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[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-phenylinidole (DAPI) (Vector Laboratory) to label cell nuclei. Negative controls were performed by omitting the primary antibodies.

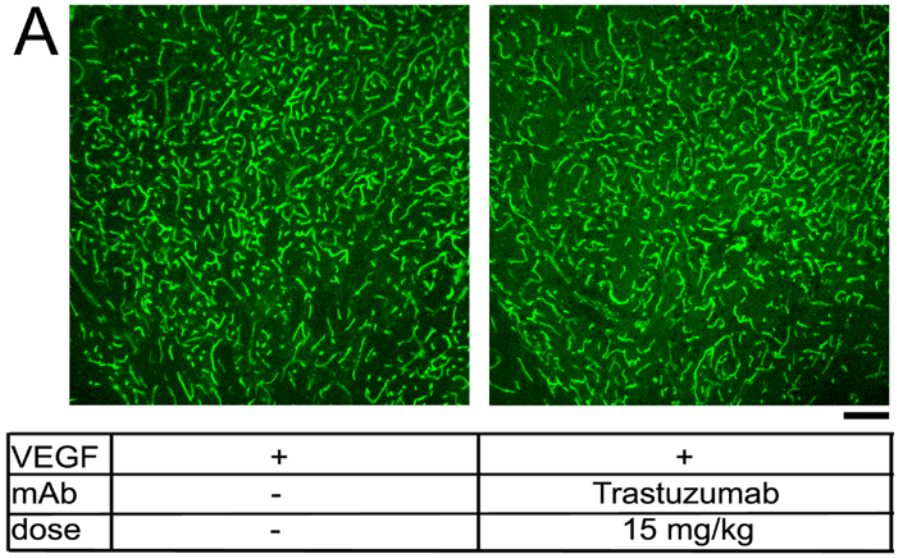
TUNEL Assay

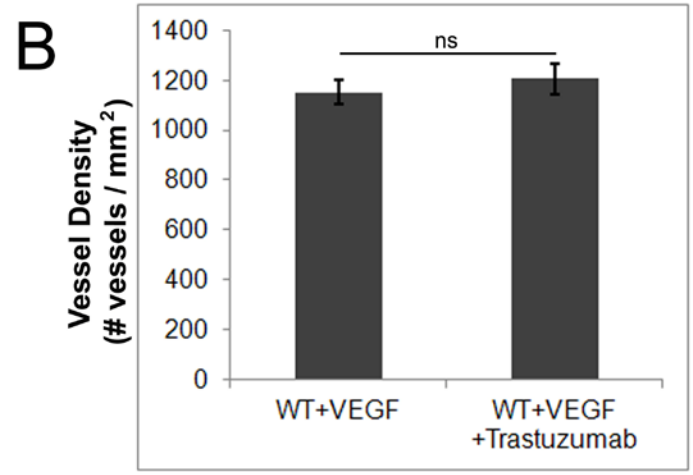
Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was done to identify the extent of DNA fragmentation, using the NeuroTACS II kit (Trevigen, Gaithersburg, MD). Brain sections were treated following the procedure specified by the manufacturer. Positive controls were 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

