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Recombinant adeno-associated viral vector encoding human $VEGF_{165}$ induces neomicrovessel formation in the adult mouse brain

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1. ABSTRACT

Delivery of therapeutic genes represents a fascinating possibility to accelerate the injury-repairing process in tissues that are otherwise difficult to treat, such as cerebral ischemia. Current studies indicate that gene transfer-induced focal angiogenesis in the brain may provide an important therapeutic strategy. In the present study, we report the efficacy of induction of angiogenesis with an adeno-associated virus (AAV) vector expressing the 165 amino acid isoform of vascular endothelial growth factor ($VEGF_{165}$). We found that AAV serotype 1 had more efficiency in transduction of the brain tissue than AAV serotype 2. Quantitative vessel counting showed that microvessels in AAV-VEGF-transduced mice significantly increased from 1 to 12 weeks compared to the control groups (AAV-VEGF: 316±58 vs. AAV-lacZ: 180±34 and saline: 152±35 vessels/mm_2, at 6 weeks, $p<0.05$). Proliferating cell nuclear antigen (PCNA) staining confirmed these microvessels were actively proliferating. Double-labeled fluorescence staining demonstrated that neurons, astrocytes, and endothelial cells could express VEGF following AAV-VEGF gene transfer. AAV vectors did not elicit a detectable inflammatory response, cell loss or neuronal damage. Our data underline the importance of angiogenesis in the brain tissue and indicate that VEGF gene transfer may present a valuable approach to treat brain ischemic disorders.

2. INTRODUCTION

Angiogenesis is a crucial prerequisite in the development of all vertebrate embryos and in the growth of many malignant tumors. Recent studies have demonstrated that angiogenic changes also exist in many disease processes, such as tumor growth (1), retina vasculization (2), wound healing (3), cerebral ischemia (4), and brain arteriovenous malformations (5). Angiogenesis is regulated by angiogenic stimulating and inhibiting factors. Vascular endothelial growth factor (VEGF) is one of the major stimulators of developmental and disease-related angiogenesis. Administration of exogenous VEGF could induce angiogenesis in different organs or tissues (6-11). In the rodent brain, VEGF increases vessel/area ratio (12) and induces endothelial cell proliferation (13). Our previous study demonstrated that adenoviral-mediated VEGF gene transfer could induce angiogenesis in the mouse brain (14). However, the effects of VEGF following direct protein injection or adenoviral transduction are limited by the shorter period of gene expression.

Recombinant AAV (rAAV) contains no viral genomes and elicits less or no inflammatory response than other viral vectors (15). rAAV can infect both dividing and non-dividing cells and mediate long-term gene expression up to months or several years *in vivo* (16-20). Furthermore, rAAV probably maintains *in vivo* as episomal genomes

extrachromosomally or as integrated concatemers, albeit at a lower frequency (21), which diminishes the mutagenesis of insert associated with random integration. rAAV has a range of applications in experimental gene therapy including tumor, inherited disorder, neurodegenerative disorder, cardinal or cerebral vascular diseases (22). rAAV-transferred BDNF or GDNF showed neuroprotection in both Huntington (23) and Parkinson's animal models (24). AAV-delivered angiostatin showed strong inhibition of tumor angiogenesis *in vivo* (25). AAV-delivered soluble fragment of VEGF receptor Flk-1 (KDR) significantly inhibited disseminated neuroblastoma growth in mice (26). Many experiments have demonstrated that application of rAAV in the CNS is feasible and may represent a therapeutic potential.

AAV-mediated VEGF (AAV-VEGF) gene transfer has been studied in many organs, and has shown that overexpression of VEGF can induce angiogenesis (27, 28), However, stable induction of angiogenesis in the mature brain for a longer time appears difficult because of the short half-life of VEGF protein and the limitation of delivery approach. To establish a reproducible and intermediate term, a brain focal angiogenesis model is necessary for future analysis of the molecular mechanisms of angiogenic processes in the normal mature brain. Induced long-lasting neovasculature by VEGF gene transfer may also provide a unique means for the treatment of brain diseases. Based on the application of AAV-mediated gene transfer technique and the function of VEGF, we expect to demonstrate: 1) which AAV serotype has better gene transduction in the brain; 2) whether AAV-mediated VEGF gene transfer induces focal non-tumor angiogenesis in the mouse brain; and 3) if so, which cell types will be involved.

