Inhibition of pathological brain angiogenesis through systemic delivery of AAV vector expressing soluble FLT1

Fanxia Shen, MD[1,2,*], **Lei Mao, MD, PhD**[1,*], **Wan Zhu, PhD**[1], **Michael T. Lawton, MD, PhD**[3], **Peter Pechan, PhD**[4], **Peter Colosi, PhD**[5], **Zhijian Wu, PhD**[5], **Abraham Scaria, PhD**[4], and **Hua Su, MD**[1]

[1]Center for Cerebrovascular Research, Department of Anesthesia and Perioperative Care, University of California, San Francisco, San Francisco, CA, USA

[2]Department of Neurology, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China

[3]Department of Neurological Surgery, University of California, San Francisco, San Francisco, CA, USA

[4]Sanofi-Genzyme R&D Center, Framingham, MA, USA

[5]Ocular Gene Therapy Core, National Eye Institute, National Institutes of Health, Bethesda, MD, USA

Abstract

The soluble vascular endothelial growth factor (VEGF) receptor 1 (sFLT1) has been tested in both animals and humans for anti-angiogenic therapies, e.g., age-related macular degeneration. We hypothesized that adeno-associated viral vector (AAV)-mediated sFLT1 expression could be used to inhibit abnormal brain angiogenesis. We tested the anti-angiogenic effect of sFLT1 and the feasibility of using AAV serotype 9 to deliver sFLT1 through intravenous injection (IV) to the brain angiogenic region. AAV vectors were packaged in AAV serotypes 1 and 2 (stereotactic injection) and 9 (IV-injection). Brain angiogenesis was induced in adult mice through stereotactic injection of AAV1-VEGF. AAV2-sFLT02 containing sFLT1 VEGF-binding domain (domain 2) was injected into the brain angiogenic region, and AAV9-sFLT1 was injected into the jugular vein at the time of or 4 weeks after AAV1-VEGF injection. We showed that AAV2-sFLT02 inhibited brain angiogenesis at both time points. Intravenous injection of AAV9-sFLT1 inhibited angiogenesis only when the vector was injected 4 weeks after angiogenic induction. Neither lymphocyte infiltration nor neuron loss was observed in AAV9-sFLT1-treated mice. Our data

Correspondence should be addressed to Dr. Su (hua.su@ucsf.edu), Department of Anesthesia and Perioperative Care, University of California, San Francisco, 1001 Potrero Avenue Box 1368, San Francisco CA 94110, USA, Phone: 415-206-3162, Fax: 415-206-8907.
*F. Shen and L. Mao contributed equally to this work.

show that systemically delivered AAV9-sFLT1 inhibits angiogenesis in the mouse brain, which could be utilized to treat brain angiogenic diseases such as brain arteriovenous malformation.

INTRODUCTION

Pathological angiogenesis plays key roles in many disorders, such as tumors[1] and vascular and cerebrovascular diseases including brain arteriovenous malformation (bAVM), in both sporadic and familiar cases, such as those in Hereditary Hemorrhagic Telangiectasia (HHT),[2,3] and dual fistula. Among known angiogenic factors, vascular endothelial growth factor-1 (VEGF-A, generally called VEGF) is one of the most important molecules that function through two main receptors, FMS-related tyrosine kinase 1 (FLT1, also called VEGFR-1), and kinase insert domain receptor (KDR, also called VEGFR-2).[1] Besides VEGF-A (hereafter VEGF), other molecules can also bind to FLT1/VEGFR-1 or KDR/VEGFR-2, such as VEGFB and PLGF (placental growth factor, or PGF) for VEGFR-1, and VEGFB, VEGFC and VEGFD for VEGFR-2.[4] While KDR is known to mediate VEGF-induced endothelial cell mitogenesis and vascular permeability, FLT1 has long been recognized as a "decoy" receptor for VEGF and does not stimulate angiogenesis.[1,5] However, evidence has emerged in the past decade showing that the FLT1-mediated signaling pathway in endothelial cells is complex and context-dependent.[1,4] In addition, FLT1 is also expressed by many tumor cells.[6] Despite the controversial findings, the fundamental molecular interaction between VEGF and its receptors has been used in the development of anti-angiogenic drugs,[7,8] including bevacizumab (Avastin, Genentech, South San Francisco, CA), an anti-VEGF antibody for inhibition of angiogenesis and tumor growth.[7–12] Bevacizumab has also been tested to inhibit abnormal angiogenesis in the brain and reduce the severity of vascular disease, i.e., AVM.[13–15] Antibody therapy, however, has some drawbacks, including concern about inducing hemorrhage[16] and the need for prolonged periods of intermittent intravenous (IV) infusions.

VEGFRs share a similar structure, which consists of seven immunoglobulin-like extracellular domains, a transmembrane region, and an intracellular domain for kinase activity.[17,18] The soluble form of FLT1 (sFLT1) is an alternative transcript of FLT1 that contains only six extra-cellular domains of FLT1.[19,20] sFLT1 has a high binding affinity to VEGF, and thus can reduce VEGF-mediated signaling through its membrane-bound receptors. It has been shown previously that domain 1-3 of sFLT1 has equal binding capacity to VEGF as the full length sFLT1, and that domain 2 is the actual binding domain to VEGF.[21] Based on this knowledge, adeno-associated viral vector (AAV) (Table 1) carrying full length sFLT1 or a chimeric protein containing domain 2 of sFLT1 and CH3 domain of IgG1 has been tested to inhibit pathogenic angiogenesis.[22–24] AAV2-sFLT01 and AAV2-sFLT02 are two AAV vectors made by Sanofi-Genzyme Corporation containing sFLT1 domain 2 with different modifications of C-terminal structure and packaged in AAV serotype 2 capsid (Table 1). They have similar levels of VEGF binding ability as sFLT1 domain 1-3.[22] AAV2-sFLT01 has been tested in mice and nonhuman primate models for the treatment of age-related macular degeneration (AMD).[22–24] In addition, AAV2-sFLT01 treatment was well tolerated, and capable of mediating long-term sFLT01 expression in a nonhuman primate model.[24] However, AAV-mediated sFLT1 expression has not been tested

in brain angiogenesis, largely due to the limited ability of AAV to penetrate the blood-brain barrier (BBB) and enter the brain parenchyma. Many AAV serotypes with different tissue-preferences have been identified.[25,26] Among those, serotype 9 (AAV9) can enter the brain parenchyma, particularly the brain angiogenic region, much more effectively than other serotypes.[27,28]

In this study, we first tested if overexpression of sFLT1 can inhibit VEGF-induced brain angiogenesis through stereotactic injection of AAV2-sFLT02[22] into the brain angiogenic foci at the time of or 4 weeks after angiogenic induction. After the effect of sFLT1 anti-brain angiogenesis was confirmed, we tested the feasibility of utilizing intravenous delivery of AAV9-sFLT1 (containing full-length sFLT1, and packaged in AAV serotype 9 capsid, Table 1)[29] to inhibit VEGF-induced brain angiogenesis.[30] We found that intravenous injection of AAV9-sFLT1 4 weeks after angiogenic induction reduced vessel densities in the brain angiogenic region.

