Published in final edited form as:
Stroke. 2014 March ; 45(3): 900–902. doi:10.1161/STROKEAHA.113.003655.

De novo cerebrovascular malformation in the adult mouse after endothelial *Alk1* deletion and angiogenic stimulation

Wanqiu Chen, PhD[1], **Zhengda Sun, MD, PhD**[2], **Zhenying Han, MD, PhD**[1], **Kristine Jun, BS**[1], **Marine Camus, MS**[1], **Mamta Wankhede, PhD**[1], **Lei Mao, MD**[1], **Tom Arnold, MD**[3], **William L. Young, MD**[1,3,4], and **Hua Su, MD**[1]

[1]Center for Cerebrovascular Research, Department of Anesthesia and Perioperative Care, University of California, San Francisco, California

[2]Department of Radiology, University of California, San Francisco, California

[3]Department of Neurological Surgery, University of California, San Francisco, California

[4]Department of Neurology, University of California, San Francisco, California

Abstract

Background and Purpose—In humans, activin receptor-like kinase 1 (*Alk1*) deficiency causes arteriovenous malformations (AVMs) in multiple organs, including the brain. Focal *Alk1* pan-cellular deletion plus vascular endothelial growth factor (VEGF) stimulation induces brain AVMs (bAVMs) in the adult mouse. We hypothesized that deletion of *Alk1* in endothelial cell (EC) alone plus focal VEGF stimulation is sufficient to induce bAVM in the adult mouse.

Methods—Focal angiogenesis was induced in the brain of eight-week-old *Pdgfb*-iCreER;*Alk1*$^{2f/2f}$ mice by injection of adeno-associated viral vectors expressing VEGF (AAV-VEGF). Two weeks later, EC-*Alk1* deletion was induced by tamoxifen (TM) treatment. Vascular morphology was analyzed, and EC proliferation and Dysplasia Index (number of vessels with diameter >15μm per 200 vessels) were quantified10 days after TM administration.

Results—Tangles of enlarged vessels resembling AVMs were present in the brain angiogenic region of TM-treated *Pdgfb*-iCreER;*Alk1*$^{2f/2f}$ mice. Induced bAVMs were marked by increased Dysplasia Index ($P<0.001$), and EC proliferation clustered within the dysplastic vessels. AVMs were also detected around the ear tag-wound and in other organs.

Conclusions—Deletion of *Alk1* in EC in adult mice leads to an increased local EC proliferation during brain angiogenesis and de novo bAVM.

Keywords

animal model; AVM; EC proliferation; hereditary hemorrhagic telangiectasia-2

Correspondence to: Hua Su, MD, University of California, San Francisco, Dept of Anesthesia and Perioperative Care, 1001 Potrero Avenue, Box 1363, San Francisco, CA 94110, Phone: 415-206-3162, Fax: 415-206-8907, hua.su@ucsf.edu.

Disclosures: None

Introduction

Brain AVMs cause life-threatening intra-cranial hemorrhage, but bAVM pathobiology is poorly understood. Previously, we induced a bAVM phenotype in adult mice by focal pan-cellular *Alk1* gene deletion and VEGF stimulation.[1] Alk1 is predominantly expressed in the arterial EC.[2] We tested the hypotheses that deletion of *Alk1* in EC is sufficient to cause bAVM after angiogenic stimulation, and that increased growth of *Alk1*-deficient EC is a key mechanism underlying brain AVM development.

Methods

Detailed methods are described in the online-only Data Supplement. Experimental procedures for using laboratory animals were approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco and conformed to the NIH Guidelines for the care and use of animals in research.

Alk1$^{2f/2f}$ mice (*Alk1* exons 4-6 flanked by loxP sites)[3] were bred with *Pdgfb*-iCreER mice that express tamoxifen (TM)-inducible cre recombinase (iCreER) in EC.[4] EC-*Alk1* deletion was induced[5] by intra-peritoneal injection of TM 14 days after intra-brain injection of AAV serotype 1-packaged AAV-VEGF with CMV promoter driving VEGF expression. Mice with *NG2* promoter (*NG2*-iCreER) driving Cre expression were used to induce pericyte-*Alk1* deletion. Vascular morphology was analyzed using latex casting and immunostaining 10 days after TM administration. EC proliferation and Dysplasia Index were quantified. Experimental design and groups are shown in Figure 1. Data are mean±SD.

