INTRODUCTION
Pathological angiogenesis has key roles in many disorders, such as tumors and vascular and cerebrovascular diseases including brain arteriovenous malformation, in both sporadic and familiar cases, such as those in Hereditary Hemorrhagic Telangiectasia, 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). 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. 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. However, evidence has emerged in the past decade showing that the FLT1-mediated signaling pathway in endothelial cells is complex and context-dependent. In addition, FLT1 is also expressed by many tumor cells. Despite the controversial findings, the fundamental molecular interaction between VEGF and its receptors has been used in the development of anti-angiogenic drugs, including bevacizumab (Avastin, Genentech, South San Francisco, CA, USA), an anti-VEGF antibody for inhibition of angiogenesis and tumor growth. Bevacizumab has also been tested to inhibit abnormal angiogenesis in the brain and reduce the severity of vascular disease, that is, AVM. Antibody therapy, however, has some drawbacks, including concern about inducing hemorrhage and the need for prolonged periods of intermittent intravenous infusions. VEGFRs share a similar structure, which consists of seven immunoglobulin-like extracellular domains, a transmembrane region and an intracellular domain for kinase activity. The soluble form of FLT1 (sFLT1) is an alternative transcript of FLT1 that contains only six extra-cellular domains of FLT1. 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 domains 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. The soluble form of FLT1 (sFLT1) is an alternative transcript of FLT1 that contains only six extra-cellular domains of FLT1. 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 domains 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. On the basis of 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. 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. AAV2-sFLT01 has been tested in mice and nonhuman primate models for the treatment of age-related macular degeneration. In addition, AAV2-sFLT01 treatment was well tolerated and capable of mediating long-term sFLT01 expression in a nonhuman primate model. However, AAV-mediated sFLT1 expression has not been tested in brain angiogenesis, largely owing 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. Among those, serotype 9 (AAV9)
can enter the brain parenchyma, particularly the brain angiogenic region, much more effectively than other serotypes. In this study, we first tested whether overexpression of sFLT1 can inhibit VEGF-induced brain angiogenesis through stereotactic injection of AAV2-sFLT02 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 IV delivery of AAV9-sFLT1 (containing full-length sFLT1, and packaged in AAV serotype 9 capsid, Table 1) to inhibit VEGF-induced brain angiogenesis. We found that IV injection of AAV9-sFLT1 4 weeks after angiogenic induction reduced vessel densities in the brain angiogenic region.

**RESULTS**

To test whether 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. AAV2-sFLT02, expressing a fusion protein sFLT02 angiogenesis, AAV1-VEGF (Table 1 and Supplementary Figure S1) was stereo-tactically 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. ELISA analysis in the AAV2-sFLT02-injected brain (349.4 ± 47.6 pg mg⁻¹ of brain protein) detected robust sFLT02 expression, compared with the AAV2-VEGF-injected brain (20.7 ± 2.7 pg mg⁻¹, P < 0.001, Supplementary Figure S2). Four weeks after AAV2-sFLT02 injection, vessel density was quantified on sections stained with lycopersicon esculentum lectin (Vector Laboratory, Burlingame, CA, USA), which binds to glycofibrin 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-VEGF-injected mice (655 ± 61 vessels per mm² vs 887 ± 177 vessels per mm², P = 0.016, Figures 2a and b), and was similar to that of AAV1-LacZ/AAV2-sFLT02-injected control mice (616 ± 99 vessels per mm², P = 0.07, Figures 2a and b). Vessel density was also significantly lower in AAV2-sFLT02-injected mice 4 weeks after angiogenic induction compared with those injected with AAV2-VEGF (747 ± 135 vessels per mm² vs 966 ± 158 vessels per mm², P = 0.027, Figures 2c and d). Therefore, AAV-mediated sFLT1 gene transfer could inhibit brain angiogenesis when injected into the angiogenic 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: IV injection. 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 IV injection of AAV9-sFLT1 4 weeks after angiogenic induction significantly reduced vessel density in the brain angiogenic foci (sFLT1 vs GFP: 912 ± 101 vessels per mm² vs 1134 ± 85 vessels per mm², P = 0.002, Figures 3a and b). However, IV 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, Figures 3c and d), consistent with our previously published data that increased permeability of the BBB is required for AAV9 to enter the adult brain parenchyma. 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. 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 IV 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-VEGF-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 terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL). Few TUNEL-positive endothelial cells were detected in the angiogenic foci of all groups (Figure 5). Most likely, therefore, sFLT1 inhibited

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**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  | Betaagalactosidase |
| sFLT  | Full-length soluble VEGF receptor 1 |
| sFLT02| Domain 2 of sFLT1 sequence and modified adjacent regions |
| GFP   | Green fluorescent protein |

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brain angiogenesis mainly through inhibition of endothelial cell proliferation. Because lymphocytic infiltration and loss of neurons have previously been reported to be associated with astrocyte transduction of AAV9, we analyzed whether IV 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 IV injection of AAV9-sFLT1 could be developed into a safe tool for treating brain angiogenic diseases.

