Increased tissue perfusion promotes capillary dysplasia in the ALK1-deficient mouse brain following VEGF stimulation

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Hao Q, Su H, Marchuk DA, Rola R, Wang Y, Liu W, Young WL, Yang GY. Increased tissue perfusion promotes capillary dysplasia in the ALK1-deficient mouse brain following VEGF stimulation. Am J Physiol Heart Circ Physiol 295: H2250–H2256, 2008. First published October 3, 2008; doi:10.1152/ajpheart.00083.2008.—Loss-of-function activin receptor-like kinase 1 gene mutation (ALK1<sup>+/−</sup>) is associated with brain arteriovenous malformations (AVM) in hereditary hemorrhagic telangiectasia type 2. Other determinants of the lesional phenotype are unknown. In the present study, we investigated the influence of high vascular flow rates on ALK1<sup>+/−</sup> mice by manipulating cerebral blood flow (CBF) using vasodilators. Adult male ALK1<sup>+/−</sup> mice underwent adeno-associated viral-mediated vascular endothelial growth factor (AAVVEGF) or lacZ (AAVlacZ as a control) gene transfer into the brain. Two weeks after vector injection, hydralazine or nicardipine was infused intraventricularly for another 14 days. CBF was measured to evaluate relative tissue perfusion. We analyzed the number and morphology of capillaries. Results demonstrated that hydralazine or nicardipine infusion increased focal brain perfusion in all mice. It was noted that focal CBF increased most in AAVVEGF-injected ALK1<sup>+/−</sup> mice following hydralazine or nicardipine infusion (145 ± 23% or 150 ± 11%; P < 0.05). There were more detectable dilated and dysplastic capillaries (2.4 ± 0.3 or 2.0 ± 0.4 dysplasia index; P < 0.01) in the brains of ALK1<sup>+/−</sup> mice treated with AAVVEGF and hydralazine or nicardipine compared with the mice treated with them individually. We concluded that increased focal tissue perfusion and angiogenic factor VEGF stimulation could have a synergistic effect to promote capillary dysplasia in a genetic deficit animal model, which may have relevance to further studies of AVMs.

activin receptor-like kinase 1; angiogenesis; vascular endothelial growth factor

BRAIN ARTERIOVENOUS MALFORMATIONS (AVM), an important cause of intracranial hemorrhage, are a nidus of abnormal vessels lacking a true capillary bed and are characterized by various degrees of arteriovenous shunting (14). The pathogenesis of brain AVM remains poorly understood. There is currently no animal model that mimics the hemodynamic characteristics of the disease and the clinical course of recurrent spontaneous hemorrhage.

There are two general sets of clues regarding the pathogenesis of the disease. First, lesion tissue displays an actively angiogenic pattern. Features of the brain AVM phenotype include overexpression of vascular endothelial growth factor (VEGF) (21, 27, 40, 44), increased endothelial proliferation (19), altered Tie-2/angiopoietin signaling (18), and increased matrix metalloproteinase (MMP)-9 activity (7, 20).

Finally, there is evidence that genetic alteration in transforming growth factor (TGF)-β signaling greatly predisposes patients to the formation of brain AVM (32). Hereditary hemorrhagic telangiectasia (HHT), a multisystemic focal vascular lesion, is an autosomal dominant vascular disorder that has a high incidence of solid organ AVMs, including brain tissue (15, 41). Activin receptor-like kinase 1 heterozygous (ALK1<sup>+/−</sup>) mice have been proposed as an animal model for HHT (25). Aged ALK1<sup>+/−</sup> animals display vascular lesions, such as multiple thin-walled blood vessels, multiple foci of hemorrhage, dilated capillaries, abnormally large vessels, and discontinuities in the vessel wall in many organs including the mouse brain (43).