RESULTS

To test if AAV-mediated sFLT1 gene transfer inhibits brain angiogenesis, AAV1-VEGF (Table 1 and Supplementary Figure S1) was injected into the basal ganglia of the brain to induce angiogenesis.[31] AAV2-sFLT02, expressing a fusion protein sFLT02 containing human FLT1 extra-cellular domain 2 and CH3 domain of IgG1,[22] (Table 1 and Supplementary Figure S1) was stereotactically injected into the angiogenic foci at the time of (Figure 1a) or 4 weeks after (Figure 1b) angiogenic induction. AAV1-LacZ was used as a vector control for AAV1-VEGF; AAV2-EV, an empty vector, was used as vector control for AAV2-sFLT02. Injection of AAV1-LacZ did not alter vessel density in the brain.[32,33] ELISA analysis in the AAV2-sFLT02-injected brain (349.4 ± 47.6 pg/mg of brain protein) detected robust sFLT02 expression, compared with the AAV2-EV-injected brain (20.7 ± 2.7 pg/mg, P<0.001, Supplementary Figure S2).

Four weeks after AAV2-sFLT02 injection, vessel density was quantified on sections stained with lycopersicin esculentum lectin (Vector Laboratory, Burlingame, CA), which binds to glycophorin and Tamm-Horsfall glycoprotein and is very effective for labeling vascular endothelium in rodents. Mice injected with AAV2-sFLT02 at the time of angiogenic induction had significantly lower vessel density in the brain angiogenic foci compared with that in AAV2-EV-injected mice (655 ± 61 vessels/mm^2 *vs*. 887 ± 177 vessels/mm^2, p=0.016, Figure 2a & b), and was similar to that of AAV1-LacZ/AAV2-sFLT02-injected control mice (616 ± 99 vessels/mm^2, p=0.07, Figure 2a & 2b). Vessel density was also significantly lower in AAV2-sFLT02-injected mice 4 weeks after angiogenic induction compared with those injected with AAV2-EV (747 ± 135 vessels/mm^2 *vs*. 966 ± 158 vessels/mm^2, p=0.027, Figure 2c & 2d). Therefore, AAV-mediated sFLT1 gene transfer could inhibit brain angiogenesis when injected into the angiognic foci at the time of or 4 weeks after angiogenic induction.

To avoid potential risk associated with direct injection of viral vectors into the brain, we tested a less invasive route: intravenous injection. Because AAV serotype 9 (AAV9) penetrates the BBB and enters the brain angiogenic region effectively,[28] AAV-sFLT1 with a full-length human *sFLT1* sequence[29,34] was packaged in AAV 9 capsid and injected into the

jugular vein at the time of (Figure 1c) or 4 weeks after (Figure 1d) angiogenic induction by intra-brain injection of AAV1-VEGF. AAV9-GFP was used as a vector control. Vessel density was quantified 4 weeks after AAV9-sFLT1 injection on lectin-stained brain sections. We found that intravenous injection of AAV9-sFLT1 4 weeks after angiogenic induction significantly reduced vessel-density in the brain angiogenic foci (sFLT1 *vs*. GFP: 912±101 vessels/mm^2 *vs*. 1134±85 vessels/mm^2, p=0.002, Figure 3a & 3b). However, intravenous injection of AAV9-sFLT1 at the time of angiogenic induction did not seem to inhibit brain angiogenesis (sFLT *vs*. GFP: 879±78 *vs*. 856±45, p=0.56, Figure 3c & 3d), consistent with our previously published data that increased permeability of the BBB is required for AAV9 to enter the adult brain parenchyma.[28] At the time of angiogenic induction, no new vessels had developed. In the normal brain, the vascular permeability is very low. Four weeks after angiogenic induction, new vessels had formed. New VEGF-induced vessels tend to have higher permeability than normal ones.[35] With low permeability of the BBB at the time of angiogenic induction, few AAV9 vectors could enter the brain parenchyma to express sFLT1. Overall, our data show that intravenous injection of AAV9-sFLT1 is capable of inhibiting brain angiogenesis.

To study how sFLT1 inhibits VEGF-induced brain angiogenesis, we assessed vascular endothelial cell proliferation in mice that received intra-brain co-injection of AAV1-VEGF and AAV2-sFLT02 4 weeks after vector injection. Double staining with CD31-specific antibody was used to label the endothelial cells, and Ki67-specific antibody to identify proliferating nuclei. Compared with AAV-EV-injected mice, those injected with AAV2-sFLT02 had fewer proliferating endothelial cells (CD31 and Ki67 positive cells, p=0.001, Figure 4). Endothelial cell apoptosis was evaluated in the same groups of mice through double labeling apoptotic endothelial cells using CD31 antibody and TUNEL. Few TUNEL positive endothelial cells were detected in the angiogenic foci of all groups (Figure 5). Most likely, therefore, sFLT1 inhibited brain angiogenesis mainly through inhibition of endothelial cell proliferation.

Since lymphocytic infiltration and loss of neurons have previously been reported to be associated with astrocyte transduction of AAV9,[36] we analyzed whether intravenous injection of AAV9-sFLT1 causes any of these side effects. As shown in Figure 6, we did not observe lymphocyte infiltration or neuronal loss in the brain angiogenic region, thus suggesting that intravenous injection of AAV9-sFLT1 could be developed into a safe tool for treating brain angiogenic diseases.

DISCUSSION

The results of our study show that intravenous injection of AAV9-sFLT1 inhibited brain angiogenesis, and that the angiogenic inhibition effect of sFLT1 was mainly through the reduction of endothelial cell proliferation. Neither lymphocytic infiltration nor neuronal loss was observed in mice that received intravenous injection of AAV9-sFLT1.

A variety of approaches to block the effects of VEGF has been established over the years, including the use of humanized VEGF antibodies, bevacizumab and newer alternatives,[8,37] or small molecule receptor tyrosine kinase inhibitors.[38] However, there are considerable side

effects associated with the use of these anti-VEGF treatments.[38] Because the anti-angiogenic therapeutic efficacy and safety of AAV2-sFLT01 treatment for Age-Related Macular Degeneration (AMD) have been tested in a nonhuman primate model,[23] using AAV-sFLT1 offers advantages.

Recently, significant strides have been made towards clinical application of AAV due to its excellent safety profile and success in several clinical trials.[39] Indeed, this has been bolstered by evidence that more than 90% of the AAV genomes remain episomal in infected cells.[40] Thus, tumor formation through insertional mutagenesis is of less concern when AAV vectors are used. Clinical trials so far have tested effectiveness and safety of AAV delivery in patients with inherited retina dystrophies (Leber's congenital amaurosis),[41,42] heart failure,[43] Duchenne muscular dystrophy,[44] Parkinson's disease,[45] and hemophilia B.[46] More importantly, two exciting developments suggest that the obstacles for AAV clinical application are lessening: (1) NIH's Recombinant DNA Advisory Committee (RAC) approved a clinical trial using systemic AAV9 survival motor neuron 1 (SMN) gene therapy for spinal muscular atrophy (SMA); and (2) an AAV product has been licensed in Europe.[47,48] AAV-mediated gene therapies, therefore, appear to have a promising future.