**DISCUSSION**

The results of our study show that IV injection of AAV9-sFLT1 inhibited brain angiogenesis, and that the angiogenic inhibition effect of sFLT1 was mainly through the reduction of endothelial cell proliferation.
cell proliferation. Neither lymphocytic infiltration nor neuronal loss was observed in mice that received IV 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, or small molecule receptor tyrosine kinase inhibitors. However, there are considerable side effects associated with the use of these anti-VEGF treatments. Because the anti-angiogenic therapeutic efficacy and safety of AAV2-sFLT01 treatment for age-related macular degeneration have been tested in a nonhuman primate model, using AAV-sFLT1 offers advantages.

Recently, significant strides have been made toward clinical application of AAV owing to its excellent safety profile and success in several clinical trials. Indeed, this has been bolstered by evidence that more than 90% of the AAV genomes remain episomal in infected cells. Thus, tumor formation through insertional mutagenesis is of less concern when AAV vectors are used. Clinical trials so far have tested the effectiveness and safety of AAV delivery in patients with inherited retinal dystrophies (Leber’s congenital amaurosis), Duchenne muscular dystrophy, Parkinson’s disease and hemophilia B. More importantly, two exciting developments suggest that the obstacles for AAV clinical application are lessening: (i) 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; and (ii) an AAV product has been licensed in Europe. 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 IV 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 (Figures 2a and b), IV injection of AAV9-sFLT at the time of angiogenic induction did not (Figures 3c and d), which is consistent with our previous observation that AAV9-mediated gene expression in the brain requires increased permeability of the BBB. We also showed previously that single-strand AAV9 mediated significant gene expression only in the angiogenic foci when the BBB permeability is increased. 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, because 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 whether the 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 the 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. It has been reported that acute withdrawal of VEGF in mice overexpressing VEGF in the airway results in endothelial cell apoptosis and shedding. We showed that blockage of VEGF signaling using bevacizumab (Avastin) reduces the number of abnormal vessels in the brain arteriovenous malformation lesion and induces endothelial cell apoptosis. 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. 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, lung and brain. Systemic delivery of AAV9 also results in high transduction in the liver. A previous study, we showed that systemic delivery of AAV9 resulted in significant gene transduction in the liver and heart. 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 brain arteriovenous malformation, in both sporadic and familiar cases such as those in Hereditary Hemorrhagic Telangiectasia, 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, USA). Mice were fed standard rodent food and water ad libitum, and were housed (five per cage) in 421 × 316 cm² sawdust-lined cages in an air-conditioned environment with 12-h light/dark cycles.

AAV vector construction and production

A cytomegalovirus 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 (Supplementary Figure S1) using the three plasmid co-transfection system. 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 centrifugation. Viral titers were determined by dot-blot analysis of DNA content and expressed as genome copies.

AAV2-sFLT02 and AAV2-EV were provided by Sanofi-Genezyme Corporation (Framingham, MA, USA). sFLT02 in AAV2-sFLT02 (Supplementary Figure S1) contains human sFLT domain 2 driven by cytomegalovirus promoter.

AAV9-sFLT1 was produced by Dr Zhijian Wu at the National Eye Institute, NIH (Bethesda, MD, USA). As described previously, pAAV-sFLT1 (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. Viral titers were determined through real-time PCR using linearized plasmid standards. AAV9-GFP (Supplementary Figure S1) was produced by Vector Biolabs (Philadelphia, PA, USA).

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, USA). 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 × 10⁹ genome copies 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.
suture. Four weeks after AAV2-sFLT02 or AAV2-EV injection, mice were killed and tissues were collected for subsequent analyses.

Intravenous injection of AAV vectors
Mice were anesthetized with isoflurane. A total of 50 μl phosphate-buffered saline containing 1 × 10^{11} genome copies 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 killed and tissues were collected for subsequent analyses.

Immunohistochemistry and immunofluorescence
Fresh brain sections were frozen directly in dry ice after the mice were killed. Coronal cryostat sections were cut at a 20 μm thickness on a Leica CM1900 Cryostat (Leica, Buffalo Grove, IL, USA). Sections were incubated at 4 °C overnight with the following primary antibodies: anti-CD31 (1:100, M20, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-ki67 (1:100, Ab 15580, Abcam, Cambridge, MA, USA), NeuN (1:500, MAB377, Chemicon, Temecula, CA, USA) 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, USA), and coverslipped with Vectashield mounting medium with 4′-6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA) 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-buffered saline, protease inhibitors, 0.1% NP-40). sFLT1 protein was quantified by ELISA assays using Human FLT1 Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions.

TUNEL assay
TUNEL assay was carried out to identify the extent of DNA fragmentation, using the NeuroTACS II kit (Trevigen, Gaithersburg, MD, USA). 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

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 hematoxylin and eosin (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 4′-6-diamidino-2-phenylindole (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.
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 min, then incubated overnight with fluorescein isothiocyanate–esculentin lectin (Vector Laboratories), 2 g/ml–1 at 4 °C, and then coverslipped with Vectashield mounting medium with 4',6-diamidino-2-phenylindole (Vector Laboratories) to label cell nuclei. Three images were taken from each section (right and left of, and below the injection site) under a ×20 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 mm2.

Statistical analyses
Data are presented as mean ± standard deviation (s.d.). One-way analysis of variance 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 two 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

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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