3. MATERIALS AND METHODS

3.1. AAV-VEGF packing, purification, and titration

We first constructed two AAV vectors with VEGF or lacZ gene. The pAAV-VEGF vector was generated by inserting the human $VEGF_{165}$ cDNA between two ITRs of pAVLL plasmid (a plasmid which has two left ITRs of AAV serotype 2). CMV promoter was used to control gene expression in this vector. To confirm VEGF expression mediated by these plasmids, 293 cells were infected. The concentration of VEGF in cell culture supernatant was determined to be approximately $3ng/10^9$ cells/24 hour culture by ELISA (Quantikine human VEGF, R&D Systems, Minneapolis, MN, USA). The pAAV-LacZ plasmid carrying a CMV promoter driving LacZ gene expression was provided by Avigen Inc. (Alameda, CA). AAV vectors were prepared by using three plasmids co-transfection system. Briefly, recombinant pAAV plasmid were co-transfected with two helper plasmids (provided by Avigen Inc.) to 293 cells using the calcium phosphate precipitate method. One helper plasmid, pLadeno5, has the adenoviral VA, E2A and E4 regions that mediate AAV vector replication. The other, pHLP19, has AAV rep and cap genes. Cell lysate was produced using three freeze-and-thaw cycles three days after the transfection. AAV vector was purified by CsCl2 centrifugation. Viral titers were determined by dot blot analysis of the DNA content. The purified vectors were tested for their infection and transgene expression by infecting cultured 293 cells.

3.2. Experimental groups

All animal procedures were carried out according to a protocol approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco. Adult male CD-1 mice weighing 30-35 g were purchased (Charles River Laboratories, Wilmington, MA). One hundred and eight mice were used for three experiments, in which 36 AAV-lacZ-transduced mice were utilized to determine the doses, viral types, and time course of AAV-lacZ expression in the brain. Based on the first experiment, AAV-VEGF (n=24) and AAV-lacZ (n=24, as a viral control) transduced and saline-injected (n=24, as a control) mice were sacrificed at 1, 3, 6 and 12 weeks to examine the number of microvessels, immunohistochemistry and Western blot analysis.

3.3. Recombinant AAV-VEGF transfer in the mouse brain

The mice were anesthetized intraperitoneally with Ketamine (50 mg/kg body weight) and xylazine (10mg/kg body weight) (Sigma, St. Louis, MO).. Following induction of anesthesia, the mice were placed in a stereotactic frame with a mouth hold (David Kopf Instruments, Tujunga, CA), and a burr hole was drilled to the pericranium 1 mm lateral to the sagittal suture and 1 mm posterior to the coronal suture. A 10 µl syringe (Hamilton Company, NV) was inserted into the lateral ventricle 3 mm under the cortex. Two µl of AAV suspension with 2×10^9 virus particles (1×10^{12} virus particles/ml) were injected stereotactically into the lateral ventricle at a rate of 0.2 µl/minute. The needle was withdrawn after 15 minutes, the hole was sealed with bone wax, and the wounds were closed with suture. The animals were then put back into their cages for recovery.

3.4. 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) staining

After sacrificing the animals, the mice were perfused with a fixation of 2% paraformaldehyde in 0.1 M PIPES pH 6.9. Twenty µm coronal sections were then cut and the sections were fixed in 0.5 % glutaraldehyde for 10 minutes, incubated for 2 hours in X-gal staining solution (5 mmol/L K3Fe (CN)6, 5 mmol/L K4Fe (CN)6, 2 mmol/L MgCl2, 0.01% sodium deoxycholate, 0.02% NP-40, and 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside in PBS), and photographed. Using NIH Image 1.63 software, the transduction area was calculated by multiplying the transduction areas by the thickness of the sections.

