**Vascular Malformations in the Adult Mouse Brain**

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**Background and Purpose**—Vascular endothelial growth factor (VEGF) expression is elevated in human brain arteriovenous malformations (bAVM). We have developed a bAVM model in the adult mouse by focal Alk1 gene deletion and human VEGF stimulation. We hypothesized that once the abnormal vasculature has been established, tonic VEGF stimulation is necessary to maintain the abnormal phenotype, and VEGF antagonism by bevacizumab (Avastin) would reduce vessel density and attenuate the dysplastic vascular phenotype.

**Methods**—Angiogenesis and bAVM were induced by injection of adeno-associated viral vector expressing human VEGF alone into the brain of wild-type mice or with adenoviral vector expressing Cre recombinase (Ad-Cre) into Alk12f/2f mice. Six weeks later, bevacizumab or trastuzumab (Herceptin, bevacizumab control) was administered. Vessel density, dysplasia index, vascular cell proliferation and apoptosis, and human IgG were assessed (n=6/group).

**Results**—Compared with trastuzumab (15 mg/kg), administration of 5, 10, and 15 mg/kg of bevacizumab to adenoviral vector expressing Cre recombinase (Ad-Cre) into Alk12f/2f mice reduced focal vessel density (P<0.05); administration of 5 mg/kg bevacizumab decreased proliferating vascular cells (P=0.04) and increased TUNEL-positive vascular cells (P=0.03). More importantly, bevacizumab (5 mg/kg) treatment reduced both vessel density (P=0.01) and dysplasia index (P=0.02) in our bAVM model. Human IgG was detected in the vessel wall and in the parenchyma in the angiogenic foci of bevacizumab-treated mice.

**Conclusions**—We provide proof-of-principle that, once abnormal AVM vessels have formed, VEGF antagonism may reduce the number of dysplastic vessels and should be evaluated further as a therapeutic strategy for the human disease. (Stroke. 2012;43:00-00.)

**Key Words:** VEGF ▪ arteriovenous malformation ▪ mouse ▪ brain ▪ angiogenesis

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Brain arteriovenous malformations (bAVM) represent a relatively infrequent, but important, source of neurological morbidity in children and young adults, primarily from new or recurrent intracranial hemorrhage. Current therapies consist of a combination of surgical resection, embolization, and stereotactic radiotherapy, all with potentially high morbidities. Specific medical therapies are needed, especially for the large fraction of bAVM patients who are currently not offered treatment because of perceived high risks associated with available therapies. Studies of surgically resected bAVM tissue suggest an active angiogenic and inflammatory lesion rather than a static congenital anomaly. We and others have shown that vascular endothelial growth factor (VEGF) level is increased in resected surgical specimens; VEGF signaling may present a target for medical intervention.

Patients with hereditary hemorrhagic telangiectasia (OMIM#187300) have a much higher prevalence of bAVM than does the generation population, on the order of 1000-fold higher in hereditary hemorrhagic telangiectasia-1 caused by endoglin (ENG) haploinsufficiency and 100-fold higher in hereditary hemorrhagic telangiectasia-2 with activin receptor-like kinase (ALK1) haploinsufficiency. Vascular lesions developed in various organs in Eng+/− and Alk1+/− mice, but spontaneous lesions in the brain are quite modest, and are only seen in older mice. Our group demonstrated greatly increased brain microvascular dysplasia after virally mediated VEGF stimulation in both Eng+/− and Alk1+/− mice.

Somatic conditional deletion of Alk1 in adult mice induced arteriovenous fistulas and hemorrhage in the lung and gastrointestinal tract, but not in the skin or brain. Importantly, on induction of skin wounding, Alk1-deleted mice developed arteriovenous fistula and AVM-like vessels around the wound. The combination of local angiogenic stimulation (Matrigel + VEGF/FGF) and Eng loss led to gross venous...
enlargement in the retina. These results suggest that angiogenic stimulation, in addition to genetic mutation, is required for the development of vascular malformations in adult mouse brain. Taken together, all these studies suggest that a paradigm for AVM pathogenesis may be an abnormal response to injury. In support of this notion, we recently developed a bAVM model in adult mice by focal Alk1 gene deletion combined with VEGF stimulation (virally mediated human VEGF-A overexpression), which resembles various aspects of human bAVM, including large irregular vasculature and arteriovenous shunting.

