Bevacizumab Attenuates VEGF-Induced Angiogenesis and 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 Alk1<sup>−/−</sup> 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 adeno-associated viral vector expressing human VEGF treated wild-type 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. 2013;43:1925-1930.)

Key Words: VEGF ■ arteriovenous malformation ■ mouse ■ brain ■ angiogenesis
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 (viraly 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.

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 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). Trastuzumab-treated (15 mg/kg) mice had a similar vessel density to untreated WT mice injected with AAV-VEGF alone (WT+VEGF, 1152±48 vessels/mm² versus WT+VEGF+trastuzumab, 1205±63 vessels/mm²; P=0.59; Supplemental Figure S1); this suggests that trastuzumab does not affect VEGF-induced brain angiogenesis. Analysis of the dose-response effect predicted the inhibitory curve (IC₅₀) of bevacizumab to inhibit brain angiogenesis is 1.06 mg/kg (95% CI, 0.36–3.10 mg/kg; Figure 2C). Based on these data, 5 mg/kg bevacizumab was selected for subsequent experiments.

Figure 1. Human antibody was detected in the brain parenchyma of mice treated with bevacizumab and trastuzumab. Vessels are labeled with lectin (green). Human IgG (red) was detected only in VEGF-stimulated regions of mice treated with bevacizumab and trastuzumab. Inserts show high magnification images of vessels. Scale bar: 100 μm.

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.

Wild-type (WT) mice received 2×10⁷ genome copies of AAV viral vectors in 2 μL PBS. Inserts show high magnification images of vessels are labeled with lectin (green). Human IgG (red) was detected only in VEGF-stimulated regions of mice treated with bevacizumab and trastuzumab. Ves-

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 meansSEM. A probability value <0.05 was considered statistically significant.

The Supplemental Methods section describes additional methods. http://stroke.ahajournals.org
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±1.7 cells/mm² versus trastuzumab, 24.8±5.4 cells/mm²; \(P=0.04\); Figure 3). WT mice with VEGF stimulation alone had 13.5±6.9 Ki67-positive vascular cells/mm². In addition, bevacizumab treatment increased TUNEL-positive vascular cells in the angiogenic region by 36% compared with trastuzumab controls (bevacizumab, 78.3±9.3 cells/mm² versus trastuzumab, 50.4±5.4 cells/mm²; \(P=0.03\); Figure 4). Thus, the reduced vessel density in bevacizumab-treated mice is caused by both a decrease in vascular cell growth and increase in vascular cells with damaged DNA.

**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 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.24,25 In this study, we used the AAV1 vector that transfects various cell types in the brain, including neurons, astrocytes, and endothelial cells.22 VEGF acts directly on endothelial cells26 and has been shown to be involved in endothelial cell proliferation and angiogenic remodeling.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.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.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. It has been reported that in human bAVM, radiotherapy-damaged endothelial cells shrink and detach from neighboring endothelial cells and basement membrane, permitting platelet infiltration with deposition of fibrin and hyaline.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.33,34 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

Figure 4. Bevacizumab treatment increased TUNEL-positive cells on vessel wall. A, Representative images show sections double-stained with lectin (green, left), TUNEL (brown, middle), and merged (right). The bevacizumab-treated group had more TUNEL-positive vascular cells (arrows) as compared with trastuzumab treated group. A few TUNEL-positive cells were not associated with a vessel (arrowheads). Insets are high-magnification images showing TUNEL-positive nuclei colocalized with lectin. Scale bar: 50 μm. B, Bar graph shows quantification of TUNEL-positive vascular cells (*P=0.03).

Figure 5. Bevacizumab treatment reduced vessel density and dysplasia in the adult mouse bAVM model. A, Representative images show lectin-stained vessels. There are fewer irregular-shaped large vessels (arrows) in the bevacizumab-treated group as compared with trastuzumab-treated. Scale bar: 50 μm. B, Bar graph shows quantification of vessel density and dysplasia index. Bevacizumab treatment reduced vessel density (*P=0.01) and dysplasia index (*P=0.02) as compared with trastuzumab treatment.
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 beva-
cizumab 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
therapy and increased hemorrhage in cancer patients.36
However, VEGF-A inhibition by bevacizumab has been shown to
have minimal neurotoxicity,37 with a low risk of central nervous
system hemorrhage.36 In fact, there has been recent interest
in VEGF inhibition for clinical management of bAVM for
controlling perilesional edema.38 However, before bevacizi-
umab 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,39 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 patho-
genesis.40 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.41 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 beva-
cizumab can penetrate the blood-brain barrier in VEGF-
induced angiogenic foci and inhibit angiogenesis; and in
our bAVM model, bevacizumab treatment can reduce ves-
sel 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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A correction is needed in the article, “Bevacizumab attenuates VEGF-induced angiogenesis and vascular malformations in the adult mouse brain” by Walker et al (Stroke. 2012;43:1925–1930). Gregory Amend, BS, was not included in the author byline. The online version of the article has been updated and is correct.
ONLINE SUPPLEMENT

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 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-lycopersicum 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-phenylinidole (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