In this study, we tested AAV-mediated sFLT1 gene expression to inhibit VEGF-induced angiogenesis in the mouse brain. AAV vectors expressing sFLT1 molecules were delivered through intra-brain angiogenic foci injection or intravenous injection. We observed some differences between these two delivery routes. Whereas intra-brain angiogenic foci injection of AAV2-sFLT01 at the time of angiogenic induction (intra-brain injection of AAV1-VEGF) completely inhibited VEGF-induced brain angiogenesis (Figure 2a & b), intravenous injection of AAV9-sFLT at the time of angiogenic induction did not (Figure 3c & d), which is consistent with our previous observation that AAV9-mediated gene expression in the brain requires increased permeability of the BBB.[28] We also showed previously that single-strand AAV9 mediated significant gene expression only in the angiogenic foci after intravenous injection[28] when the BBB permeability is increased.[49] The quiescent vessels in the normal adult brain have very low vascular permeability, and the newly formed vessels are likely to have a higher BBB permeability than quiescent vessels. Therefore, better angiogenic inhibition was observed when AAV9-sFLT1 was intravenously injected 4 weeks after angiogenic induction when new vessels were formed, rather than at the time of angiogenic induction. Comparing the effects of intravenous injection versus intra-arterial injection would be a productive research topic in future studies.

Intra-brain angiogenic foci injection of AAV2-sFLT02 4 weeks after angiogenic induction resulted in slightly better anti-angiogenic effect (22% reduction of vessel-density) than intravenous injection of AAV9-sFLT (19% reduction of vessel-density). However, since different viral doses were used for different injection routes, it is hard to make any conclusion based on this study. Nevertheless, our goal was to test if delivery of sFLT1 expressing AAV vector through a non-invasive route could inhibit brain angiogenesis. We showed that intravenous delivery of AAV9-sFLT1 can effectively reduce vessel density in the brain angiogenic foci.

We have also shown that sFLT1 inhibits VEGF-induced angiogenesis mainly through inhibition of endothelial cell proliferation. This is consistent with previous reports that sFLT1 binding with VEGF may inhibit VEGF downstream signals for endothelial cell proliferation.[1,50,51] It has been reported that acute withdrawal of VEGF in mice overexpressing VEGF in the airway[52] and in tumors[53] result in endothelial cell apoptosis and shedding. We showed that blockage of VEGF signaling using bevacizumab (Avastin) reduces the number of abnormal vessels in the bAVM lesion and induces endothelial cell apoptosis.[13] Therefore, we chose to analyze endothelial apoptosis in our study. We did not detect significant endothelial apoptosis in the AAV1-VEGF and AAV2-sFLT02 co-injected brain. The minimal number of apoptotic endothelial cells detected in this study could have been due to the slow onset of AAV-mediated gene expression, which takes time to reach a plateau. Luciferase expression peaked at 100 days after intra-tail vein injection of an AAV2 or AAV9 vector carrying the luciferase gene.[26] Similarly, sFLT1 gene expression in our study most likely increased gradually, resulting in gradual and constant inhibition of VEGF-induced brain angiogenesis. No massive acute vessel regression occurred in our model. Therefore, the number of apoptotic endothelial cells was limited at any given time.

In this study, we not only showed the feasibility and efficacy of systemic delivery of AAV9-sFLT1 in inhibiting brain angiogenesis, but also demonstrated that systemic delivery of AAV9-sFLT1 does not cause neural inflammation and neuronal death. It has been reported that AAV9 can transduce the heart,[54] lung,[55] and brain.[27,56,57] Systemic delivery of AAV9 also results in high transduction in the liver.[26] In a previous study, we showed that systemic delivery of AAV9 resulted in significant gene transduction in the liver and heart.[28] Understanding the influence of systemic delivery of AAV9-sFLT1 to non-targeted vital organs is important for clinical applications, and therefore, we will analyze this in a future study.

Most AAV therapies tested for the treatment of central nervous system diseases used direct intra-brain injection. Direct injection, however, is an invasive procedure that can cause unexpected side effects. Most vascular lesions have dense blood vessels, and injection of a viral vector into these lesions can cause massive bleeding. Direct injection is not feasible when multiple lesions are present. We tested a less invasive method in this study, and showed that intravenous delivery is a feasible route to deliver therapeutic AAV vector into brain lesions that have active angiogenesis. This study establishes a foundation for exploring the possibility of using AAV9-sFLT1 through intravenous delivery to treat cerebral vascular diseases including bAVM, in both sporadic and familiar cases such as those in HHT, brain tumor, and dual fistula.

MATERIALS AND METHODS

Ethics statement

Animal experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California, San Francisco (UCSF), and conformed to NIH Guidelines. Animal husbandry was made available by the staff of the Animal Core Facility, and by the staff of the IACUC of UCSF, under the guidance of supervisors who are

certified Animal Technologists. Veterinary care was provided by IACUC faculty members and veterinary residents located on the San Francisco General Hospital campus.

Animals

Adult wild-type male mice (C57BL/6J, 8–10 weeks of age) were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were fed standard rodent food and water ad libitum, and were housed (5 per cage) in 421×316 cm^2 sawdust-lined cages in an air-conditioned environment with 12-hour light/dark cycles.

AAV vector construction and production

A cytomegalovirus (CMV) promoter was used in all vectors. VEGF, sFLT02 and full-length sFLT are all of human origin.

AAV1-VEGF and AAV1-LacZ were made as previously described[58,59] (Supplementary Figure S1) using the three plasmid co-transfection system.[60] VEGF in AAV1-VEGF vector is the cDNA of human VEGF$_{165}$ [58,59]. Two helper plasmids, one with adenoviral VA, E2A, and E4 regions, and the other with the AAV rep and cap genes, were co-transfected with AAV plasmids into HEK 293 cells to package the AAV vector. AAV vectors were purified using CsCl$_2$ centrifugation. Viral titers were determined by dot-blot analysis of DNA content and expressed as genome copies (gcs).

AAV2-sFLT02 and AAV2-EV[22] were provided by Sanofi-Genzyme Corporation (Framingham, MA). sFLT02 in AAV2-sFLT02 (Supplementary Figure S1) contains human sFLT doman 2 driven by CMV promoter.[22]

AAV9-sFLT1 was produced by Dr. Zhijian Wu at the National Eye Institute, NIH (Bethesda, MD). As described previously, pAAV-sFLT1[29] (Supplementary Figure S1) containing the full-length human sFLT1 gene was packaged in AAV9 capsid through a three-plasmid co-transfection system. The virus was purified with polyethylene glycol precipitation followed by cesium chloride density gradient fractionation.[29,34] Viral titers were determined through real-time PCR using linearized plasmid standards. AAV9-GFP (Supplementary Figure S1) was produced by Vector Biolabs (Philadelphia, PA).

Stereotactic injection of AAV vectors into the basal ganglia

Mice were randomly assigned to different groups and were anesthetized using isoflurane and placed in a stereotactic frame with a holder (David Kopf Instruments, Tujunga, CA). A burr hole was drilled in the pericranium to allow injection with a needle into the center of the basal ganglia, 2 mm lateral to the sagittal suture, 1 mm posterior to the coronal suture, and 3 mm into the cortex. A total of 2 μl viral suspension containing 2×10^9 genome copies (gcs) of AAV1-VEGF, AAV1-LacZ, AAV2-sFLT02 or AAV2-EV was stereotactically injected into the right basal ganglia at a rate of 0.2 μl per minute using a Hamilton syringe. AAV2-sFLT02 or AAV2-EV were injected together with AAV1-VEGF or AAV1-LacZ (Figure 1a), or injected 4 weeks after AAV1-VEGF injection (Figure 1b). The needle was withdrawn 10 min after completion of the injection, and the wound was closed with a suture.