Because human brain AVM vessels are exposed to high flow rates, we wanted to address the question of whether flow could interact with the other identified components that may be involved in brain AVM pathogenesis. We first examined the capillary density after hydralazine treatment in the adeno-associated viral-mediated (AAV)VEGF-transduced brain of ALK1<sup>+/−</sup> or wild-type (WT) mice. The capillary densities were similar in these two groups. However, capillary dysplasia was detected in the ALK1<sup>+/−</sup> mouse brain but not in the WT mouse brain. Thus we conducted further studies on vascular changes in the ALK1<sup>+/−</sup> mice. We hypothesized that increased focal perfusion and angiogenic stimulation would have a synergistic effect in the promotion of capillary dysplasia in a genetic deficit animal model.

MATERIALS AND METHODS

Animals and treatment. Experimental procedures for using laboratory animals were approved by the Institutional Animal Care and Use Committee of the University of California San Francisco (UCSF). ALK1<sup>+/−</sup> mice were developed in Dr. Douglas Marchuk’s laboratory at Duke University and bred at the UCSF animal facility. There were no observable phenotype abnormalities in these animals. Adult male ALK1<sup>+/−</sup> mice weighing 25–30 g (8–10 wk old) were used for the experiments. Animals were allowed free access to food and water with a 12-h:12-h alternating light-dark cycle.

Experimental groups. Adult male ALK1<sup>+/−</sup> mice underwent AAVVEGF or lacZ gene (AAVlacZ) transduction. Following 14 days...
of AAVVEGF or AAVlacZ gene transfer, the mice received a microcromotic pump implantation. Hyalurazime, nicardipine, or saline (as control) was continuously infused into the cerebral left ventricle through a minipump. These ALK1+/− mice were divided into six experimental groups based on different treatments: 1) saline infusion in AAVlacZ-transduced mice, 2) saline infusion in AAVVEGF-transduced mice, 3) hyalurazime infusion in AAVlacZ-transduced mice, 4) hyalurazime infusion in AAVVEGF-transduced mice, 5) nicardipine infusion in AAVlacZ-transduced mice, and 6) nicardipine infusion in AAVVEGF-transduced mice.

**AAVVEGF gene transfer.** Adult male ALK1+/− mice were anesthetized using ketamine-xylazine (100/10 mg/kg body wt) intraperitoneally. The mice were placed in a stereotactic frame with a mouth holder (David Kopf Instruments, Tujunga, CA). A burr hole was drilled in the pericranium 2 mm lateral to the sagittal suture and 1 mm posterior to the coronal suture (Fig. 1). A 10-μl Hamilton syringe was inserted into the right parenchyma about 3.0 mm under the cortex.

We chose lectin staining because our previous studies demonstrated that this method is reproducible and reliable. Sections were fixed with 4% paraformaldehyde (PFA) for 30 min, blocked with 5% normal blocking serum for another 30 min, and incubated overnight with 2 μg/ml lectin at 4°C. Sections were then incubated overnight with anti-α-smooth muscle antibody actin (α-SMA; 1:1,000 dilution; Sigma, St. Louis, MO) at 4°C. The secondary antibody was Alexa 594 anti-mouse IgG (1:1,000 dilution; Molecular Probe, Carlsbad, CA). Three adjacent areas of capillaries (left, right, and bottom within ~0.5 mm from the needle track) were chosen in two separate sections, and pictures were taken at low magnification (10×). As a surrogate for vessel counting, capillary density was determined by lectin optical density measurements using National Institutes of Health ImageJ software. The number of capillaries was calculated as the mean of the numbers obtained from the six images, as previously described (16).

