement membrane.

The role of Alk1 in angiogenesis is controversial. Based on the observation of severe dilution of blood vessels, defective differentiation of vascular smooth muscles, and upregulation of angiogenic factors in Alk1−/− embryos, we have previously suggested that Alk1 signaling plays an important role in the resolution phase of angiogenesis. Recently, Goumans et al suggested that Alk1 signaling promotes endothelial cell migration and proliferation by up-regulating Id1, a transcription factor promoting vascular growth through repressing an inhibitor of angiogenesis, thrombospondin-1. Gene expression profiling studies on human umbilical vein endothelial cells expressing a constitutively active form of Alk1 confirmed the data that Id1 and Id2 are specifically upregulated by Alk1 transfection.
In contrast, Lamouille et al. have shown that transfection of a constitutively active form of Alk1 inhibits the proliferation and migration of endothelial cells. The microarray experiment in the same study suggests that Alk1 inhibits endothelium proliferation by upregulating p21\(^{waf1}\) and downregulating c-myc protooncogene. Consistent with this result, a zebrafish Alk1 mutant, vbg, whose vessel dilation phenotype is reminiscent of that of the Alk1\(^{-/-}\) mice, showed an increased number of endothelial cells within the affected vessels, suggesting the role of Alk1 in the inhibition of endothelial cell proliferation and in the stabilization of vessel structure integrity.

Alk1 expression patterns during wound healing in the present study also support this notion. During the early stages of wound healing, a tremendous number of capillaries had formed in the rim area surrounding the wound. If Alk1 signaling were important for the activation phase of angiogenesis, high expression of Alk1 would be expected in these capillary vessels. Contrary to this, the Alk1 expression in these capillaries was almost undetectable (Figures 3a and 3b), supporting the role of Alk1 in the resolution phase rather than the activation phase of angiogenesis.

**Role of Alk1 Signaling in Arterialization and Vascular Remodeling**

Molecular mechanisms underlying the determination of arterial and venous vessels have been a fascinating subject. Recent identification of genes that are differentially ex-

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**Figure 5.** Alk1 induction is correlated with the increased blood flow. a, X-gal-stained skin showing induced Alk1 expression in preexisting feed arteries, but not veins, converging on the periphery of the wound (w) at day 5 after injury. Note that the branching arteries (blue arrowheads), but neither veins (v) nor trunk arteries (red arrowheads), are X-gal positive. b through d, Mesenteric artery ligation model, showing Alk1 expression in the arteries corresponding to feed arteries to the hypoxic area. Ligation was made at a second-order branch of mesenteric artery supplying ileum, indicated by an arrow. Predicted blood flow pattern of before and after the ligation is indicated by arrowheads in the diagram (b). Ligation at a second-order branch causes ischemic injury to the downstream ilium area and may lead to increased blood flow (blue arrow) in the arteries feeding from the cecum side. Alk1 expression was detected in the feed arteries from the cecum side (blue arrowheads in panel c), whereas mesenteric arteries in other areas were X-gal negative (d).
pressed in arteries over veins sheds light on this subject. These genes include notch ligandDll4, basic helix-loop-helix transcription factor gridlock, and membrane-bound ligand ephrin-B2. It was shown that gridlock favors the differentiation of common angioblasts to prearterial angioblasts. Notch signaling, which is associated with cell fate determination through lateral specification, may also be involved in the differentiation of arterial versus venous endothelium. Membrane-bound ephrine-B2 and its receptor EphB4 show restricted expression in arteries and veins, respectively, and their bidirectional interactions are required for proper angiogenesis.