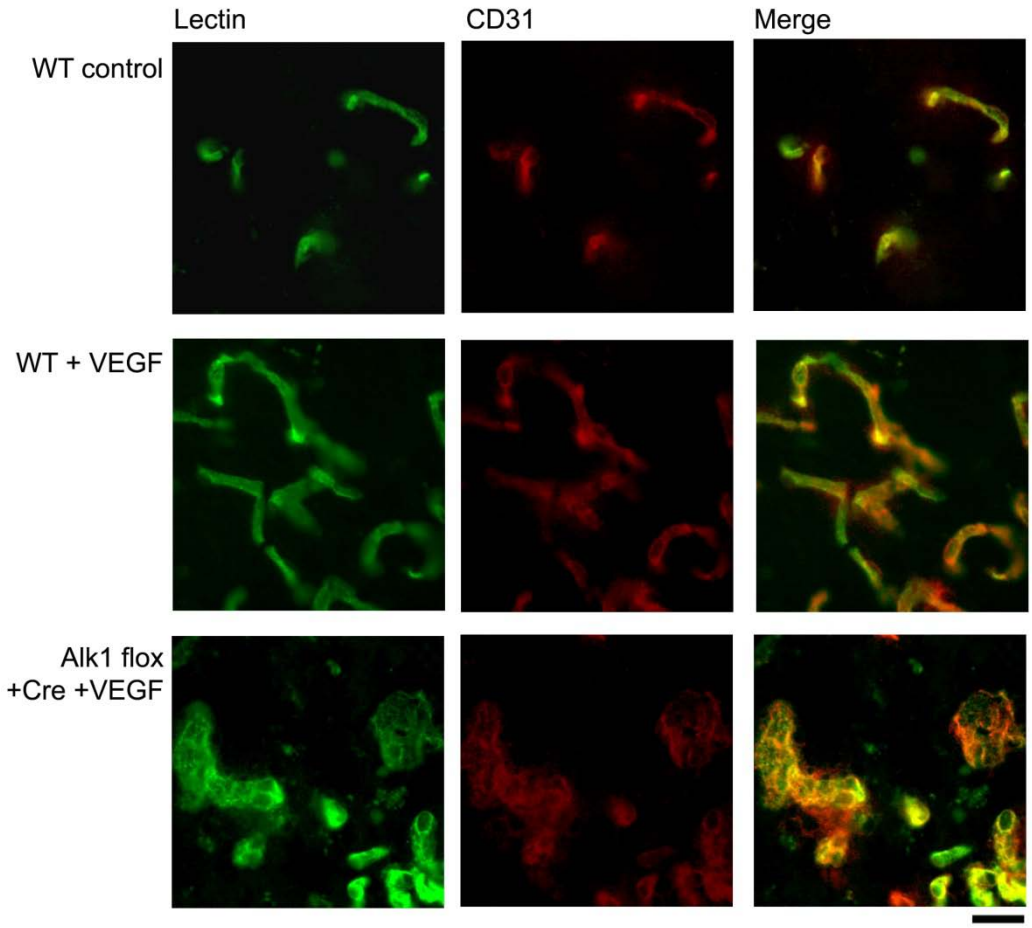
Coronal sections of lectin perfused brain were used for vessel quantification as previously described.[2] Briefly, two sections per mouse, 0.5 mm rostral and 0.5 mm caudal of the injection site, were chosen. Three areas (to the right, left, and below the injection site) of each section were captured 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.[3] Dysplasia index was defined as total vessels >15 μm per 200 vessels.[2, 4]

Supplemental Figures

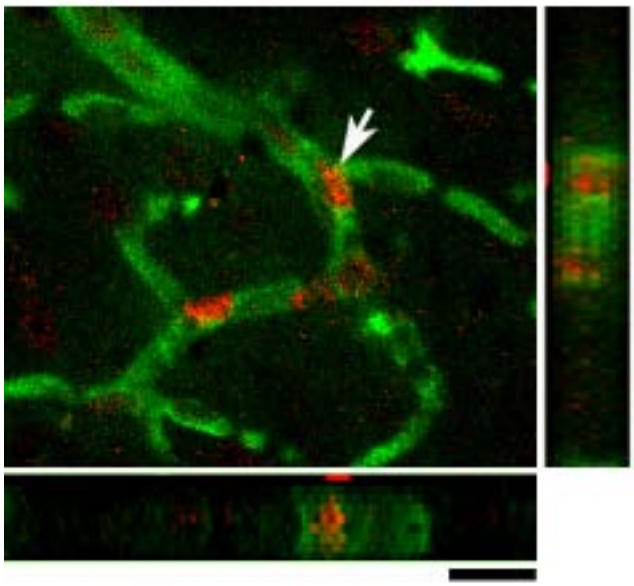




Supplemental Figure S1: The vascular density of trastuzumab treated group is similar to untreated group. A) Images of angiogenic foci taken from lectin-perfused brain samples. Scale Bar: 100 µm. B) Quantification of vessel density showed no significant difference between groups (p=0.59).



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

1. Park SO, Lee YJ, Seki T, Hong KH, Fliess N, Jiang Z, et al. ALK5- and TGFBR2-independent role of ALK1 in the pathogenesis of hereditary hemorrhagic telangiectasia type 2 (HHT2). *Blood*. 2008;111:633-642.
2. Hao Q, Su H, Marchuk DA, Rola R, Wang Y, Liu W, et al. Increased tissue perfusion promotes capillary dysplasia in the ALK1-deficient mouse brain following VEGF stimulation. *Am J Physiol Heart Circ Physiol*. 2008;295:H2250-2256.
3. Yang GY, Xu B, Hashimoto T, Huey M, Chaly T, Jr., Wen R, et al. Induction of focal angiogenesis through adenoviral vector mediated vascular endothelial cell growth factor gene transfer in the mature mouse brain. *Angiogenesis*. 2003;6:151-158.
4. Walker EJ, Su H, Shen F, Choi EJ, Oh SP, Chen G, et al. Arteriovenous malformation in the adult mouse brain resembling the human disease. *Ann Neurol*. 2011;69:954-962.