3.5. Microvessel counting

The method of microvessel counting has been described previously (29, 14, 30). Briefly, 20 µm thick frozen coronal sections were fixed with 100% ethanol at 20°C for 20 minutes, then incubated with fluorescein-lycopersicin esculentum lectin (Vector Lab, Burlingame, CA) 2 g/ml at 4°C overnight. Two coronal sections from the lectin staining brain, 1 mm anterior and 1 mm posterior from the needle track, were chosen. The microvessel

Table 1. List of Primary Antibodies

Primary antibodies	Dilution	Vendor	Markers
Rabbit anti-VEGF	1:50	Neomarker, Fremont, CA	VEGF protein
Rat anti-CD-31	1:50	Pharmingen, San Diego, CA	Endothelial cells
Rat anti-GFAP	1:500	Calbiochem, La Jolla, CA	Astrocytes
Sheep anti-NeuN	1:50	Capricorn, Scarborough, ME	Neurons
PCNA	1:100	Nova Castra Lab, New Castle,	Proliferating cells

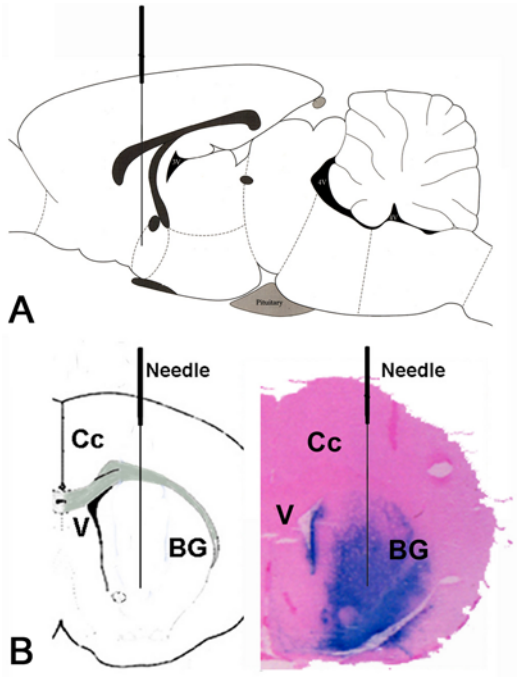

Figure 1. Injection of AAV vector into the mouse brain. The pictures illustrate the position of AAV-vector injection into the mouse brain. Sagittal section (a) and coronal section (b) indicate the position of AAV vector injection. Distribution of X-gal positive staining 1 week following the injection of $2x10^9$ particles of AAV-lacZ (C). Cc= Corpus callosum; V= ventricle; and BG= basal ganglia.

density was quantified by counting the total number of three areas of microscopic fields per tissue section immediately adjacent (left, right, and bottom) to the needle track, which were chosen with a low power objective (10X) by two independent investigators. As a surrogate of vessel counting, vessel density was determined by lectin optical density measurements using NIH Image 1.63 software.

3.6. Double-labeled fluorescent staining

The brain sections were fixed with acetone at –20°C for 10 min, and then incubated with 2.5% normal blocking serum for one hour for double-labeling immunostaining. The sections were double-labeled with mouse endothelial cell (CD-31)/VEGF, myosin/VEGF, GFAP/VEGF, NeuN/VEGF, and lectin/PCNA. The primary antibodies used are listed in Table 1. The secondary antibody is fluorescent anti-rabbit IgG or anti-rat IgG Texas red anti-rabbit IgG (Vector Labs). The staining sections were evaluated using a fluorescence microscope (Nikon Microphoto-SA) with a filter cube (excitation filter, 450-490 nm) for fluorescence, and a filter cube (excitation filter, 515-560 nm) for Texas-Red. Appropriate positive and negative controls were run for each batch of slides.

