VEGF Induces More Severe Cerebrovascular Dysplasia in Eng+/- than in Alk1+/- Mice

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Abstract Brain arteriovenous malformations (BAVMs) are an important cause of intracranial hemorrhage (ICH) in young adults. A small percent of BAVMs is due to hereditary hemorrhagic telangiectasia 1 and 2 (HHT1 and 2), which are caused by mutations in two genes involved in transforming growth factor-β signaling: endoglin (Eng), and activin-like kinase 1 (Alk1). The BAVM phenotype has incomplete penetrance in HHT patients, and the mechanism is unknown. We tested the hypothesis that a "response-to-injury" triggers abnormal vascular (dysplasia) development, using Eng and Alk1 haploinsufficient mice. Adeno-associated virus (AAV) expressing vascular endothelial growth factor (VEGF) was used to mimic the injury conditions. VEGF overexpression caused a similar degree of angiogenesis in the brain of all groups, except that the cortex of Alk1+/- mice had a 33% higher capillary density than other groups. There were different levels of cerebrovascular dysplasia observed in haploinsufficient mice (Eng+/->Alk1+/-), which simulates the relative penetrance of BAVM in HHT patients (HHT1>HHT2). Few dysplastic capillaries were observed in AAV-LacZ-injected mice. Our data indicate that both angiogenic stimulation and genetic alteration are necessary for the development of vascular dysplasia, suggesting that anti-angiogenic therapies might be adapted to slow the progression of the disease and decrease the risk of spontaneous ICH.

Keywords Intracranial hemorrhage · Hereditary hemorrhagic telangiectasia 1 and 2 · Brain AVM · Angiogenic stimulation

Introduction

Brain arteriovenous malformations (BAVM) are an important cause of intracranial hemorrhage (ICH) in young adults. Roughly 2–5% of all brain AVMs is due to hereditary hemorrhagic telangiectasia (HHT), in which the main subtypes (HHT1 and 2) are caused by loss-of-function mutations in two genes involved in transforming growth factor-β signaling: endoglin (Eng) and activin-like kinase 1 (Alk1). HHT is an autosomal dominant disorder, but the BAVM lesion has incomplete penetrance, and the mechanism is unknown [1]. The BAVM lesional phenotype includes an active angiogenic and inflammatory component that is inconsistent with a static congenital anomaly [2], suggesting that a "response-to-injury" may explain incomplete penetrance of BAVM in HHT patients. There is important new evidence from extracranial tissues that such response-to-injury takes place in AVM development [3].

To explore this general hypothesis, we overexpressed vascular endothelial growth factor (VEGF), an angiogenic factor upregulated in injured tissue and BAVM lesional tissue, in the brain of Eng+/- and Alk1+/- mice. We showed that vascular dysplasia occurs only after VEGF stimulation and is more pronounced in Eng+/- than in Alk1+/- mice.
Materials and Methods

Animals

Experimental procedures for using laboratory animals were approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco (UCSF). Eng$^{+/−}$ and Alk1$^{+/−}$ mice were obtained from Dr. Douglas Marchuk’s laboratory at Duke University and bred at UCSF’s animal facility. These mice have been back-crossed with C57BL mice for more than ten generations in our laboratory. Thus, they are in C57BL/6 background. C57BL wild type (WT) mice were used as control. Adult male Eng$^{+/−}$, Alk1$^{+/−}$, Eng/Alk1$^{+/−}$ (Eng and Alk1 double heterozygous knockout), and WT mice weighing 25–30 g (8 to 10 weeks old) were used for the experiments.

AAV Gene Transfer

Mice were anesthetized using ketamine/xylazine (100/10 mg/kg body weight) intraperitoneally and placed in a stereotactic frame with a mouth holder (David Kopf Instruments, Tujunga, CA, USA). A burr hole was drilled in the pericranium 2 mm lateral to the sagittal suture and 1 mm posterior to the coronal suture. A 10 µl Hamilton syringe was inserted into the right basal ganglia. For cortical injection, the needle was inserted 1 mm into the cortex and advanced 2 mm parallel to the surface. Two microliters of viral suspension containing $2\times10^9$ genome copies of adeno-associated virus (AAV)-VEGF or AAV-LacZ, AAV vectors with cytomegalovirus (CMV) promoter driving VEGF or LacZ gene expression, was slowly injected at a rate of 0.2 µl per minute.

![Fig. 1](image-url) In the striatum. A Injection site (gray square). B Angiogenic foci and dysplastic capillaries (arrows). Inserts are enlarged images of dysplastic capillaries. Scale bars are 100 (top panel) and 50 µm (bottom panel). C, D The capillary density and dysplasia index.

Asterisk, $p<0.05$ vs. AAV-LacZ group. Number sign, $p<0.05$ vs. AAV-VEGF-transduced WT or Alk1$^{+/−}$ mice. VEGF: AAV-VEGF-injected mice, LacZ: AAV-LacZ-injected mice. N=6
Lectin Staining, Capillary Density Counting, and Microvascular Morphology

Brain specimens were collected 6 weeks after the vector injection. The brain was removed, snap-frozen with dry ice, and stored at −80°C. A series of 20 µm cryostat coronal sections were collected. Sections were fixed with 4% paraformaldehyde for 30 min and incubated overnight with fluorescein-lycopersicin esculentum lectin (Vector Laboratories, Burlingame, CA, USA), 2 µg/ml at 4°C.