Dilated vessels and dysplastic vessels were counted manually using the same images used for capillary counts. Studies have shown that the normal diameter of a capillary is between 3 and 8 μm (49). We scored a capillary as dilated if its diameter was >8 μm and the vascular wall had no smooth muscle layer. Normal capillaries have minimal change in vessel caliber between the vessels at the origin branch point and those at the termination of a vascular segment. Furthermore, the vascular wall is smooth. We scored vessels as dysplastic if 1) there was a deviation from the normal arborization pattern resulting in a visible morphology change in vessel caliber between the microvessels at the origin branch point and those at the termination of a vascular segment, 2) the microvessel had a diameter >15 μm, and 3) they lacked a smooth muscle layer. This definition encompasses the loss of normal smooth, tubular capillary structure and the primary observed patterns of irregular vascular wall or contour or focal aneurysmal dilations, either single or multiple and spiral, twisted vascular shapes.

To better observe dysplastic vessel morphology, the section was examined using Nikon ECLIPSE E1000 confocal microscopy (Nikon, Melville, NY). A section image of 1 μm was captured and reconstructed for three-dimensional observation if abnormal capillaries were detected. All capillary counting was performed blindly.

**Immunofluorescent staining.** To label capillary wall cells, sections were fixed with 4% PFA for 30 min and incubated with 5% normal blocking serum for another 30 min, followed by fluorescent-lectin staining. Sections were then incubated with CD31 antibody (1:200 dilution; BD Pharmingen, San Jose, CA) and α-SMA overnight at 4°C. The secondary antibodies were Alexa 594 anti-rat IgG and Alexa 350 anti-mouse IgG (1:1,000 dilution; Molecular Probe, Carlsbad, CA).

Before the animals were euthanized, BrdU (Sigma-Aldrich, St. Louis, MO) was injected intraperitoneally daily at a dose of 100 μg per mouse per day for 7 days (46). For BrdU/CD31/α-SMA staining, sections were incubated with CD31, α-SMA (1:500 dilution; Abcam, Cambridge, MA), and BrdU antibody (1:500 diluted in mouse-on-mouse dilute working solution; Sigma-Aldrich) overnight, and Alexa Fluor 594 anti-rat IgG, 350 anti-rabbit, and 488 anti-mouse IgG were used as secondary antibodies. Sections were evaluated using a fluorescence microscope (Nikon Microphoto-SA, Melville, NY).

**CBF measurement.** Local CBF adjacent to the needle track was measured before euthanizing the mice, using a laser Doppler flowmetry monitor (Model BPM2 System; Vasamedics, St. Paul, MN) equipped with a small-caliber probe (P-433; Vasamedics) that generated a beam of 740-nm wavelength light and penetrated the brain tissue ~1 to 2 mm beyond the probe ending (11). The probe was placed on the thinned bone of the mouse skull. To standardize subsequent measurements, this site was marked with a spot drilled in the bone. Changes in local CBF were recorded as percentages of the
baseline. The CBF in the same region in the contralateral hemisphere was monitored as a control.

Statistical analysis. Parametric data in the different groups were compared using one-way ANOVA, followed by Fisher protected least-significant difference test. For AAVVEGF injection versus drug, two-way ANOVA was applied. All data are presented as means ± SD. A probability value of <5% was considered statistically significant.

RESULTS

No visible abnormal behavioral changes were detected in the animals following AAVVEGF injection and minipump implantation. Animals moved freely and ate and drank normally. No significant differences in mean artery blood pressure, PaO₂, PaCO₂, and blood pH were noted in the various groups of mice. This indicated that AAV-mediated gene transfer and minipump implantation did not affect general physiological parameters.

Hydralazine or nicardipine increased local CBF in the ALK1−/− mouse brain. To determine whether hydralazine or nicardipine increased local CBF, we measured CBF before euthanizing the animals. There was no difference in CBF change in both the ipsilateral and contralateral hemispheres in AAVlacZ-transduced mice after the hydralazine or nicardipine infusion, suggesting that the minipump was accurately inserted in the lateral ventricle and contributed equally to both hemispheres. All the groups treated with hydralazine or nicardipine had a statistically relevant increase of CBF than their saline-treated counterparts (P < 0.05; Fig. 2). We noted that local CBF increased up to 145% and 150% in the AAVVEGF-transduced mice following hydralazine or nicardipine infusion compared with the AAVVEGF-transduced mice following saline infusion (P < 0.05), indicating that hydralazine or nicardipine infusion plus VEGF stimulation could maximize local CBF.