3.7. Fluoro-Jade B staining

The frozen sections were fixed with 2% paraformaldehyde in 0.1 M PIPES pH 6.9 for 20 minutes. After washing, the sections were stained with 0.06% Potassium Permanganate ($KMnO_4$) for 30 minutes at room temperature, then immersed in a 0.001% fluoro-Jade staining solution (Chemicon, Temecula, CA) in 0.1% acetic acid for 20 minutes. The staining sections were evaluated using a fluorescence microscope.

3.8. Statistical analysis

All data are presented as mean ± SD. Parametric data among the AAV-VEGF, AAV-lacZ-transduced, and saline control groups were compared using a one-way ANOVA followed by Fisher's PLSD test. A probability value of less than 5% was considered to be statistically significant.

4. RESULTS

4.1. Distribution of lacZ expression following AAV-lacZ gene transfer

Efficient gene transfer into mouse CNS has been achieved by injecting AAV-lacZ, an rAAV containing human cytomegalovirus (CMV) promoter driving lacZ gene, directly into the brain. To examine which AAV serotype we would use for our study, one µl (1×10^9 particles) or 2 µl (2×10^9 particles) of AAV-lacZ packaged in AAV serotype 1 or 2 capsid were stereotactically injected into the right basal ganglia of CD-1 mice. We detected X-gal positive staining in the basal ganglia areas, occasionally extending into the cerebral cortex distant from the site of injection, after 1 week of AAV-lacZ transduction (Figure 1). The X-gal positive staining region in the AAV type 1 vector-injected brain was larger than in the AAV type 2 (Figure 2A). Furthermore, 2 µl of AAV-lacZ injected brains had larger x-gal positive areas compared to the 1 µl of AAV-lacZ injected brains (Figure 2B, $p<0.05$). This result demonstrated that the AAV serotype 1 was superior to AAV serotype 2 in delivering genes into the brain. We then examined the time course of lacZ gene expression following AAV-lacZ serotype 1 mediated gene transfer. The brains were collected at 1, 3, 6, and 12 weeks following AAV-lacZ injection. LacZ expression was detected in the transducted hemisphere after 1 week of AAV-lacZ injection. The expression peaked at 6 weeks and was sustained to 12 weeks after the experiment stopped (Figure 3, $p<0.05$). This result indicated that AAV

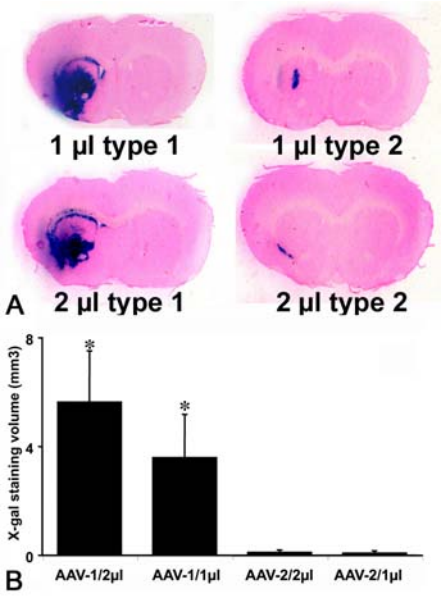

Figure 2. Distribution of X-gal positive staining following AAV-lacZ transduction. A. Photomicrographs show the distribution of X-gal positive staining 1 week following the injection of 1 or 2 x10^9 particles of AAV-lacZ vector. Dark blue X-gal staining area is much larger in the AAV serotype 1 injected brains than in AAV serotype 2 vectors injected brains. B. Bar graph is the quantification of x-gal staining areas. The result demonstrates that AAV serotype 1 is a good candidate for gene delivery into the brain tissue.