Results

EC-*Alk1* deletion resulted in AVM in the brain angiogenic region and other organs

Alk1^{iECKO}+VEGF mice developed tangles of irregular vessels in the brain angiogenic region, whereas *Alk1*^{iECKO}+LacZ or WT+VEGF did not (Figures 2A & B). The Dysplasia Index was higher in the *Alk1*^{iECKO}+VEGF group (11.5±4.1) than in the WT+VEGF (1.2±0.4, P<0.001) and *Alk1*^{iECKO}+LacZ (1.8±1.2, P<0.001) groups (Figure 2C). Macrophage infiltration and microhemorrhage were observed around the malformed vessels (Supplementary Figure I). EC-specificity of *PDGFb*-iCreER was confirmed in the brain of *Alk1*^{iECKO}+VEGF mice (Figure 1B) using Ai14 cre reporter (Supplementary Figure II).

AVMs also developed in the intestine, lung, and around ear-tag wounds of *Alk1*^{iECKO}+VEGF and *Alk1*^{iECKO}+LacZ mice. *Alk1*^{iECKO} mice had pale paws and darkened feces indicating anemia and gastrointestinal bleeding, and died 6-13 days after TM administration (Supplementary Figures III and IV).

EC proliferation increased in bAVM

Compared to WT+VEGF mice, *Alk1*^{iECKO}+VEGF mice had more newly proliferated ECs ($BrdU^+/ERG^+$, 285±48 vs. 91±25 cells/mm^2, P<0.001, Figure 3) and a higher vessel density in the brain angiogenic region (P<0.001, Supplementary Figure V). The newly proliferated ($BrdU^+$) and proliferating ($ki67^+$) ECs were clustered around dysplastic vessels (Figure 3).

Pericyte-*Alk1* deletion did not cause AVM

NG2-iCreER was used to delete pericyte-*Alk1*. The cre activity in pericytes was confirmed using Ai14 reporter (Supplemental Figure VI-A). No AVM was detected in adult *NG2*-iCreER;$Alk1^{2f/2f}$ mice (Supplementary Figure VI-B).

Discussion

Previously, we reported that injection of AAV-VEGF into the brain of adult $Alk1^{+/-}$ mice induces a capillary level of dysplasia,[6] which served as a surrogate model for bAVM until we developed a macroscopic level of a bAVM model through focal homozygous *Alk1* deletion plus VEGF stimulation.[1] In that model, an adenoviral vector that has CMV promoter driving cre expression (Ad-Cre) was used to induce focal pan-cellular *Alk1* deletion.[1] Here, we show that deletion of *Alk1* in adult EC results in a bAVM phenotype similar to that in the pan-cellular *Alk1* deletion model. Both have macrophage infiltration and microhemorrhage.[7] EC-specific *Alk1* deletion in the adult mouse has also resulted in AVM in the small intestine and lung and around the skin wound, which recapitulated the phenotype of global *Alk1* deletion in adult mice.[5] Thus, deletion of *Alk1* in adult EC is sufficient to induce AVMs.

SM22α—Cre-driven *Alk1* deletion has been reported to result in brain and spinal cord AVMs,[8] suggesting that loss of ALK1 from pericytes/vascular smooth-muscle cells can cause AVMs. In contrast, we find that conditional deletion of *Alk1* in adult pericytes did not trigger AVMs in any organ, around the ear-tag wound, or in the VEGF-stimulated brain. As revealed by Rosa-LacZ cre reporters, SM22α promoter driving cre expression results in non-specific recombination in EC-lineage.[8] Therefore, we propose that *Alk1* deletion in pericytes alone is not sufficient and that gene deletion in EC is required to initiate AVM development.

We found that deletion of *Alk1* in ECs in the adult brain increased EC proliferation in response to VEGF stimulation. Although the expression of Ai14 reporter indicated that cre was activated in ECs of all vessels in the angiogenic region, only a few dysplasia vessels were formed. The dysplastic vessels have more proliferating ECs than the surrounding normal capillaries. As it is statistically unlikely that the dominance of the proliferating cells in dysplastic vessels is the result of multiple independent Cre recombination events, we propose that ECs with homozygous *Alk1* deletion undergo clonal expansion and have a higher proliferation rate than WT EC or $Alk1^{+/-}$ EC, which results in unevenly enlarged abnormal vessels. This hypothesis needs to be further validated.