Bevacizumab (Avastin) is a humanized monoclonal antibody that is directed against human VEGF-A. Bevacizumab binds to and neutralizes all VEGF-A isoforms and bioactive cleavage fragments. Bevacizumab sequesters VEGF, preventing the interaction of VEGF with its cell surface receptor. Bevacizumab has been used extensively to inhibit angiogenesis in cancer and other diseases with pathological angiogenesis.

In this study, we sought to test the hypothesis that, once the abnormal phenotype has been established in our bAVM mouse model, antagonism of VEGF by bevacizumab would attenuate the dysplastic vascular phenotype.

Materials and Methods

Viral Vector Transduction in the Mouse Brain

The mice were placed in a stereotactic frame (David Kopf Instruments). A burr hole was drilled to the pericranium 2 mm lateral to the sagittal suture and 1 mm posterior to the coronal suture. A 10-μL syringe (Hamilton Company) was inserted into the basal ganglia 3 mm under the cortex. Viral vectors were injected at a rate of 0.2 μL/min. Ad-Cre (adenoviral vector with CMV promoter driving Cre recombinase expression) was purchased from Vector Biolabs. Adeno-associated viral vector expressing human VEGF (AAV-VEGF) and AAV-LacZ have been previously described.

Antibody Treatment and Dosage

Six weeks after viral vector injections, WT and bAVM mice received intraperitoneal (IP) injections every other day for 10 days of either bevacizumab (Avastin, Genentech/Roche Inc; 1, 5, 10, or 15 mg/kg), an anti-VEGF humanized monoclonal antibody, or trastuzumab (Herceptin, Genentech/Roche Inc; 5 or 15 mg/kg), an anti-HER2 humanized monoclonal antibody that will not affect endogenous mouse VEGF expression or the human VEGF expression mediated by AAV-VEGF.

Statistical Analysis

Analysis of vessel density was performed using 1-way ANOVA, followed by Bonferroni posthoc analysis to compare the means between groups. A dose-inhibitory analysis was performed with GraphPad Prism (GraphPad Software, Inc) to obtain a dose-inhibitory curve and IC50 value. Sample sizes were n=6 per group. Data are presented as mean±SEM. A probability value <0.05 was considered statistically significant.

Bevacizumab Inhibits Human VEGF-Induced Angiogenesis in Mouse Brain

To determine the appropriate dose to use for our disease model, we treated WT mice with 1, 5, 10, and 15 mg/kg bevacizumab every other day for 10 days 6 weeks after the injection of AAV-VEGF and assessed vessel density. AAV-VEGF stimulated angiogenesis compared with AAV-LacZ-injected controls (WT+VEGF, 1152±48 vessels/mm² versus WT+LacZ, 736±65 vessels/mm², P<0.005). Administration of 5, 10, and 15 mg/kg of bevacizumab resulted in a reduction of vessel density in the AAV-VEGF-stimulated angiogenic foci compared with the trastuzumab-treated group (15 mg/kg trastuzumab versus 5 mg/kg bevacizumab, P=0.04; 10 mg/kg bevacizumab, P=0.01; 15 mg/kg bevacizumab, P=0.03). Administration of 1 mg/kg bevacizumab resulted in a trend toward reduction of vessel density compared with trastuzumab treatment (P=0.07; Figure 2A and 2B).

Results

Humanized Antibody Present in the Vessel Wall and Parenchyma at Angiogenic Foci of Bevacizumab-Treated Mice

To test whether IP injected bevacizumab and trastuzumab would reach the VEGF-stimulated angiogenic foci in the mouse brain, we stained the mouse brain with an antibody specific to human IgG. Positive staining was observed in the vessel wall and brain parenchyma in the VEGF-stimulated angiogenic region. No staining was detected in untreated mice (Figure 1). This indicates that the humanized antibodies can penetrate the blood-brain barrier in a VEGF-induced angiogenic focus.