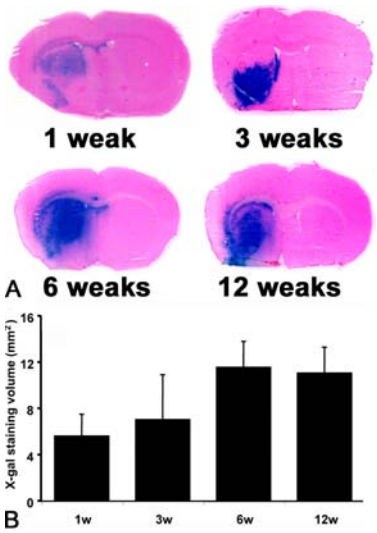

Figure 3. Time course of X-gal positive staining following AAV-lacZ transduction. A. Photomicrographs show X-gal positive staining, 1 to 12 weeks following the brain parenchyma injection of 2x10^9 particles of AAV-lacZ vector. Blue X-gal staining area was detected from 1 week to 12 weeks. B. Bar graph shows the X-gal positive staining volume after gene transfer, suggesting that AAV gene can express at least 12 weeks in the adult mouse brain.

serotype 1 could induce gene expression in the brain for a longer period (12 weeks), and also suggested that using AAV serotype 1 to deliver genes into the adult mouse brain could be feasible and reproducible. Thus, we used AAV-VEGF and LacZ packaged in AAV serotype 1 capside in the following experiments.

4.2. Increased microvessels in the AAV-VEGF-transduced mouse brain

To test if lectin could be used as a surrogate to endothelial cells, we performed double staining using lectin and an endothelial marker, CD31. The result showed that lectin stained the same endothelial cells as CD31 (Figure 4). We therefore used lectin to stain brain tissues for vessel counting. We counted the number of microvessels in the AAV-VEGF, AAV-lacZ-transduced, and the saline-treated mouse brains. There was no difference in the number of microvessels between the control mice and AAV-lacZ-transduced or the saline-treated mice (Figure 5A). However, the number of microvessels greatly increased in the AAV-VEGF-transduced brains, peaked at 6 weeks, and was sustained to 12 weeks after the experiment stopped (Figure 5B, $p<0.05$).

To identify whether these increased microvessels were newly formed vasculature, we examined cell proliferation using PCNA immunostaining. There were few PCNA positive endothelial cells in AAV-lacZ-transduced and the saline-treated brain. The PCNA positive cells were greatly increased in the AAV-VEGF-transduced brains. PCNA positive endothelial cells were mainly located in the basal ganglia regions adjacent to the AAV-VEGF injection site and needle track (Figure 6). This result demonstrated that new microvessels were formed following AAV-VEGF gene transfer.

To identify which cell types were transduced and expressed VEGF protein following AAV-VEGF gene transfer, we performed double-labeled fluorescent staining with antibodies staining specific cell types and anti-VEGF antibody. Our results revealed that the NeuN, GFAP and CD-31 were co-localized with VEGF in the cell bodies following AAV-VEGF gene transfer (Figure 7). VEGF positive cells were located in the striatum regions of the AAV-VEGF injecting site; some positive cells extended to the cortex region. VEGF positive astrocytes end feet were often surrounded with microvessels (Figure 7d). Endothelial cells and neurons also expressed VEGF in this area (Figure 7a and 7g). We demonstrated that AAV vectors could transduce in neurons, astrocytes, and endothelial cells, and might be the brain source of VEGF protein after AAV-VEGF transduction.

4.3. Histological assessment of neuronal damage after AAV-VEGF gene transfer

In general, no visible neurological deficits, body weight loss and hyperthermia were observed during 12 weeks of virus injection in AAV-VEGF, AAV-lacZ and saline-treated mice. To examine the potential neuronal damage after AAV vector injections, we performed fluoro-jade B staining method (31), and found that there was no detectable neuronal injury or death in all three groups,

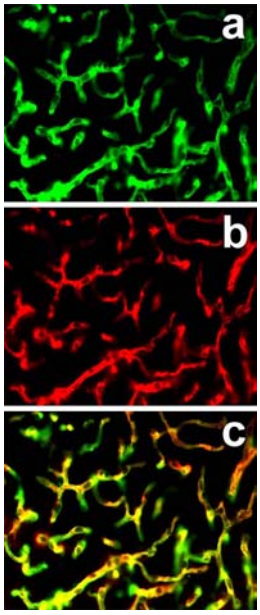

Figure 4. Establishing microvessel counting. Photomicrographs show the lectin (a, green color) and CD-31 (b, red color) double-labeled immunostaining. Yellow color shows that CD-31 and lectin-stained microvessels are merged completely (c), indicating that the lectin stains brain endothelial cell specifically.