Four weeks after AAV2-sFLT02 or AAV2-EV injection, mice were euthanatized and tissues were collected for subsequent analyses.

Intravenous injection of AAV vectors

Mice were anesthetized with isoflurane. A total of 50 µl PBS containing 1×10^{11} gcs or AAV9-sFLT1 or AAV9- GFP vectors was injected through the left jugular vein at the time of (Figure 1c) or 4 weeks after (Figure 1d) intra-brain injection of AAV1-VEGF. The wound was closed with a suture. Four weeks after AAV9-sFLT1 injection, mice were euthanatized and tissues were collected for subsequent analyses.

Immunohistochemistry and immunofluorescence

Fresh brain sections were frozen directly in dry ice after the mice were sacrificed. Coronal cryostat sections were cut at a 20 µm thickness on a Leica CM1900 Cryostat (Leica). Sections were incubated at 4°C overnight with the following primary antibodies: anti-CD31 (1:100, M20, Santa Cruz Biotechnology, Santa Cruz, CA), anti-ki67 (1:100, Ab 15580, Abcam, Cambrige, MA), NeuN (1:500, MAB377, Chemicon, Temecula, CA) or anti-CD3 (1:100, Ab 16044, Abcam). Sections were incubated for 90 min with secondary antibodies Alexa Fluor 594-conjugated (1:500) or Alexa Fluor 488-conjugated IgG (1:500) (Invitrogen, Carlsbad, CA), and coverslipped with Vectashield mounting medium with 4′-6-diamidino-2-phenylinidole (DAPI) (Vector Laboratories, Burlingame, CA) to label cell nuclei.

ELISA assay

To examine sFLT1 expression in the brain, brain tissue around the vector injection side was collected from mice that received intra-brain co-injection of AAV1-VEGF and AAV2-sFLT02 or AAV1-VEGF and AAV2-EV 4 weeks after vector injection. Six mice were used in each group. Brain tissue was homogenized in a tissue lysis buffer (Tris buffed saline, proteases inhibitors, 0.1% NP-40). sFLT1 protein was quantified by ELISA assays using Human FLT1 Quantikine ELISA kit (R&D systems Inc., Minneapolis, MN) according to the manufacturer's instructions.

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 by nuclease treatment according to the manufacturer's instructions. As a negative control, slides were prepared in a labeling reaction mix without the TdT enzyme resulting in no TUNEL staining.

Vessel density and dysplasia index quantification

Two sections, 0.5 mm apart per brain within the injection site, were stained with fluorescent-labeled lectin. Sections were fixed with 100% ethanol at 20°C for 20 minutes, then incubated overnight with fluorescein lycopersicin esculentum lectin (Vector Laboratories), 2 g/ml at 4°C, and then coverslipped with Vectashield mounting medium with DAPI (Vector Laboratories) to label cell nuclei. Three images were taken from each section (right and left

of, and below the injection site) under a 20X objective. The number of vessels was counted using NIH Image 1.63 software by three investigators who had no knowledge about the experimental groups, and vessel density was expressed as the number of vessels per mm^2.

Statistical analyses

Data are presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to determine statistical significance among multiple groups, followed by pairwise multiple comparisons using the post-hoc Tukey test. Student's t-test was used to compare 2 groups. A *p* value of <0.05 was considered statistically significant. Sample sizes were n=6 for each group. The sample size was determined based on our previous study.[13]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

References

1. Chung AS, Ferrara N. Developmental and pathological angiogenesis. Annu Rev Cell Dev Biol. 2011; 27:563–584. [PubMed: 21756109]

2. Kim H, Su H, Weinsheimer S, Pawlikowska L, Young WL. Brain arteriovenous malformation pathogenesis: a response-to-injury paradigm. Acta Neurochir Suppl. 2011; 111:83–92. [PubMed: 21725736]

3. Chen W, Choi EJ, McDougall CM, Su H. Brain arteriovenous malformation modeling, pathogenesis and novel therapeutic targets. Transl Stroke Res. 2014; 5:316–329. [PubMed: 24723256]

4. Olsson AK, Dimberg A, Kreuger J, Claesson-Welsh L. VEGF receptor signalling - in control of vascular function. Nat Rev Mol Cell Biol. 2006; 7:359–371. [PubMed: 16633338]

5. Shibuya M. Vascular endothelial growth factor receptor-1 (VEGFR-1/Flt-1): a dual regulator for angiogenesis. Angiogenesis. 2006; 9:225–230. discussion 231. [PubMed: 17109193]

6. Al-Maghrabi J, Gomaa W, Buhmeida A, Qari Y, Al-Qahtani M, Al-Ahwal M. Prognostic significance of VEGFR1/Flt-1 immunoexpression in colorectal carcinoma. Tumour Biol. 2014; 35:9045–9051. [PubMed: 24908415]

7. Heist RS, Duda DG, Sahani DV, Ancukiewicz M, Fidias P, Sequist LV, et al. Improved tumor vascularization after anti-VEGF therapy with carboplatin and nab-paclitaxel associates with survival in lung cancer. Proc Natl Acad Sci U S A. 2015; 112:1547–1552. [PubMed: 25605928]

8. Liu L, Yu H, Huang X, Tan H, Li S, Luo Y, et al. A novel engineered VEGF blocker with an excellent pharmacokinetic profile and robust anti-tumor activity. BMC Cancer. 2015; 15:170. [PubMed: 25881012]

9. Ferrara N, Hillan KJ, Novotny W. Bevacizumab (Avastin), a humanized anti-VEGF monoclonal antibody for cancer therapy. Biochem Biophys Res Commun. 2005; 333:328–335. [PubMed: 15961063]

10. Mitchell A, Adams LA, MacQuillan G, Tibballs J, vanden Driesen R, Delriviere L. Bevacizumab reverses need for liver transplantation in hereditary hemorrhagic telangiectasia. Liver Transpl. 2008; 14:210–213. [PubMed: 18236396]

11. Flieger D, Hainke S, Fischbach W. Dramatic improvement in hereditary hemorrhagic telangiectasia after treatment with the vascular endothelial growth factor (VEGF) antagonist bevacizumab. Ann Hematol. 2006; 85:631–632. [PubMed: 16807748]

12. Andreoli CM, Miller JW. Anti-vascular endothelial growth factor therapy for ocular neovascular disease. Curr Opin Ophthalmol. 2007; 18:502–508. [PubMed: 18163003]

13. Walker EJ, Su H, Shen F, Degos V, Amend G, Jun K, et al. Bevacizumab attenuates VEGF-induced angiogenesis and vascular malformations in the adult mouse brain. Stroke. 2012; 43:1925–1930. [PubMed: 22569934]

14. Williams BJ, Park DM, Sheehan JP. Bevacizumab used for the treatment of severe, refractory perilesional edema due to an arteriovenous malformation treated with stereotactic radiosurgery. J Neurosurg. 2012; 116:972–977. [PubMed: 22324417]

15. Dupuis-Girod S, Ginon I, Saurin JC, Marion D, Guillot E, Decullier E, et al. Bevacizumab in patients with hereditary hemorrhagic telangiectasia and severe hepatic vascular malformations and high cardiac output. JAMA. 2012; 307:948–955. [PubMed: 22396517]