In summary, deletion of *Alk1* in EC leads to increased focal EC proliferation during brain angiogenesis and de novo AVM development. Knowledge of the importance of EC in AVM development will help in understanding AVM pathogenesis and in designing specific therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

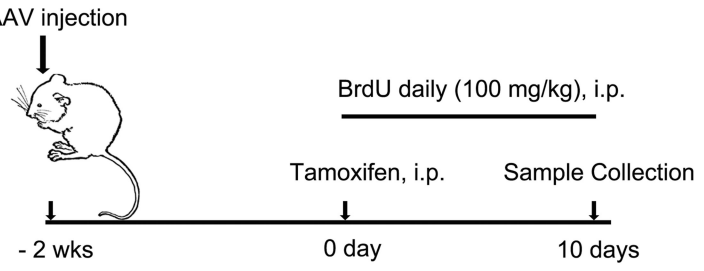
We thank Voltaire Gungab for assistance with manuscript preparation, S. Paul Oh at the University of Florida for providing *Alk1*$^{2f/2f}$ mice, and members of the UCSF BAVM Study Project (http://avm.ucsf.edu) for their support.

Sources of Funding: Supported by grants to H.S. from the National Institutes of Health (R01NS027713 and P01NS044155), and from the Leslie Munzer Foundation. Additional support was provided by a grant to W.L.Y. from the Michael Ryan Zodda Foundation.

References

1. Walker EJ, Su H, Shen F, Choi EJ, Oh SP, Chen G, et al. Arteriovenous malformation in the adult mouse brain resembling the human disease. Ann Neurol. 2011; 69:954–962. [PubMed: 21437931]
2. Seki T, Yun J, Oh SP. Arterial endothelium-specific activin receptor-like kinase 1 expression suggests its role in arterialization and vascular remodeling. Circ Res. 2003; 93:682–689. [PubMed: 12970115]
3. Park SO, Lee YJ, Seki T, Hong KH, Fliess N, Jiang Z, et al. ALK5- and TGFBR2- independent role of ALK1 in the pathogenesis of hereditary hemorrhagic telangiectasia type 2 (HHT2). Blood. 2008; 111:633–642. [PubMed: 17911384]
4. Claxton S, Kostourou V, Jadeja S, Chambon P, Hodivala-Dilke K, Fruttiger M. Efficient, inducible Cre-recombinase activation in vascular endothelium. Genesis. 2008; 46:74–80. [PubMed: 18257043]
5. Park SO, Wankhede M, Lee YJ, Choi EJ, Fliess N, Choe SW, et al. Real-time imaging of de novo arteriovenous malformation in a mouse model of hereditary hemorrhagic telangiectasia. J Clin Invest. 2009; 119:3487–3496. [PubMed: 19805914]
6. Hao Q, Zhu Y, Su H, Shen F, Yang GY, Kim H, et al. VEGF induces more severe cerebrovascular dysplasia in Endoglin$^{+/-}$ than in Alk1$^{+/-}$ mice. Transl Stroke Res. 2010; 1:197–201. [PubMed: 20640035]
7. Chen W, Guo Y, Walker EJ, Shen F, Jun K, Oh SP, et al. Reduced mural cell coverage and impaired vessel integrity after angiogenic stimulation in the *Alk1*-deficient brain. Arterioscler Thromb Vasc Biol. 2013; 33:305–310. [PubMed: 23241407]
8. Milton I, Ouyang D, Allen CJ, Yanasak NE, Gossage JR, Alleyne CH Jr, et al. Age-dependent lethality in novel transgenic mouse models of central nervous system arteriovenous malformations. Stroke. 2012; 43:1432–1435. [PubMed: 22328553]

A



B

Group	Genotype	Tamoxifen	Viral injection
Alk1^{iECKO}+LacZ	*Pdgfb*-iCreER(+);*Alk1*$^{2f/2f}$	+	AAV-LacZ
WT+VEGF	*Pdgfb*-iCreER(-);*Alk1*$^{2f/2f}$	+	AAV-VEGF
Alk1^{iECKO}+VEGF	*Pdgfb*-iCreER(+);*Alk1*$^{2f/2f}$	+	AAV-VEGF

Figure 1. Design and groups

(A) Design: Adult mice were injected with AAV-VEGF or AAV-LacZ into the brain. Two weeks later, TM was injected i.p. to induce *Alk1* gene deletion. Samples were collected 10 days later. To track proliferating cells, BrdU was injected i.p. daily starting on the day of TM injection and continuing for 10 days. (B) Groups. iECKO: inducible EC *Alk1* knockout. Twelve mice per group: 6 for latex perfusion and 6 for immunostaining.