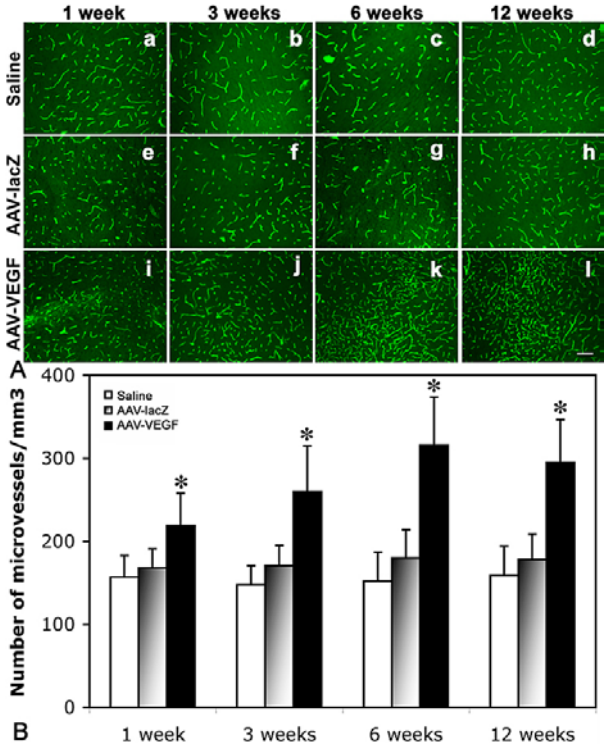

Figure 5. Increase of microvessel counts following AAV-VEGF gene transfer. A. Photomicrographs show lectin staining microvessels in the saline (a to d) AAV-lacZ (e to h), and AAV-VEGF (i to l) transduced mouse brain after 1 to 12 weeks of AAV gene transfer. Bar = 100 μm. There are more microvessels in the AAV-VEGF transduced mice than in the AAV-lacZ and saline-treated mice. B. Bar graph shows the number of lectin staining microvessels after 1 to 12 weeks of AAV gene transfer. Values are mean±SD; N = 6 in each group. * $p<0.05$, AAV-VEGF vs. AAV-lacZ and saline groups.

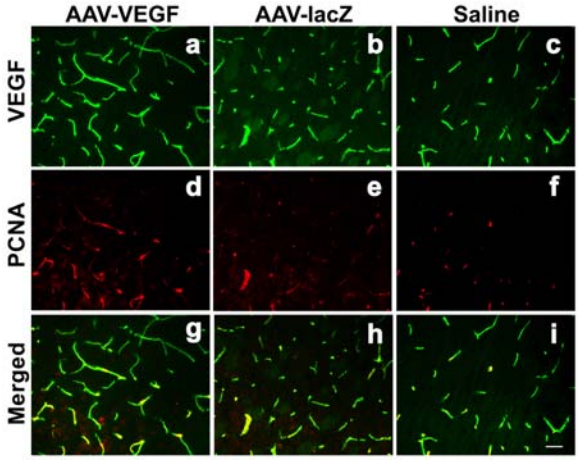

Figure 6. Increase of endothelial cell proliferation following AAV-VEGF gene transfer. Photomicrographs show lectin and PCNA double-labeled staining in the mouse brain following AAV-VEGF gene transfer. Green color lectin staining identifies microvessels in the AAV-VEGF (a), AAV-lacZ-transduced (b), and saline-treated (c) mice. Red color PCNA positive staining (d, e, and f) was detected in the same sections in the ipsilateral hemisphere adjacent to the injected region, especially close to the needle track. Many yellow co-localized staining cells were detected in Figure g, indicating that endothelial cell proliferation actively occurred in the AAV-VEGF-transduced brain endothelial cells compared to the other two groups of mice. Bar = 50 μm.