Bevacizumab Treatment Decreased Proliferating Vascular Cells and Increased TUNEL-Positive Vascular Cells in VEGF-Stimulated Angiogenic Foci

To determine the mechanism of the inhibition effect of bevacizumab on VEGF-induced brain angiogenesis, we as-
cessed vascular cell proliferation and apoptosis within the VEGF-stimulated angiogenic foci. We found a 54% decrease of Ki67-positive cells colocalized with lectin-positive vascular cells in the angiogenic foci of bevacizumab-treated (5 mg/kg) WT mice compared with trastuzumab-treated controls (bevacizumab, 11.5±7.1 cells/mm² versus trastuzumab, 24.8±5.4 cells/mm²; P<0.05). Bev. = Bevacizumab, Tras. = Trastuzumab. C. Dose-inhibitory curve. The predicted IC₅₀ for bevacizumab antiangiogenic effect is 1.1 mg/kg (dotted black line). Graph is plotted on a log scale, control value not shown.

Figure 2. Bevacizumab treatment reduced vessel density in VEGF-induced angiogenic foci. A, Representative images of vessels labeled with lectin (green). Scale bar: 100 μm. B, Bar graphs show quantification of vessel density. Administration of 5, 10, and 15 mg/kg bevacizumab significantly reduced vessel density as compared with trastuzumab-treated control (*P<0.05). Bev. = Bevacizumab, Tras. = Trastuzumab. C, Dose-inhibitory curve. The predicted IC₅₀ for bevacizumab antiangiogenic effect is 1.1 mg/kg (dotted black line). Graph is plotted on a log scale, control value not shown.

Figure 3. Bevacizumab treatment reduced vascular cell proliferation. A, Representative images of sections costained with lectin (green) and Ki67 for proliferating nuclei (red). Proliferating cells on vessel walls (arrows) were reduced in bevacizumab-treated group as compared with trastuzumab-treated group. A few Ki67-positive cells were not associated with a vessel (arrowheads). Inset is a confocal image showing colocalization of a lectin-positive vascular cell and Ki67-positive nucleus. Scale bar: 20 μm. B, Quantification demonstrated a significant reduction in proliferating vascular cells in the bevacizumab-treated group as compared with trastuzumab (*P=0.04).

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Bevacizumab Treatment Reduced Vessel Density and Vascular Dysplasia in Mouse bAVM Model

In our mouse bAVM model, we tested whether bevacizumab treatment could reduce dysplastic vessels. We treated mice with bevacizumab (5 mg/kg) 6 weeks after the induction of the model (injection of Ad-Cre and AAV-VEGF into the brain) through IP injection every other day for 10 days. Compared with trastuzumab controls, bevacizumab treatment resulted in a 30% reduction of vessel density (trastuzumab, 952±51 vessels/mm² versus bevacizumab, 662±73 vessels/mm²; P=0.01) and a 54% reduction of dysplasia index (trastuzumab, 2.97±0.5 versus bevacizumab, 1.37±0.3; P<0.02; Figure 5), suggesting that reducing VEGF levels in the lesion leads to regression of dysplastic vessels.

Discussion

We demonstrate here that bevacizumab, a VEGF-specific antibody, is able to cross the blood-brain barrier in angiogenic foci following IP injection entering into the brain parenchyma. Bevacizumab treatment in WT mice attenuated VEGF-stimulated angiogenesis, reduced vascular cell proliferation, and increased TUNEL-positive vascular cells. Most importantly, bevacizumab treatment reduced vessel density and the number of dysplastic vessels in our adult mouse bAVM model. These observations provide proof-of-principle that, once the abnormal AVM vessels have been formed, VEGF antagonism may reduce the number of dysplastic vessels. The implication is that tonic VEGF stimulation is needed for maintenance of the abnormal phenotype in the mouse model. Whether the abnormal vessels in the lesion have regressed or have been remodeled into normal vessels after bevacizumab treatment needs to be analyzed in future studies. However, our data indicate that an anti-VEGF strategy could be a potential therapy for bAVM and should be evaluated further for the treatment of the human disease.
There are many questions that remain to be addressed in additional studies. It is not currently known whether bevacizumab can cross the luminal surface in human bAVMs. We do not yet understand the extent to which our \textit{Alk1}-deleted, VEGF-stimulated model of bAVM replicates the human disease in terms of natural history. Even if bevacizumab can penetrate the luminal surface in human bAVM and our bAVM model faithfully represents the human disease, it is still not clear whether the reversal of the phenotype will favorably alter the natural history of the human disease. Nonetheless, this is the first report of an approach to medical therapy in a disorder that is currently treatable only by surgical ablation, either by excision, irradiation, or embolization.