16. Tanvetyanon T, Murtagh R, Bepler G. Rupture of a cerebral arteriovenous malformation in a patient treated with bevacizumab. J Thorac Oncol. 2009; 4:268–269. [PubMed: 19179909]

17. Shibuya M, Yamaguchi S, Yamane A, Ikeda T, Tojo A, Matsushime H, et al. Nucleotide sequence and expression of a novel human receptor-type tyrosine kinase gene (flt) closely related to the fms family. Oncogene. 1990; 5:519–524. [PubMed: 2158038]

18. Quinn TP, Peters KG, De Vries C, Ferrara N, Williams LT. Fetal liver kinase 1 is a receptor for vascular endothelial growth factor and is selectively expressed in vascular endothelium. Proc Natl Acad Sci U S A. 1993; 90:7533–7537. [PubMed: 8356051]

19. Kendall RL, Thomas KA. Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor. Proc Natl Acad Sci U S A. 1993; 90:10705–10709. [PubMed: 8248162]

20. Kendall RL, Wang G, Thomas KA. Identification of a natural soluble form of the vascular endothelial growth factor receptor, FLT-1, and its heterodimerization with KDR. Biochem Biophys Res Commun. 1996; 226:324–328. [PubMed: 8806634]

21. Davis-Smyth T, Chen H, Park J, Presta LG, Ferrara N. The second immunoglobulin-like domain of the VEGF tyrosine kinase receptor Flt-1 determines ligand binding and may initiate a signal transduction cascade. EMBO J. 1996; 15:4919–4927. [PubMed: 8890165]

22. Pechan P, Rubin H, Lukason M, Ardinger J, DuFresne E, Hauswirth WW, et al. Novel anti-VEGF chimeric molecules delivered by AAV vectors for inhibition of retinal neovascularization. Gene Ther. 2009; 16:10–16. [PubMed: 18633446]

23. Lukason M, Dufresne E, Rubin H, Pechan P, Li Q, Kim I, et al. Inhibition of choroidal neovascularization in a nonhuman primate model by intravitreal administration of an AAV2 vector expressing a novel anti-VEGF molecule. Mol Ther. 2011; 19:260–265. [PubMed: 20978476]

24. Maclachlan TK, Lukason M, Collins M, Munger R, Isenberger E, Rogers C, et al. Preclinical safety evaluation of AAV2-sFLT01- a gene therapy for age-related macular degeneration. Mol Ther. 2011; 19:326–334. [PubMed: 21119620]

25. Mori S, Wang L, Takeuchi T, Kanda T. Two novel adeno-associated viruses from cynomolgus monkey: pseudotyping characterization of capsid protein. Virology. 2004; 330:375–383. [PubMed: 15567432]

26. Zincarelli C, Soltys S, Rengo G, Rabinowitz JE. Analysis of AAV serotypes 1-9 mediated gene expression and tropism in mice after systemic injection. Mol Ther. 2008; 16:1073–1080. [PubMed: 18414476]

27. Foust KD, Nurre E, Montgomery CL, Hernandez A, Chan CM, Kaspar BK. Intravascular AAV9 preferentially targets neonatal neurons and adult astrocytes. Nat Biotechnol. 2009; 27:59–65. [PubMed: 19098898]

28. Shen F, Kuo R, Milon-Camus M, Han Z, Jang L, Young WL, et al. Intravenous delivery of adeno-associated viral vector serotype 9 mediates effective gene expression in ischemic stroke lesion and brain angiogenic foci. Stroke. 2013; 44:252–254. [PubMed: 23250995]

29. Ramirez M, Wu Z, Moreno-Carranza B, Jeziorski MC, Arnold E, Diaz-Lezama N, et al. Vasoinhibin gene transfer by adenoassociated virus type 2 protects against VEGF- and diabetes-induced retinal vasopermeability. Invest Ophthalmol Vis Sci. 2011; 52:8944–8950. [PubMed: 22003113]

30. Shen F, Su H, Liu W, Kan YW, Young WL, Yang GY. Recombinant adeno-associated viral vector encoding human VEGF165 induces neomicrovessel formation in the adult mouse brain. Front Biosci. 2006; 11:3190–3198. [PubMed: 16720385]

31. Shen F, Su H, Fan Y, Zhu Y, Chen Y, Kan YW, et al. Induction of focal angiogenesis through adeno-associated viral vector mediated VEGF165 gene transfer in the mature mouse brain [Abstract]. Stroke. 2006; 37:685.

32. Hao Q, Liu J, Pappu R, Su H, Rola R, Gabriel RA, et al. Contribution of bone marrow-derived cells associated with brain angiogenesis is primarily through leucocytes and macrophages. Arterioscler Thromb Vasc Biol. 2008; 28:2151–2157. [PubMed: 18802012]

33. 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. [PubMed: 21437931]

34. Grimm D, Zhou S, Nakai H, Thomas CE, Storm TA, Fuess S, et al. Preclinical in vivo evaluation of pseudotyped adeno-associated virus vectors for liver gene therapy. Blood. 2003; 102:2412–2419. [PubMed: 12791653]

35. Shen F, Walker EJ, Jiang L, Degos V, Li J, Sun B, et al. Coexpression of angiopoietin1 with VEGF increases the structural integrity of the blood-brain barrier and reduces atrophy volume. J Cereb Blood Flow Metab. 2011; 31:2343–2351. [PubMed: 21772310]

36. Ciesielska A, Hadaczek P, Mittermeyer G, Zhou S, Wright JF, Bankiewicz KS, et al. Cerebral infusion of AAV9 vector-encoding non-self proteins can elicit cell-mediated immune responses. Mol Ther. 2013; 21:158–166. [PubMed: 22929660]

37. Sullivan LA, Carbon JG, Roland CL, Toombs JE, Nyquist-Andersen M, Kavlie A, et al. r84, a novel therapeutic antibody against mouse and human VEGF with potent anti-tumor activity and limited toxicity induction. PLoS One. 2010; 5:e12031. [PubMed: 20700512]

38. Simons M, Eichmann A. "On-target" cardiac effects of anticancer drugs: lessons from new biology. J Am Coll Cardiol. 2012; 60:626–627. [PubMed: 22703925]

39. Xiao PJ, Lentz TB, Samulski RJ. Recombinant adeno-associated virus: clinical application and development as a gene-therapy vector. Ther Deliv. 2012; 3:835–856. [PubMed: 22900466]

40. Kay MA. State-of-the-art gene-based therapies: the road ahead. Nat Rev Genet. 2011; 12:316–328. [PubMed: 21468099]

41. Smith AJ, Bainbridge JW, Ali RR. Gene supplementation therapy for recessive forms of inherited retinal dystrophies. Gene Ther. 2012; 19:154–161. [PubMed: 22033465]

42. Jacobson SG, Cideciyan AV, Ratnakaram R, Heon E, Schwartz SB, Roman AJ, et al. Gene therapy for leber congenital amaurosis caused by RPE65 mutations: safety and efficacy in 15 children and adults followed up to 3 years. Arch Ophthalmol. 2012; 130:9–24. [PubMed: 21911650]

43. Hajjar RJ, Zsebo K, Deckelbaum L, Thompson C, Rudy J, Yaroshinsky A, et al. Design of a phase 1/2 trial of intracoronary administration of AAV1/SERCA2a in patients with heart failure. J Card Fail. 2008; 14:355–367. [PubMed: 18514926]