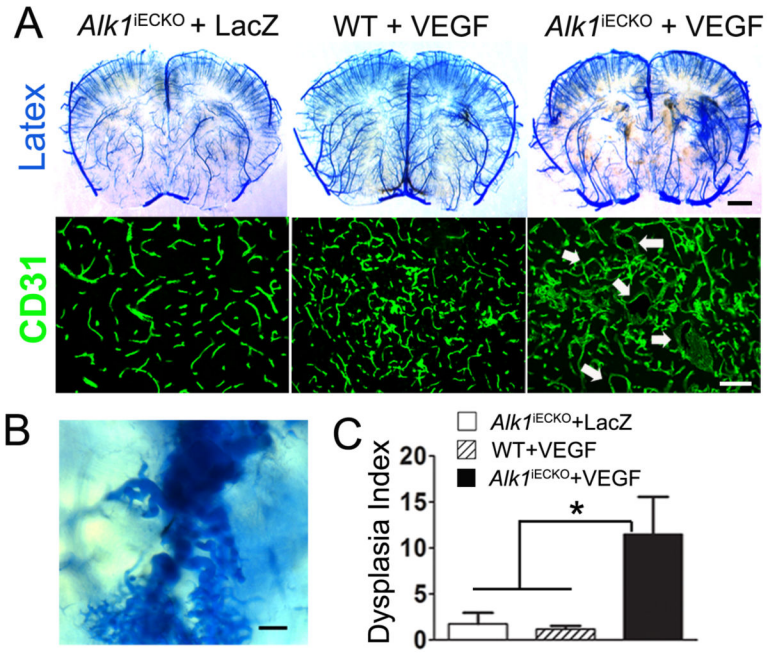


Figure 2. Malformed vessels in the brain of *Alk1*^{iECKO}+VEGF mice

(A) Latex-casted vessels and CD31-stained brain sections. Arrows indicate dilated dysplasia vessels. Scale bar: 1 mm (upper panel) and 100 μm (lower panel). (B) Enlarged image shows tortuous and dilated abnormal vessels. Scale bar: 100 μm. (C) Quantifications of dysplasia index.

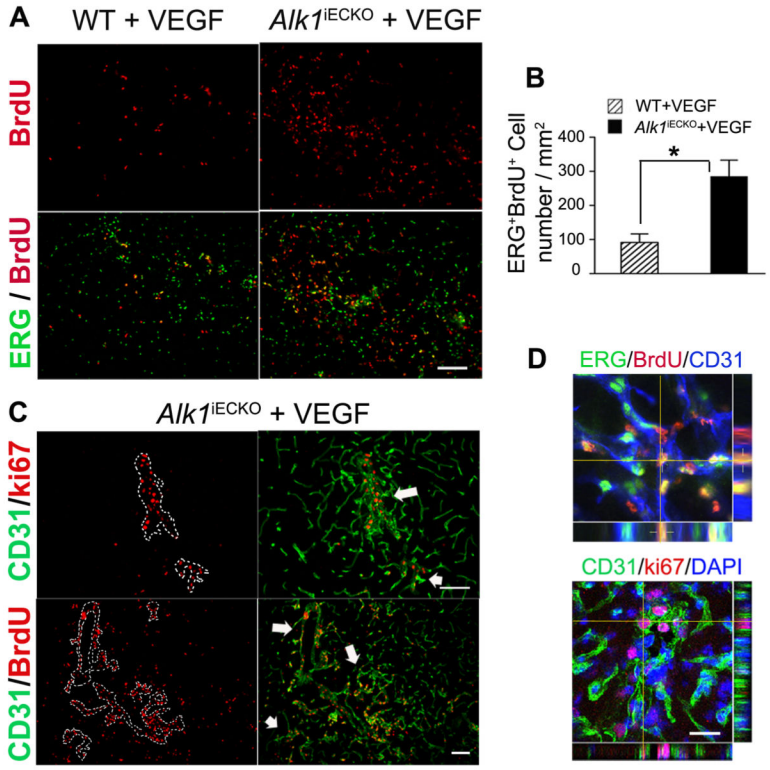


Figure 3. Increased EC proliferation in the angiogenic foci of *Alk1*^{iECKO}+VEGF mice

(A) BrdU^{+} (red) EC nuclei (ERG^{+}, green) in the angiogenic foci of WT+VEGF and *Alk1*^{iECKO}+VEGF mice. Scale bar: 100 μm. (B) Quantification of ERG^{+}/BrdU^{+} ECs. (C) Proliferating ECs (Ki67^{+} or BrdU^{+}) are clustered on the dysplastic vessels (outlined by dotted lines in the images on the left and indicated by arrows in the images on the right). Scale bar: 100 μm. (D) Confocal image showing colocalization of BrdU (red) and ERG (green, top), and ki67 (red) and CD31 (green, bottom) positive staining. The nuclei were counterstained with DAPI (blue). Scale bar: 20 μm.