Hydralazine or nicardipine increased VEGF-induced angiogenesis. To determine whether hydralazine or nicardipine increased VEGF-induced angiogenesis, we first counted capillary numbers in the six groups of mice (Fig. 3A). We found that capillary density increased in the AAVVEGF-transduced mice compared with the AAVlacZ-transduced mice following saline infusion (254 ± 33 vs. 190 ± 25 capillary density; P < 0.05; Fig. 3B). Treatment of hydralazine or nicardipine did not

![Image](http://ajpheart.physiology.org/)

**Fig. 3.** VEGF induces angiogenesis in the brain. *A:* photomicrographs show capillaries in the 6 groups of mice with different treatments. Scale bar = 50 μm. *B:* bar graph quantifies the number of capillaries in the 6 groups. Data are means ± SD; n = 6/group. *P < 0.05 vs. AAVlacZ-transduced group. *C:* photomicrographs show CD31 and BrdU double staining of dysplastic capillaries. Red is CD31-positive endothelial cells, green is BrdU-positive staining, and merged yellow color indicates that the endothelial cell is in the proliferating stage. Scale bar = 50 μm. *D:* bar graph shows the number of dilated capillary counts in the same groups above. Data are means ± SD; n = 6/group. *P < 0.05, AAVVEGF-transduced mice treated with hydralazine or nicardipine vs. AAVVEGF-transduced mice treated with saline.
further increase capillary density in AAVVEGF-transduced mice. We then counted the number of dilated capillaries. As indicated in the MATERIALS AND METHODS section, a capillary with >8 μm diameter would be defined as a dilated capillary. We found that dilated capillaries increased in the AAVVEGF-transduced mice treated with hydralazine or nicardipine compared with the AAVVEGF-transduced mice treated with saline (P < 0.05; Fig. 3D). The average diameter of dilated capillaries is 11 μm. To determine whether these dilated capillaries were under active angiogenesis, CD31/BrdU fluorescent staining was performed. The results showed that the CD31-positive cells were well merged with BrdU-positive staining (Fig. 3C), indicating that the endothelial cells actively proliferated following hydralazine infusion in the AAVVEGF-transduced mice.

**Hydralazine or nicardipine enhanced capillary dysplasia in the AAVVEGF-transduced ALK1+/− mouse brain.** To determine whether hydralazine or nicardipine enhanced capillary dysplasia (e.g., massive or single enlargement or node and clustered, twisted, or spiral capillaries) in the AAVVEGF-transduced ALK1+/− mouse brain, hydralazine or nicardipine infusion plus AAVVEGF injection was further examined (Fig. 4A). We chose dysplastic capillary per 200 capillaries as the dysplasia index because this value approximates the average number of capillary counts in one 10× field. There were a few smooth muscle-positive stained small arteries or small veins in the mouse brain section. Most of them had normal morphology, although occasionally a change was observed. However, we focused only on capillary dysplasia in this study. We counted lectin-positive vessels that were negative for α-SMA.

![Image](http://ajpheart.physiology.org/)

**Fig. 4.** Hydralazine or nicardipine induces capillary dysplasia in the AAVVEGF-transduced mouse brain. A: photomicrographs show the different patterns of capillary dysplasia morphology following hydralazine or nicardipine infusion in the AAVVEGF-transduced activin receptor-like kinase 1 gene mutation mice (a–t); n1–n4 are the controls (normal capillary morphology). Scale bar = 25 μm. B: bar graph shows dysplasia index in the treated groups of mice as above. Data are means ± SD; n = 6/group. *P < 0.01, AAVVEGF-transduced mice treated with hydralazine or nicardipine vs. AAVVEGF-transduced mice treated with saline.
to exclude small arteries or small veins. All the AAVVEGF-transduced mouse groups had a higher dysplasia index than the AAVlacZ-transduced groups ($P < 0.01$; Fig. 4B). There were more dysplastic capillaries in the AAVVEGF-transduced mouse brain treated with hydralazine or nicardipine than that treated with saline ($P < 0.01$; Fig. 4B). The average diameter for the dysplastic capillaries that we observed was 21 μm. These dysplastic capillaries usually developed in the ipsilateral cortex and caudate putamen, adjacent to the needle track and with abundant capillary growth.