Arterial endothelium-specific expression of Alk1 suggests that Alk1 signaling may play an important role in arterialization. It is plausible that Alk1 as the TGF-β receptor in endothelium may mediate the TGF-β signal, which involves in synthesis of perivascular matrix proteins and stimulation of the differentiation and recruitment of periendothelial cells, such as pericytes and vascular smooth muscle cells. Supporting this view, Alk1−/− mice have defects in vascular smooth muscle cell differentiation and recruitment. Zebrafish Alk1 mutant, vbg, exhibited vascular dilation reminiscent of the vascular phenotype of mouse Alk1−/−. Because oxygen supply in Zebrafish embryos is not dependent on blood vessels, this vessel dilation phenotype is not secondary to hypoxia. Interestingly, the vascular dilation in vbg embryos is reportedly concomitant to the time of the strengthening of heartbeats. It may indicate that the mutant blood vessels contain an intrinsic defect in accommodating hemodynamic challenges. Restricted expression of Alk1 to large arteries only during rapid growth stages also indicates that Alk1 signaling plays a role in the maturation of arterial vessels.

Various physiological factors, such as blood flow (shear stress) or pressure (cyclic strain), have been shown to trigger the remodeling of blood vessels, which involves the synthesis of extracellular matrix proteins and the proliferation of vascular smooth muscle cells. Induced Alk1 expression in preexisting feed arteries distant from the wound and tumor during wound healing and tumorigenesis (Figures 3 and 4), as well as in the small mesenteric arteries corresponding to the increase of blood flow (Figure 5), suggest that Alk1 signaling is also involved in the vascular remodeling of arteries. Our data are consistent with the findings that TGF-β and Alk1 expression was elevated in the pulmonary vessels with increased blood flow, and that increased TGF-β1 mRNA and protein from endothelial cells were stimulated by laminar shear stress. Detailed pharmacological studies with isolated arteries from wild-type and Alk1−/− mice would provide further insights on the mechanism of Alk1 upregulation as well as the function of Alk1 signaling in vascular remodeling.

Implication of the Role of Alk1 in HHT

Multiple mutant alleles in endoglin and Alk1 genes have been identified for both HHT1 and HHT2, respectively. HHT1 is generally considered more severe than HHT2, as the frequency of pulmonary arteriovenous malformation is significantly higher in HHT1 than in HHT2. However, the highly variable age of onset and expressivity of the disease make it difficult clinically to distinguish one type of HHT from the other. Variability of disease manifestations among HHT patients cannot be explained by allelic heterogeneity alone, because a similar extent of variability is also found among HHT patients within single kindreds. This observation indicates that genetic modifiers may play an important role in disease manifestation. However, endoglin heterozygous mice on 129/Ola inbred strain display HHT phenotypes in a highly variable pattern, indicating that genetic modifier may play a limited role in variable disease manifestations. In addition, the fact that only selective vascular beds in a HHT patient develop telangiectasis or AVM lesions while other vascular beds remain unaffected suggests that the haploinsufficiency of ENG or ALK1 genes is required but not sufficient for the development of vascular lesions in HHT patients.

What makes the blood vessels reduced in activity of ENG or ALK1 vulnerable to develop vascular lesions? This is an important question that remains to be answered. Based on our expression studies, we may dare to speculate as follows: if Alk1 signaling is involved in arterialization, it is conceivable that an HHT patient’s normal-appearing vascular beds may harbor some hidden defects. The subnormal response of these imperfect vessels to environmental factors (or “second hits”), which require vascular remodeling or stabilization, might lead to the development of telangiectasia or AVM. Epistaxis and mucocutaneous telangiectasia are the most common symptoms among variable disease manifestations of HHT. This is suggestive that the superficial vessels in direct contact with the environment are more prone to “second hits” compared with other occult vessels. Such “second hits” may include infections and minor injuries, which subsequently give rise to inflammation, immune response or hypoxia, and which eventually result in hemodynamic challenges.