In the normal vasculature, VEGF is required for the survival and maintenance of new vessels.\textsuperscript{24-25} In this study, we used the AAV1 vector that transfects various cell types in the brain, including neurons, astrocytes, and endothelial cells.\textsuperscript{22} VEGF acts directly on endothelial cells\textsuperscript{26} and has been shown to be involved in endothelial cell proliferation and angiogenic remodeling.\textsuperscript{27} Without this tonic stimulation, the vessels regress. After VEGF stimulation, diminution of VEGF level abruptly induces vascular tree regression in chick chorioallantoic membrane by intussusceptive vascular pruning.\textsuperscript{28} It may be the case for the human disease that tonic VEGF stimulation is needed for maintenance of the phenotype. This notion is consistent with the emerging view that bAVM is a dynamic, primarily postnatal, disease process, rather than a static congenital anomaly.\textsuperscript{13,29} BrdU pulse-chase technique will be used to study the dynamic of VEGF stimulation on endothelial cell proliferation and turnover in the bAVM model.

In our model of angiogenesis, we found more TUNEL-positive vascular cells concurrent with a decrease of proliferating vascular cells in bevacizumab-treated mice, suggesting that blocking VEGF in an angiogenic foci increased DNA damage in vascular cells.\textsuperscript{30-32} We did not analyze platelet infiltration and fibrin deposition in this study. The fact that we detected more TUNEL-positive cells in the bevacizumab-treated group than in the control group suggests that tonic VEGF is important in maintaining a normal angiogenic process in the brain. Inhibition of VEGF in our bAVM model with bevacizumab led to a reduction in vascular density and irregular vessels, indicating that tonic VEGF stimulation is also necessary to maintain the abnormal vascular phenotype in our bAVM model. Thus, reducing VEGF levels in human bAVM may reduce the lesional burden.

VEGF has various effects on the cerebrovasculature in addition to increased angiogenesis through endothelial cell proliferation and migration. One of these effects includes breakdown of the blood-brain barrier.\textsuperscript{35,34} With VEGF stimulation in our bAVM model, the blood-brain barrier is likely to be compromised, allowing therapeutic antibodies to get into the brain. The presence of the human IgG in the parenchyma in VEGF-induced angiogenic foci infers passage of IP-injected bevacizumab and trastuzumab across the blood-brain barrier; this facilitates the antibody to interact with VEGF protein in the brain parenchyma.
In this study, we induced angiogenesis in the mouse brain with AAV-VEGF expressing human VEGF, thus replicating the VEGF elaboration in the human disease and allowing assessment of the effect of bevacizumab, an antihuman VEGF antibody in a mouse model. Although our bAVM model was induced by VEGF stimulation, we started bevaci-zumab treatment 6 weeks after Alk1 deletion and VEGF stimulation, when the lesion had already developed. We found reduction of VEGF in the lesion with bevacizumab treatment leads to regression of the established dysplastic vessels. There have been potential complications associated with the use of VEGF inhibition, including bleeding, hypertension, and hemorrhage, with bevacizumab therapy having increased hemorrhage in cancer patients. However, VEGF-A inhibition by bevacizumab has been shown to have minimal neurotoxicity, with a low risk of central nervous system hemorrhage. In fact, there has been recent interest in VEGF inhibition for clinical management of bAVM for controlling perilesional edema. However, before bevacizumab can be developed as a therapeutic agent for bAVM, additional preclinical safety and efficacy studies will be necessary.

Some studies have shown reduced levels of VEGF–R1 and VEGF–R2 in human resected bAVM, and others describe a higher expression of VEGF in partially embolized AVMs; this might imply elevated VEGF in response to the embolic material rather than uniquely as a part of the disease pathogenesis. The expression of VEGF appears to be related to patient age, as younger patients have higher expression levels; those AVMs that recur after resection also appear to have higher VEGF. Taken together, there may be a range of VEGF expression in the lesional tissue that would result in varying degrees of responsiveness to bevacizumab therapy. Thus, in a possible future clinical study, the bevacizumab dose might need to be adjusted to suit different groups of patients. We show that antagonism of the VEGF effect in the angiogenic foci or in a bAVM lesion reduced vessel number and dysplasia in the bAVM. Our results are consistent with the supposition that tonic VEGF stimulation is an important factor in maintaining the lesional phenotype in the nidus of the bAVM.