44. Bowles DE, McPhee SW, Li C, Gray SJ, Samulski JJ, Camp AS, et al. Phase 1 gene therapy for Duchenne muscular dystrophy using a translational optimized AAV vector. Mol Ther. 2012; 20:443–455. [PubMed: 22068425]

45. Herzog RW, Cao O, Srivastava A. Two decades of clinical gene therapy--success is finally mounting. Discov Med. 2010; 9:105–111. [PubMed: 20193635]

46. Nathwani AC, Tuddenham EG, Rangarajan S, Rosales C, McIntosh J, Linch DC, et al. Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. N Engl J Med. 2011; 365:2357–2365. [PubMed: 22149959]

47. Pollack, A. New York Times. New York Times; New York: 2012. European agency backs approval of a gene therapy; p. B1

48. Mingozzi F, High KA. Immune responses to AAV vectors: overcoming barriers to successful gene therapy. Blood. 2013; 122:23–36. [PubMed: 23596044]

49. Shen F, Hao Q, Walker E, Sun B, Heriyanto F, Young WL, et al. AAV-mediated co-expression of angiopoietin-1 and VEGF in the ischemic brain results in reduced vessel leakage and infarct size than VEGF expression alone [Abstract]. Stroke. 2010; 41:e139. (P549).

50. Hiratsuka S, Minowa O, Kuno J, Noda T, Shibuya M. Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice. Proc Natl Acad Sci U S A. 1998; 95:9349–9354. [PubMed: 9689083]

51. Meyer S, van Liempt E, Imberty A, van Kooyk Y, Geyer H, Geyer R, et al. DC-SIGN mediates binding of dendritic cells to authentic pseudo-LewisY glycolipids of Schistosoma mansoni cercariae, the first parasite-specific ligand of DC-SIGN. J Biol Chem. 2005; 280:37349–37359. [PubMed: 16155001]

52. Baluk P, Lee CG, Link H, Ator E, Haskell A, Elias JA, et al. Regulated angiogenesis and vascular regression in mice overexpressing vascular endothelial growth factor in airways. Am J Pathol. 2004; 165:1071–1085. [PubMed: 15466375]

53. Benjamin LE, Keshet E. Conditional switching of vascular endothelial growth factor (VEGF) expression in tumors: induction of endothelial cell shedding and regression of hemangioblastoma-like vessels by VEGF withdrawal. Proc Natl Acad Sci U S A. 1997; 94:8761–8766. [PubMed: 9238051]

54. Bish LT, Morine K, Sleeper MM, Sanmiguel J, Wu D, Gao G, et al. Adeno-associated virus (AAV) serotype 9 provides global cardiac gene transfer superior to AAV1, AAV6, AAV7, and AAV8 in the mouse and rat. Hum Gene Ther. 2008; 19:1359–1368. [PubMed: 18795839]

55. Limberis MP, Wilson JM. Adeno-associated virus serotype 9 vectors transduce murine alveolar and nasal epithelia and can be readministered. Proc Natl Acad Sci U S A. 2006; 103:12993–12998. [PubMed: 16938846]

56. Duque S, Joussemet B, Riviere C, Marais T, Dubreil L, Douar AM, et al. Intravenous administration of self-complementary AAV9 enables transgene delivery to adult motor neurons. Mol Ther. 2009; 17:1187–1196. [PubMed: 19367261]

57. Gong Y, Mu D, Prabhakar S, Moser A, Musolino P, Ren J, et al. Adenoassociated virus serotype 9-mediated gene therapy for x-linked adrenoleukodystrophy. Mol Ther. 2015; 23:824–834. [PubMed: 25592337]

58. Su H, Lu R, Kan YW. Adeno-associated viral vector-mediated vascular endothelial growth factor gene transfer induces neovascular formation in ischemic heart. Proc Natl Acad Sci U S A. 2000; 97:13801–13806. [PubMed: 11095751]

59. Su H, Huang Y, Takagawa J, Barcena A, Arakawa-Hoyt J, Ye J, et al. AAV serotype-1 mediates early onset of gene expression in mouse hearts and results in better therapeutic effect. Gene Ther. 2006; 13:1495–1502. [PubMed: 16775632]

60. Matsushita T, Elliger S, Elliger C, Podsakoff G, Villarreal L, Kurtzman GJ, et al. Adeno-associated virus vectors can be efficiently produced without helper virus. Gene Ther. 1998; 5:938–945. [PubMed: 9813665]
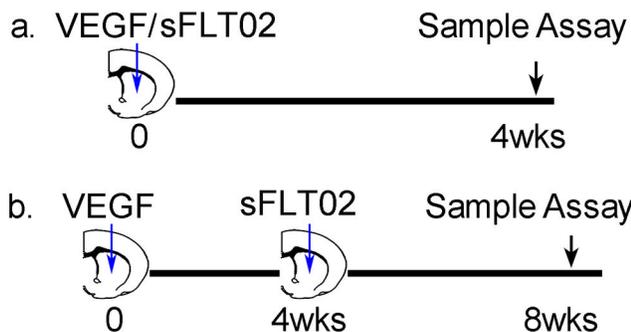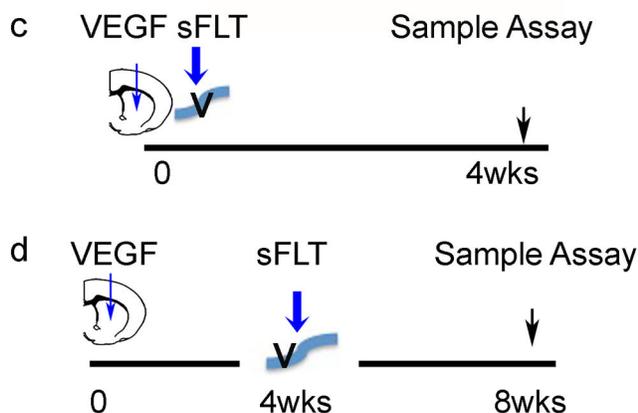
Figure 1. Experimental design

AAV2-sFLT02 (sFLT02) was injected into the brain at the time of (**a**) or 4 weeks after (**b**) AAV1-VEGF (VEGF) injection. AAV9-sFLT1 (sFLT) was injected into the jugular vein at the time of (**c**) or 4 weeks after (**d**) AAV1-VEGF (VEGF) injection. Brain samples were collected 4 weeks after injection of sFLT vectors. Thin blue arrows in **a** and **b** indicate stereotactic injection of AAV1-VEGF and AAV2-sFLT02 vectors into the right basal ganglia of the brain. Thick blue arrows in **c** and **d** indicate injection of AAV9-sFLT1 (sFLT) into the jugular vein. V: jugular vein; I.C.: intra-brain injection; I.V.: intra-jugular vein injection; wks: weeks.
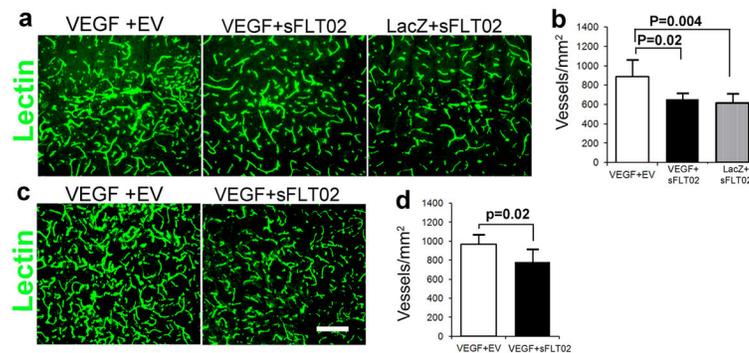
Figure 2. Intra-brain injection of AAV2-sFLT02 reduces vessel density in the brain angiogenic foci