Dilation of the postcapillary venules observed early in the pathogenesis of HHT has favored the veins over the arteries as the origin of vessel defect. However, the arterial-specific Alk1 expression in our study suggests that the primary vessels affected in HHT2 could be arteries rather than veins and the venous dilation could be secondary to defects in arteries. The expression of endoglin was observed in the endothelium of all vessel types, including venous and sinusoidal endothelial cells where Alk1 expression was undetectable in normal conditions. What would be the function of endoglin in these vessels without Alk1? Perhaps this difference suggests that endoglin may have functions in the endothelium other than mediating the TGF-β signal. If this is so, the question of whether these other functions of endoglin are involved in the pathogenesis of HHT remains to be answered.

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References


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Both Alk1\textsuperscript{neo} and Alk1\textsuperscript{lacZ} alleles are null mutations.

We previously demonstrated that a targeted insertion of the neomycin resistant gene into exon 8 of the \textit{Alk1} gene (Alk1\textsuperscript{neo}), which encodes the intracellular kinase domain, resulted in embryonic lethality around embryonic day 10.5 (E10.5) with severe vascular malformations\textsuperscript{1}. Alk1\textsuperscript{+/neo} mice exhibited HHT-like vascular abnormalities, suggesting that this mutant strain could be a useful animal model to study HHT. Since extracellular and transmembrane domains are intact in the Alk1\textsuperscript{neo} allele (Figure 1a), however, a question was raised as to whether the Alk1\textsuperscript{neo} allele is truly null. Urness et al. demonstrated similar embryonic lethal phenotype of Alk1-null mice with Alk1\textsuperscript{neo/neo}, supporting that Alk1\textsuperscript{neo} is a null mutation\textsuperscript{2}. However, due to differences in approaches used for characterizing the vascular phenotype of these two mutant strains, it is difficult to conclude that these two mutant strains had identical phenotypes. To ensure that Alk1\textsuperscript{neo} was null, we established a novel null mutant mouse line for Alk1 in which 2.3 kb of Alk1 genomic DNA was replaced with the bacterial β-galactosidase (lacZ) gene (Figure 1a). Since the deleted region includes exons 4-8 of the Alk1 gene encoding ligand binding, transmembrane and type I receptor-specific GS domains, the Alk1\textsuperscript{lacZ} allele is likely to be a null allele. Homologous recombination in embryonic stem cells was confirmed by genomic Southern blot, and a targeted Alk1\textsuperscript{lacZ} mouse line was established by blastocyst injection (Figure 1b).

Homozygous knockout mice, Alk1\textsuperscript{lacZ/lacZ}, die around E9.5-E10.5 with severe vascular defects indistinguishable from Alk1\textsuperscript{neo/neo} embryos\textsuperscript{1} (Figure 1c, d, f and g), suggesting that Alk1\textsuperscript{neo} is also a null allele. To further confirm this notion, we analyzed compound heterozygous mice, Alk1\textsuperscript{neo/lacZ}, by crossing Alk1\textsuperscript{+/lacZ} and Alk1\textsuperscript{+/neo} mice.
(Figure 1b). We found that $Alk1^{neo/lacZ}$ embryos were also embryonic lethal around E9.5-E10.5 and were indistinguishable to $Alk1^{lacZ/lacZ}$ or $Alk1^{neo/neo}$ embryos (Figure 1c, d). Therefore, we concluded that both $Alk1^{lacZ}$ and $Alk1^{neo}$ are null mutant alleles of the $Alk1$ gene.