Conclusions

We present evidence that in WT mice, IP-injected bevacizumab can penetrate the blood-brain barrier in VEGF-induced angiogenic foci and inhibit angiogenesis; and in our bAVM model, bevacizumab treatment can reduce vessel density and the number of dysplastic vessels. Our data suggest that bevacizumab, or a related strategy to abrogate VEGF signaling, merits additional evaluation in preclinical models as a potential approach for the medical therapy of bAVM.

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Disclosures

None.

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Disclosures

None.

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Bevacizumab Attenuates VEGF-induced Angiogenesis and Vascular Malformations in the Adult Mouse Brain
Supplemental 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. Adult (8 to 10-week old) Alk12f/2f with loxP sites flanking exons 4-6\(^1\) and C57BL/6 mice (WT mice, Jackson Laboratory, Bar Harbor, ME) were used.

Lectin Perfusion for Vessel Labeling

Mice were anesthetized through isoflurane inhalation. The jugular vein was exposed and 100 µl of fluorescein-lycopersicin esculentum lectin (Vector Laboratory, Burlingame, CA) was injected to label the vessels and allowed to circulate for 20 minutes. The heart was then exposed and the mouse was intracardially perfused with PBS plus heparin (1 unit/ml) to remove blood. The brain was removed and frozen. Twenty µm coronal sections were cut on a Leica CM1900 Cryostat (Leica Microsystems, Wetzlar, Germany) and images taken with a Leica DMLS fluorescent microscope with Spot Insight Software (Diagnostic Instruments, Inc., Sterling Heights, MI) to visualize vessel morphology.

Immunohistochemistry

Immunohistochemical staining was performed on lectin perfused 20-µm thick coronal sections. Briefly, sections were incubated with the following primary antibodies at 4°C overnight: anti-human IgG (1:200, Vector Laboratories, Burlingame, CA) to detect leakage of the humanized antibody into mouse brain parenchyma and rabbit anti-Ki67 (1:200, Abcam, Cambridge, MA) to assess proliferating cells. Sections were incubated 90 min with secondary antibody Alexa 488 anti-mouse IgG (1:500 dilution; Invitrogen, Carlsbad, CA) and coverslipped with Vectashield mounting medium with 4’-6-diamidino-2-phenylindole (DAPI) (Vector Laboratory) to label cell nuclei. Negative controls were performed by omitting the primary antibodies.

TUNEL Assay

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was done to identify the extent of DNA fragmentation, using the NeuroTACS II kit (Trevigen, Gaithersburg, MD). Brain sections were treated following the procedure specified by the manufacturer. Positive controls were generated with nuclease treatment as instructed by the kit. As a negative control, slides were prepared in a labeling reaction mix without the TdT enzyme resulting in no TUNEL stain.
Vessel Density and Dysplasia Index Quantification

Coronal sections of lectin perfused brain were used for vessel quantification as previously described. Briefly, two sections per mouse, 0.5 mm rostral and 0.5 mm caudal of the injection site, were chosen. Three areas (to the right, left, and below the injection site) of each section were captured under a 20X microscope objective lens. Vessel density in each picture were counted using NIH Image J 1.63 software. Values for each animal were calculated as the mean vessel count obtained from six images taken under the 20X objective. Dysplasia index was defined as total vessels >15 µm per 200 vessels.
Supplemental Figure S1: The vascular density of trastuzumab treated group is similar to untreated group. A) Images of angiogenic foci taken from lectin-perfused brain samples. Scale Bar: 100 µm. B) Quantification of vessel density showed no significant difference between groups (p=0.59).
Supplemental Figure S2: Co-localization of Lectin perfused vessels (green) with CD-31 immunostaining for endothelial cells (red). Groups include contralateral uninjected region (WT control), angiogenic focus (WT +VEGF), and dysplastic vessels in the bAVM model (Alk1 flox+Cre+VEGF). Scale bar: 20 µm.
**Supplemental Figure S3**: A confocal image showing co-localization of CD31-positive endothelial cells (green) and Ki67-positive nuclei (red). Scale bar: 20 µm.
Supplemental References