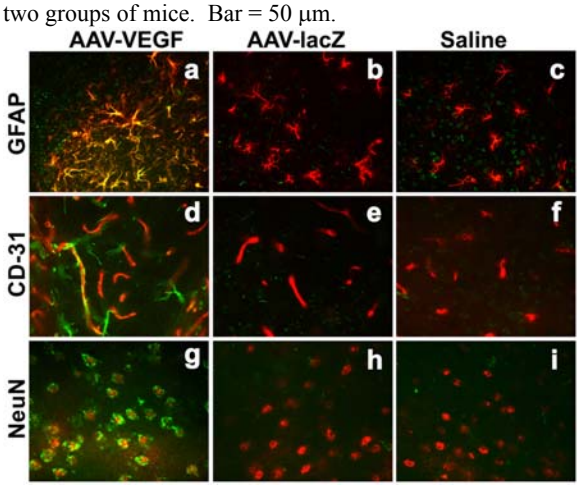

Figure 7. VEGF expressed in astrocytes, endothelial cells, and neurons. Photomicrographs show merged cell marker (red) and VEGF (green) double-labeled staining in the mouse brain following 7 days of AAV-VEGF (a, d, g), AAV-lacZ (b, e, h), and saline (c, f, I) treatment. Green color shows VEGF positive and red color shows GFAP, CD-31, and NeuN positive staining. Yellow color shows merged green and red colors, indicating that astrocytes (a) endothelial cells (d), and neurons (g) were all expressing VEGF in the AAV-VEGF-transduced mice. Bar = 50 μm. No VEGF expression was detected in AAV-lacZ and saline-injected brains by the anti-human VEGF antibody used in this experiment (b, c, e, f, h, and i).

indicating that AAV vector itself did not damage neurons (Figure 8). This result paralleled the findings from the H&E stained slides, further confirming that AAV vector did not cause inflammatory response in the AAV vector injection region.

5. DISCUSSION

Our results demonstrate that: 1) AAV serotype 1 has higher gene transduction efficiency compared to AAV serotype 2 in the brain tissue; 2) AAV-mediated VEGF gene transduction into the mouse brain induces long-lasting focal angiogenesis (at least 12 weeks); 3) astrocytes, neurons, and endothelial cells can all be infected by AAV serotype 1 vector and be the source of VEGF overexpression; and 4) AAV vector mediated gene transfer did not cause detectable neuronal damage. Our data suggest that neo-microvasculature formation can be achieved in the mature brain through AAV vector mediated VEGF gene transfer. This novel method of inducing angiogenesis in the mature brain can allow us to further study the molecular mechanisms of angiogenesis in the adult brain independently from the influence of confounding effects of upstream inciting stimuli, such as cerebral ischemia or brain tumor.

The efficiency of transgene delivery is a key factor in potential clinical gene therapy. Although both AAV serotype 1 and 2 showed capability in transducing brain tissue, AAV serotype 1 traveled farther from the injection site and transduced a larger area of the brain, particularly in the striatum, than AAV serotype 2. AAV 2 was only able to transduce certain restricted regions. Wang et al have reported a similar result (32). The data above suggest that the distribution of the receptors for AAV 1 and AAV 2 in the brain tissue is different. Transduction efficiency of rAAV packaged in serotypes 1 to 5 varies according to species (mouse, rat, gerbil and cat), brain regions (white matter or gray matter), and cell types (33, 34). In addition to the serotypes, many other factors, such as promoters, transgenes, and ITRs (35, 36), will also influence transduction efficiency.