Materials and Methods

Generation of the $Alk1^{lacZ}$ mouse line

A mouse ES-129/SvJ BAC clone containing the $Alk1$ gene was identified and purchased (Genome Systems, St. Louis, MO). A HindIII-HindIII 2 kb fragment containing exon 3, the SIBN cassette and a 5.8 kb XhoI-BamHI fragment containing exons 8-10 were subcloned into pBluescript II. The SIBN cassette contains SV40 splice donor and acceptor signals, internal ribosomal entry sequence, $\beta$-galactosidase cDNA with poly(A) signal, and neomycin resistant gene driven by the PGK promoter. Electroporation, G418 selection, and the Southern hybridization screening were done as previously described. 12 of positive ES clones were obtained from 192 of G418 resistant clones. ES cells from a positive line were injected into blastocysts on C57BL/6 (B6) strain. Chimeric male mice were mated with females on either B6 or 129S4/SvJae (129) backgrounds. Heterozygous mice from the 129 strain were kept as a 129 inbred line. We found that there was no difference in expression pattern and mutant phenotype between 129 inbred and 129/B6 hybrid lines. We used 129/B6 hybrid lines for expression studies except the tumor angiogenesis studies.
FIGURE LEGEND

FIGURE 1  Alk1\textsuperscript{neo} and Alk1\textsuperscript{lacZ} are null alleles. (a) Schematic diagram of wild-type allele, LacZ replacement vector, Alk1\textsuperscript{lacZ} allele and Alk1\textsuperscript{neo} allele. Exons and their numbers are indicated by grey boxes and arabic numerals, respectively. We found that exon 1 of the human ALK1 gene is split into two exons (1 and 2) in the mouse Alk1 gene. Exons 3-11 of mouse Alk1 correspond to exons 2-10 of human ALK1. The translation start site in murine exon 3 was indicated by the asterisk mark. Exons 4-8 were deleted and replaced with the IRES-lacZ/PGK-neo\textsuperscript{c} cassette. Note that exon 5 encoding the transmembrane domain (arrow head) is deleted in the Alk1\textsuperscript{lacZ} allele to produce null mutation. The 5’ external probe recognizes different sizes of EcoRI digested DNA in the Southern blot analysis: wild type, 9.7kb; Alk1\textsuperscript{lacZ}, 2.8kb; Alk1\textsuperscript{neo}, 7.2kb. B, BamHI; E, EcoRI; H, HindIII; Xh, XhoI. (b) Genomic Southern analysis of individual embryos from a Alk1\textsuperscript{+/lacZ} cross (left panel) and Alk1\textsuperscript{+/lacZ} with Alk1\textsuperscript{+/neo} crosses (right panel). EcoRI digested genomic DNAs were hybridized with the 5’ external probe. Genotypes of individual E9.5 embryos are indicated on the top of each lane. The compound heterozygous embryo (Alk1\textsuperscript{neo/lacZ}) is indicated with asterisk. (c, d) Gross morphology of wild-type and mutant E9.5 embryos with (c) or without (d) the yolk sac. Note that the compound heterozygous embryo (Alk1\textsuperscript{neo/lacZ}) is indistinguishable from the Alk1\textsuperscript{lacZ/lacZ} embryo. (e-g) Histological sections of X-gal stained Alk1\textsuperscript{+/+}; Flk\textsuperscript{+/lacZ} (e), Alk1\textsuperscript{neo/neo}; Flk\textsuperscript{+/lacZ} (f), and Alk1\textsuperscript{lacZ/lacZ} (g) E9.5 embryos. Endothelial cells in Alk1\textsuperscript{+/+}; Flk\textsuperscript{+/lacZ} and Alk1\textsuperscript{neo/neo}; Flk\textsuperscript{+/lacZ} embryos are stained in blue by β-galactosidase driven by the Flk1 promoter. Note the dilated dorsal aorta (da) in both Alk1\textsuperscript{neo/neo} (f) and
Alk1lacZ/lacZ (g) embryos. fg, foregut. Scale bar in (e) is equal to 100 µm and applicable to (f) and (g).

FIGURE 2. Alk1 expression in neovascular arteries in day 10 post-wounding. (a, b) X-gal stained skin containing a wound site before (a) and after (b) clearing with the organic solvent. Arrows indicate the connection of arterial vessels. a, artery; v, vein. (c) A higher magnification of the wound area indicated by the red box in (b), showing X-gal positive newly-formed vessels are arterial vessels. The scale bar in each panel is equal to 1 mm.

REFERENCES


