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

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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 *Pdgfr-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 quantified 10 days after TM administration.

Results—Tangles of enlarged vessels resembling AVMs were present in the brain angiogenic region of TM-treated *Pdgfr-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

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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 induced⁵ 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/mm², 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.

SM22 α —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, *SM22 α* 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

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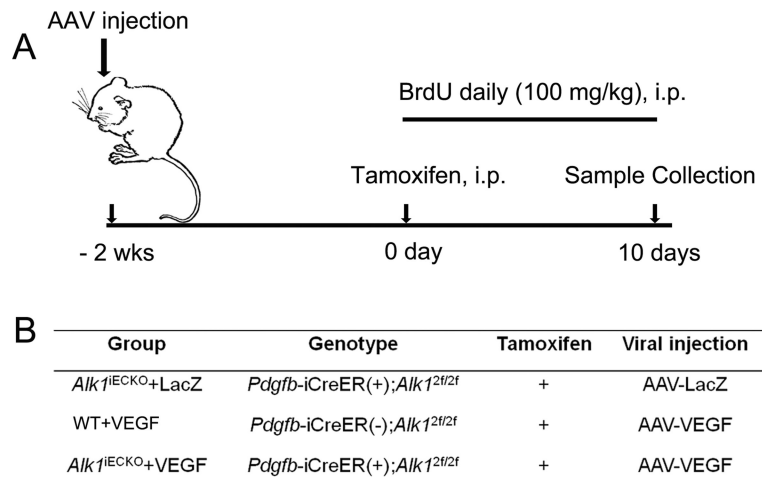


Figure 1. Design and groups

(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.

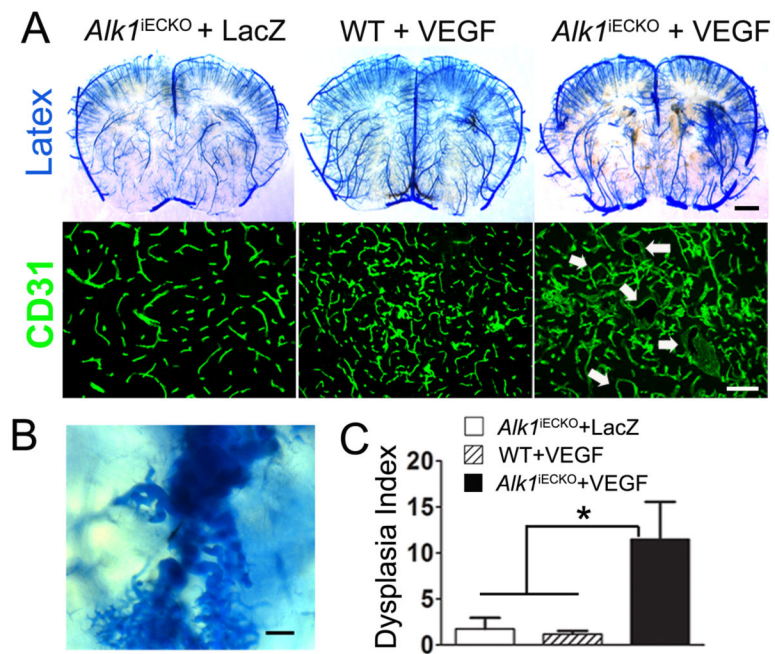


Figure 2. Malformed vessels in the brain of *Alk1*^{IECKO}+VEGF mice

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

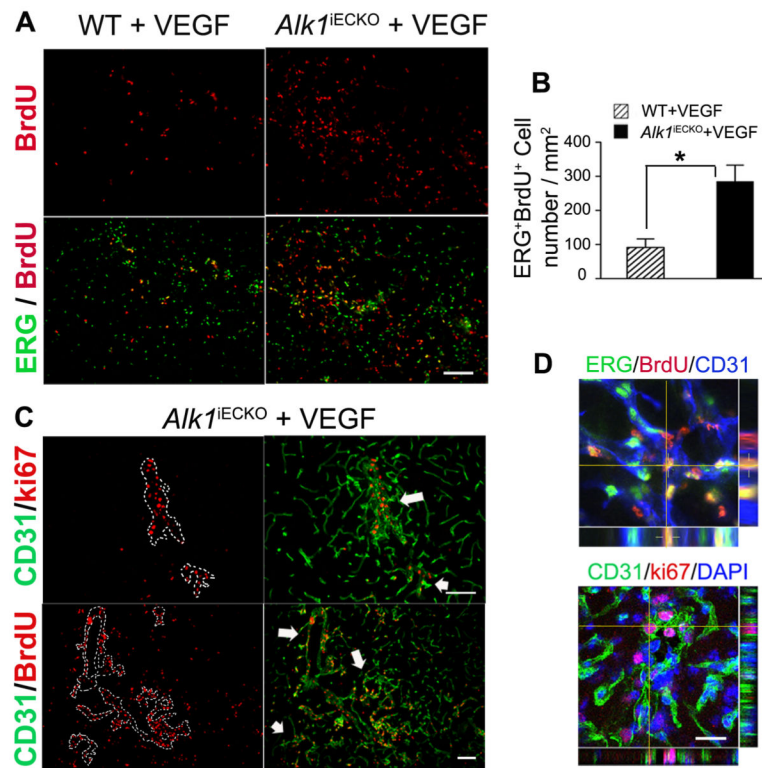


Figure 3. Increased EC proliferation in the angiogenic foci of *Alk1*^{iECKO}+VEGF mice
 (A) BrdU⁺ (red) EC nuclei (ERG⁺, green) in the angiogenic foci of WT+VEGF and *Alk1*^{iECKO}+VEGF mice. Scale bar: 100 μ m. (B) Quantification of ERG⁺/BrdU⁺ ECs. (C) Proliferating ECs (Ki67⁺ or BrdU⁺) are clustered 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 μ m. (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 μ m.