**Capillary dysplasia detection under three-dimensional confocal microscope.** In the three-dimensional reconstruction sections, the dysplastic capillary morphology could be clearly identified (Fig. 5A). Immunostaining confirmed that CD31 and lectin double positively stained the dysplastic capillary but stained negative for α-SMA (Fig. 5B). This result suggested that VEGF stimulation, along with hydralazine- or nicardipine-induced vessel dilation, triggered abnormal capillary formation in the adult ALK1$^{-/-}$ mouse brain.

**DISCUSSION**

In the present study, we demonstrated that in the AAVVEGF-transduced ALK1$^{-/-}$ mouse brain 1) a continuous infusion of hydralazine or nicardipine enhanced local CBF, 2) vasodilator infusion alone did not affect capillary counts, and 3) the vasodilator enhanced VEGF-induced capillary dilation and capillary dysplasia. Our results indicated that at least three factors, VEGF stimulation, increased blood flow, and ALK1 gene haploinsufficiency, act synergistically to promote brain capillary dysplasia.

We chose laser Doppler to measure focal CBF because this method is simple and not invasive. We did not quantitatively measure focal CBF using isotopes in this study. However, for experimental purposes, absolute quantification was not necessary, since we were interested in demonstrating as proof of principle that relative increases in perfusion could affect vascular morphology.

VEGF is a well-known angiogenic factor. The vessels resulting from VEGF stimulation are often large, dilated, and leaky (4, 35). Injection of a high dosage of VEGF plasmid to the ischemic heart causes complications such as angioma formation (37). This complication has also been observed when VEGF-engineered myoblasts are implanted in the ischemic rat leg (42) and ischemic rat heart (30), indicating that the proper dose of VEGF is key to maintaining normal capillary phenotype. We use $2 \times 10^9$ genome copies of AAV in this experiment because our previous experiment indicated that injection of $2 \times 10^9$ genome copies of AAVVEGF can induce

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**Fig. 5.** Three-dimensional confocal image indicates capillary dysplasia. **A:** photomicrographs show a series of confocal images of capillary dysplasia morphology at different levels. **B:** photomicrographs show CD31 and lectin double-labeled immunostaining of dysplasia capillary. Red is CD31-positive endothelial cells, green is lectin-positive capillary walls, and merged yellow color indicates both CD31- and lectin-positive staining. Scale bar = 50 μm.
reproducible focal angiogenesis in the mature mouse brain, including the ischemic brain (16, 38). In the AAVVEGF-transduced mice, the number of capillaries increased up to 133% and dilated capillaries up to 160%, and there were more visible dysplastic capillaries compared with those of the AAVlacZ-transduced group. Therefore, VEGF is still a main factor for angiogenesis in the adult brain tissue.

Hydralazine (1, 24, 34, 36) and nicardipine (26, 45) have been widely used in treating arterial hypertension and are well-studied experimental means of manipulating cerebral perfusion. Our results indicated that both drugs dilate microvessels and increase CBF. There are no reported data indicating that hydralazine or nicardipine has intrinsic, direct dysmorphic effects, even with their long history of use. The doses of vessel dilators were selected based on the criteria that can cause a clinically relevant increase in flow rates without adverse effects, such as systemic hypotension during recirculation of a locally infused drug, or perhaps hyperemia resulting in increased intracranial pressure. Another study found that cerebral microvascular abnormality induced by chronic hypertension in rats can be reversed using hydralazine (17). However, indirect effects may be operative. Because these drugs cause vasodilation, they may enhance pressure-induced extravasation of protein into the brain, resulting in leakage at lower pressure levels (23). Increased vascular leakage is a prerequisite for inducing angiogenesis (13) although whether vasodilators increase vascular leakage needs to be further studied. One important mechanistic consideration to follow up on in future studies would be to distinguish the effect of increased vascular shear at the endothelial surface from effects caused by changes in barrier function from vasodilation.