Representative images of lectin (green)-stained sections taken from brain injected with AAV2-sFLT02, AAV2-EV or AAV2-LacZ at the time of (**a**) or 4 weeks after (**c**) injection of AAV1-VEGF. Quantifications of vessel-density (**b**) and (**d**) using brain sections from mice injected with AAV2-sFLT02, AAV2-EV or AAV2-LacZ at the time of (**b**) or 4 weeks after (**d**) injection of AAV1-VEGF. Abbreviations: VEGF: AAV1-VEGF; EV: AAV2-EV; LacZ: AAV1-LacZ; and sFLT02: AAV2-sFLT02. Scale bar: 100μm.
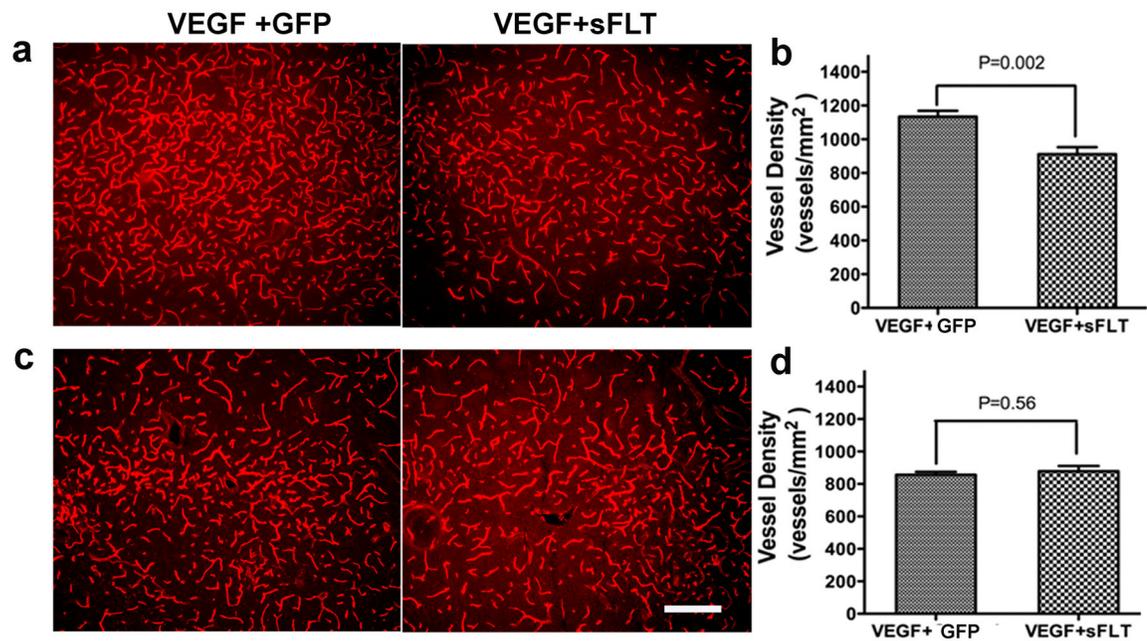
Figure 3. Intravenous injection of AAV9-sFLT1 4 weeks after angiogenic induction reduces vessel density in the brain angiogenic foci

Representative images of lectin (red)-stained sections taken from brain receiving intra-jugular vein injection of AAV9-sFLT1 or AAV9-GFP 4 weeks after (**a**) or at the time of (**c**) intra-brain injection of AAV1-VEGF. Quantifications of vessel-density (**b**) and (**d**) using brain sections from mice injected with AAV9-sFLT1 or AAV9-GFP 4 weeks after (**b**) or at the time of (**d**) intra-brain injection of AAV1-VEGF. Abbreviations: VEGF: AAV1-VEGF; GFP: AAV9-GFP; and sFLT: AAV9-sFLT1. Scale bar: 100μm.
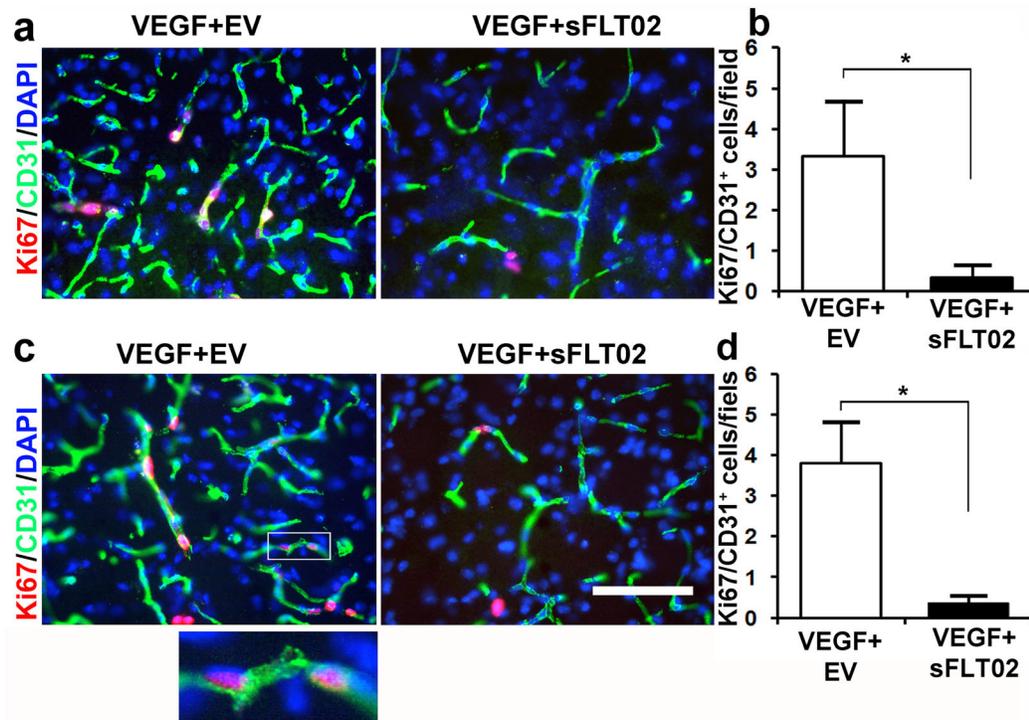
Figure 4. Intra-brain injection of AAV2-sFLT02 inhibits endothelial cell growth in the brain angiogenic foci