Two coronal sections, located 0.5 mm rostral to and 0.5 mm caudal to the injection site, were chosen for vessel quantification. Pictures were taken from three areas (left, right, and bottom of the injection site) of each section using ×10 microscope objective lens. Capillary density (CD, the number of capillaries per ×10 objective field) and the dysplasia index (the number of capillaries with lumen >15 µm per 200 capillaries) were quantified using Image J [4]. Two investigators without knowledge of treatment conditions performed the quantification separately.

Data are mean±SD. Differences among groups were analyzed by one-way analysis of variance followed by Fisher's protected least significant difference test.

Results

In the striatum, the AAV-VEGF-injected mice had higher CD than the AAV-LacZ-injected mice ($P<0.05$): Eng+/− (237±30 vs. 174±24), Alk1+/− (246±37 vs. 173±32), or WT (249±33 vs. 179±28). There were no differences among the groups ($P>0.05$). The dysplasia index was higher in the striatum of AAV-VEGF-injected Eng−/− mice (3.0±1.0) than in Alk1+/− mice (0.7±0.5; $P<0.05$). Few dysplastic capillaries were detected in the striatum of AAV-LacZ-injected Eng−/− and Alk1+/− mice and AAV-VEGF-injected WT mice (Fig. 1).

![Fig. 2](image-url) In the cortex. A Injection site (gray square). B Angiogenic foci and dysplastic capillaries (arrows). Inserts are enlarged images of dysplastic capillaries. Scale bars are 100 (top panel) and 50 µm (bottom panel). C, D The capillary density and dysplasia index. Asterisk (in C), $p<0.05$ vs. AAV-LacZ group. Asterisk (in D), $p<0.05$ vs. AAV-VEGF-transduced WT. Number sign, $p<0.05$ vs. AAV-VEGF-transduced Alk1+/− mice. VEGF: AAV-VEGF-injected mice, LacZ: AAV-LacZ-injected mice. N=3
In the cortex, CD was similar in all groups, except that Alk1⁺/− mice (342±76) had a 33% higher CD than Eng/Alk1⁺/− (258±56) and Eng⁺/− (259±46) mice (P<0.05). Vascular dysplasia was also similar to that seen in the striatum, but differences among groups were less marked. The dysplasia index was higher in Eng⁺/− mice (2.3±1.2) than in Alk1⁺/− (1.2±0.8) (P<0.05; Fig. 2).

The Eng/Alk1⁺/− mice displayed a dysplasia index similar to the Alk1⁺/− group (1.5±1.1). The dysplasia index trended higher in the cortex of 6-month-old (3.7±1.4) than in 3-month-old Eng⁺/− mice after AA-V-VEGF injection (P=0.16).

**Discussion**

We demonstrated that (1) VEGF stimulated similar CD increases in the striatum or cortex of Eng⁺/−, Alk1⁺/−, and WT mice (except in Alk1⁺/− mice, which had a higher cortical CD than the other groups); (2) VEGF induced different levels of cerebrovascular dysplasia in Eng⁺/− and Alk1⁺/− mice (Eng⁺/−>Eng/Alk1⁺/−>Alk1⁺/−); and (3) minimal cerebrovascular dysplasia was observed in haploinsufficient mice without VEGF stimulation.

There is no good model of BAVM that mimics the human disease [5]. We have developed an intermediate phenotype—cerebrovascular dysplasia—that is not a direct disease model but may be useful in studying vascular responses in Eng⁺/− and Alk1⁺/− mice separately [4, 6]. Here, we provided the first direct comparison of the CD and dysplastic index in the VEGF-stimulated brain of Eng⁺/− and Alk1⁺/− mice, and showed that the degree of dysplasia in Eng⁺/− and Alk1⁺/− mice simulated the prevalence of BAVM in HHT patients. Although we used VEGF, it is likely that other factors upregulated during tissue injury, such as cytokines involved in inflammatory reaction, might also play primary, synergistic, and/or permissive roles.

Satomi et al. found that three out of ten Eng⁺/− mice spontaneously develop various types of vascular dysplasia [7]. The abnormal morphology we observed is similar to theirs, but no good model of BAVM that mimics the human disease [5]. Although other groups found a reduced angiogenic response in adult Eng⁺/− mice in ischemic hind limb and tumor models [8, 9], we induced a similar degree of angiogenesis in the brain of mice with different genetic backgrounds, except that Alk1⁺/− mice had slightly higher CD in the cortex than other groups. Gene expression assays in the local microenvironment of VEGF-stimulated brain would help clarify these observations.

**Summary**

We have provided evidence that the development of vascular dysplasia in Eng⁺/− or Alk1⁺/− mice is greatly magnified by VEGF overexpression, the degree of which simulates the relative prevalence of BAVM in HHT 1 and 2. If dysplasia can be taken as an intermediate phenotype for BAVM, then our data support a “response-to-injury” hypothesis. Although the clinical nature of such an injury or acquired change in vascular microenvironment is unknown, identification of pathways and modifiers may lead to the discovery of ways to manipulate signaling to decrease the prevalence of BAVM formation in HHT patients and reduce the incidence of ICH.

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