In the present study, we found that after hydralazine and nicardipine infusion in the AAVlacZ-transduced ALK1+/− mice, dilated capillaries increased to 160% and 170%, respectively, and CBF to 120% of baseline (Fig. 3). Interestingly, after hydralazine and nicardipine infusion in AAVVEGF-transduced ALK1+/− mice, dilated capillaries markedly increased to 250% and 260%, respectively (Fig. 3), and CBF to 145% and 150% (Fig. 2) of baseline, respectively. These results suggest vasodilator and VEGF stimulation have an additive effect. Most importantly, no dysplastic capillary was found after hydralazine or nicardipine infusion alone but was occasionally found after VEGF stimulation, suggesting vasodilators alone do not induce capillary dysplasia (Fig. 4). However, hydralazine or nicardipine infusion greatly increased capillary dysplasia (4-fold) in the AAVVEGF-transduced ALK1+/− mice compared with that with VEGF stimulation alone (Fig. 4). It is possible that higher brain perfusion contributed to the development of brain capillary dysplasia in the setting of VEGF excess and abrogated ALK1 function. We used two unrelated vasodilators in this study to rule out some unknown synergism between the drugs and the VEGF stimulation against the ALK1 gene deletion to cause dysplasia.

ALK1 is a receptor for the TGF-β ligands superfamily (3) and regulates TGF-β action in endothelial cells through binding TGF-β1 and TGF-β3 (8, 31). Decrease in TGF-β1 or TGF-β1 receptors leads to unstable cellular interactions in the vessel wall, dilated vessels, and vascular abnormalities (5). These data also suggest a potential mechanism for the role of this pathway in AVM pathogenesis. ALK1 is required for endothelial cell maturation (10, 28). Disruption of this signalizing pathway by mutation or possibly through physiological perturbation will result in a block in the maturation process, leading to inappropriate endothelial cell migration and proliferation. This suggests that aberrant endothelial cell migration and proliferation may be one of the earliest events in the development of an AVM, either sporadic or as a result of familial mutation.

ALK1 homozygous mice die at embryo on day 10.5 and display vascular developmental defects such as AVM (33, 47). Development of vascular lesions in ALK1+/− mice is age dependent and has only been reported in ALK1+/− mice older than 7 mo (43). The factors that initiate lesion formation and those that influence disease progression remain unknown. We used young ALK1+/− mice (8–10 wk old) to explore the factors causing vascular dysplasia development; there is no visible abnormal vascular structure in the ALK1+/− mouse brain at this age (data not shown here).

Alterations in vascular flow rates can induce vessel remodeling, and MMPs and nitric oxide/nitric oxide synthase appear to be critical in flow-induced vessel remodeling (6, 9, 12). These effects need to be further studied in the future.

One of the limitations for this line of experiments is the lack of strictly quantitative objective criteria to use as the index of vascular dysplasia. Nonetheless, the rather striking and reproducible effects we observed show that our index is sufficient to demonstrate the principle of synergism between flow, VEGF, and genetic background. The visual representation shown in Fig. 4A suggests a biological effect worthy of further quantitative study. Future longitudinal time course studies might clarify the progression from normal to enlarged capillaries.

In summary, locally increased VEGF concentration together with increased CBF may alter the balance of vascular signaling in ALK1+/− mice, which leads to what appears to be pathological capillary remodeling. This experimental vascular phenotype holds promise for modeling the phenotype seen in human brain AVM.

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