Representative images of Ki67 (red) and CD31 (green) antibody-stained brain sections from mice receiving injection of AAV2-sFLT02 or AAV2-EV in the brain angiogenic regions at the time of (**a**) or 4 weeks after (**c**) intra-brain injection of AAV1-VEGF. Cell nuclei were stained using DAPI (blue). Quantifications of Ki67 and CD31 double positive cells (proliferating endothelial cells) (**b**) and (**d**) in the angiogenic foci of brain injected with AAV2-sFLT02 or AAV2-EV at the time of (**b**) or 4 weeks after (**d**) angiogenic induction mediated by intra-brain injection of AAV1-VEGF. The small picture under (**c**) is a close-up of the rectangular region showing 2 endothelial cells that was stained positively by an Ki67 antibody. Scale bar: 100μm. *: p=0.001.
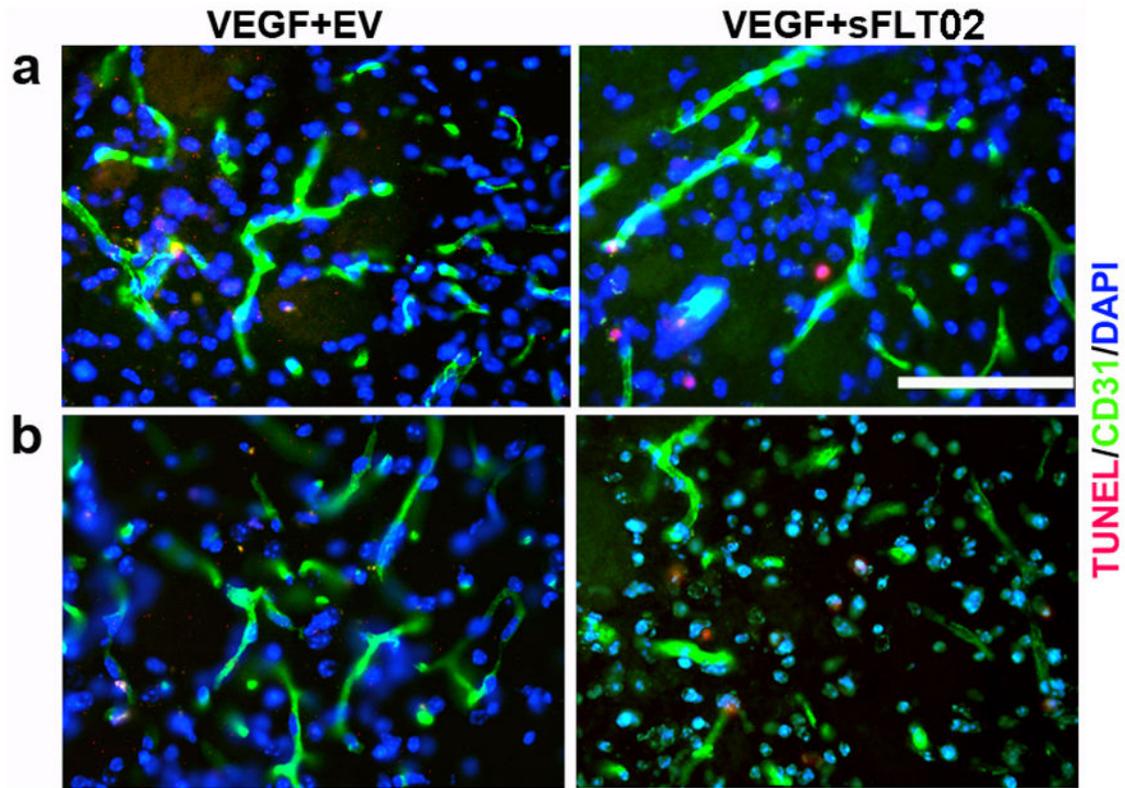
Figure 5. Intra-brain injection of AAV2-sFLT02 does not increase TUNEL positive endothelial cells

Representative images of TUNEL (red) and CD31 (green) antibody-stained brain sections from mice receiving injection of AAV2-sFLT02 or AAV2-EV in brain angiogenic regions at the time of (**a**) or 4 weeks after (**b**) angiogenic induction mediated by AAV1-VEGF. Cell nuclei were stained using DAPI (blue). Scale bar: 100μm.
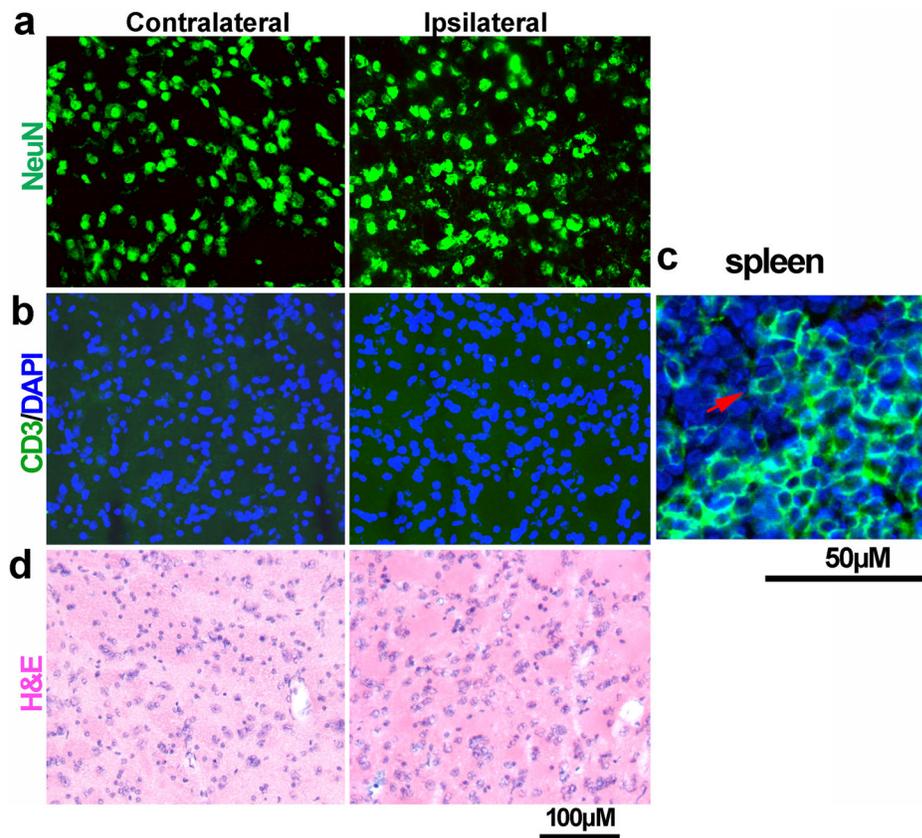
Figure 6. Intravenous injection of AAV9-sFLT1 does not cause neuronal death or lymphocyte inflammation

Representative images of brain sections stained with antibodies specific to NeuN (**a**, green) or CD3 (**b**, green), or hemotoxilin and erosin (H & E) (**d**). An image of spleen section (**c**) shows positive CD3 staining (green). This picture shows a positive control for CD3 staining, which indicates that the negative CD3 staining in (**b**) is due to the lack of lymphocyte in the brain, instead of a stain failure. Cell nuclei in (**b**) and (**c**) were stained using DAPI (blue). Scale bars: 100 μm for brain sections and 50 μm for spleen section. Ipsilateral: the side of the brain injected with AAV1-VEGF; Contralateral: the hemisphere that is opposite to the AAV1-VEGF-injected hemisphere.

Table 1

Nomenclature of AAV vectors

AAV	Adeno-associated viral vector
AAV1	AAV vectors packaged in serotype 1 capsid
AAV2	AAV vectors packaged in serotype 2 capsid
AAV9	AAV vectors packaged in serotype 9 capsid
VEGF	Vascular endothelial growth factor
LacZ	Betagalactosidase
sFLT	Full-length soluble VEGF receptor 1
sFLT02	domain 2 of sFLT1 sequence and modified adjacent regions {Pechan, 2009 #26583}
GFP	Green fluorescent protein