Numerous studies have demonstrated that VEGF plays a fundamental role in embryonic vasculogenesis and angiogenesis (37). Maintaining a constant level of VEGF is necessary to stimulate focal angiogenesis. VEGF recombinant protein infusion is one of the ways to induce brain angiogenesis (12, 13). However, VEGF protein has to be infused continuously due to its short half-life and reduced bioavailability (38). Brain angiogenesis can only be observed for a few days. Subdural placement of gelatin sponge containing VEGF is another way to induce angiogenesis in the surface of the brain (13). However, it is difficult to maintain the animals in an acceptable physiological condition after the sponge placement. Our previous study showed that adenoviral vector mediated VEGF gene transfer could induce focal angiogenesis (14). The limitation of using AdVEGF is the short duration of VEGF expression, which lasted only 4 weeks. Here we found that VEGF could express for at least 12 weeks by AAV-mediated VEGF gene transfer. Importantly, the

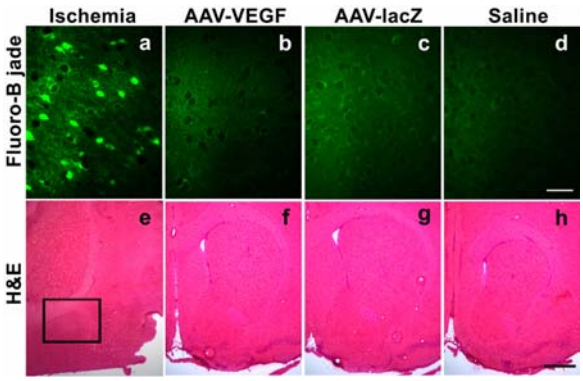

Figure 8. Fluoro-Jade B positive staining in ischemic mouse brains. The photomicrographs show the fluoro-Jade B staining following 5 days of AAV-VEGF (b) AAV-lacZ (c), and saline (d) treated mouse brain. Green positive staining cells represent neuronal death (a, ischemic brain section as a positive control). We could not detect the fluoro-Jade B positive cells following AAV vector transduction as well as saline. Bar=50 mm. Parallel H&E staining (e to h) shows that no detectable inflammation occurred in the AAV-injected hemisphere in these three groups of mouse brains, suggesting that AAV vector is feasible for brain gene transfer. Bars =500 mm.

microvessels continuously increased in the AAV-VEGF-transduced mice compared to the control mice, suggesting that maintaining a consistent level of VEGF locally is necessary for the development of neovasculature in the brain. Our data indicated that using AAV to mediate VEGF gene transfer into the brain could be feasible and reproducible, and could have a potential for future clinical application.

AAV vectors are excellent candidates as tools for gene therapy in brain diseases because they are capable of transducing non-divided cells such as neurons (39, 40), astrocytes (41), and oligodendrocytes (42). Studies have shown that in the normal brain, both endothelial cells and non-endothelial cell types, such as smooth muscle cells, fibroblasts, and Schwann's cells, are capable of producing VEGF (43, 44). Our present study demonstrated that VEGF could be extensively expressed in the astrocytes, neurons, and endothelial cells in the AAV-VEGF injected hemisphere, which were sustained for a long period. A high level of VEGF protein was detected in the astrocytes and their end feet adjacent to the microvessels (Figure 7d). These results indicate that compared to other cell types, astrocytes are more easily transduced by AAV-VEGF. Astrocytes deliver VEGF to the active growing microvessels through their end feet, and therefore may play a crucial role in brain angiogenesis induced by AAV vector-delivered VEGF gene.

In summary, we have successfully developed focal angiogenesis in the mature mouse brain through AAV-mediated VEGF gene transfer. Our results further emphasize the relevance of the angiogenic process in the mature brain tissue and the potent role of VEGF in triggering focal angiogenesis. The results also demonstrate the feasibility of using a gene therapy approach for brain ischemic injury by potential and restored angiogenesis. Finally, the prolonged gene expression, achieved by using AAV vector delivery system, should also be considered as a novel and powerful tool to assess the biological consequences of long-term overexpression of other angiogenic factors or angiogenic factor combinations, which may be relevant to the treatment of brain ischemic injury.

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