Intravenous Delivery of Adeno-Associated Viral Vector Serotype 9 Mediates Effective Gene Expression in Ischemic Stroke Lesion and Brain Angiogenic Foci

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Background and Purpose—Adeno-associated viral vector (AAV) is a powerful tool for delivering genes to treat brain diseases. Intravenous delivery of a self-complementary but not single-stranded AAV9 (ssAAV9) mediates robust gene expression in the adult brain. We tested if ssAAV9 effectively mediates gene expression in the ischemic stroke lesion and angiogenic foci.

Methods—Focal ischemic stroke was induced by permanent occlusion of the left middle cerebral artery (MCAO) and focal angiogenesis was induced by injecting an AAV expressing vascular endothelial growth factor (AAV-VEGF) into the basal ganglia. ssAAV vectors that have cytomegalovirus (CMV) promoter driving (AAV-CMVlacZ) or hypoxia response elements controlling (AAV-H9LacZ) LacZ expression were packaged in AAV9 or AAV1 capsid and injected into mice through the jugular vein 1 hour after MCAO or 4 weeks after the induction of angiogenesis. LacZ gene expression was analyzed in the brain and other organs 5 days after LacZ vector injection.

Results—LacZ expression was detected in the peri-infarct region of AAV9-CMVlacZ and AAV9-H9LacZ–injected MCAO mice and the brain angiogenic foci of AAV9-CMVlacZ–injected mice. Minimum LacZ expression was detected in the brain of AAV1-CMVlacZ–injected mice. Robust LacZ expression was found in the liver and heart of AAV-CMVlacZ–injected mice, but not in AAV9-H9LacZ–injected mice.

Conclusions—ssAAV9 could be a useful tool to deliver therapeutic genes to the ischemic stroke lesion or brain angiogenic foci.

Key Words: adeno-associated viral vector serotype 9 ■ angiogenesis ■ brain ■ intravenous delivery ■ mouse ■ peri-infarct region

A dено-associated viral vector (AAV) is an ideal vector for delivering genes into the brain because it effectively infects neurons and astrocytes. It has been used to deliver genes to various brain disease models. In most studies, the AAVs were delivered to the brain via stereotactic injection. Direct injection, however, is an invasive procedure that can cause additional damage to a critically ill patient.

Recombinant AAV packaged in a serotype 9 (AAV9) capsid effectively passes through the blood–brain barrier (BBB). However, only self-complementary AAV9 (scAAV9), not single-stranded AAV9 (ssAAV9), robustly mediates transgene expression in the adult brain after intravenous (IV) injection. Many therapeutic genes are too big to be packaged as scAAV. We demonstrate in this study that IV-injected ssAAV9 (AAV-CMVlacZ) can effectively deliver genes into the adult brain in the ischemic peri-infarct region and angiogenic foci. AAV9 with hypoxia response elements (HREs) (AAV-H9LacZ) restricts gene expression specifically in the peri-infarct region of the brain with focal ischemic injury.

Materials and Methods
All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco, and conformed to National Institutes of Health Guidelines for use of animals in research. CD1 male mice at age 8–10 weeks (Charles River, Wilmington, MA) were used.

Focal Ischemic Stroke Model and Brain Angiogenic Model
Focal ischemic stroke was created by permanent occlusion of the left distal middle cerebral artery (MCAO). Brain focal angiogenesis was induced by stereotactic injection of AAV expressing vascular endothelial growth factor (VEGF; AAV-VEGF), 2 × 10⁹ genome copies (gcs) into the basal ganglia.

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IV Injection of AAV

Into the jugular vein, AAV-H9LacZ and AAV-CMVLacZ (2 x 10¹¹, 8 x 10¹¹, 1 x 10¹² gcs) in 200-µL phosphate-buffered saline were injected 1 hour after MCAO (N=6) and 4 weeks after induction of angiogenesis (N=6).

Detailed Methods are provided in the online-only Data Supplement.

Results

Stroke Model

AAV9-CMVLacZ or AAV1-CMVLacZ was injected into the jugular vein 1 hour after MCAO (Figure 1A). Brain samples were collected 5 days later. Infarct region was visualized on Nissl-stained and NeuN antibody-stained sections (Figure 1B).

Figure 1. LacZ expression in the peri-infarct region. A, Experimental design. B, Representative Nissl-stained sections showing infarct area. C, Representative image of X-gal-stained sections showing LacZ expression in the peri-infarct region. No LacZ expression was detected in the brain of adeno-associated viral vector 1 (AAV1)-CMVLacZ–injected mice. Scale bar: 1 mm.

Figure 2. LacZ expression in angiogenesis foci. A, Experimental design. Representative images of a X-gal (left) and lectin (middle) costained brain section collected from AAV1-CMVLacZ (B) and AAV9-CMVLacZ (C) vector-injected mice with brain angiogenesis. To visualize both vessels and LacZ expression, lectin-positive vessels were converted to pink in the merged picture (right). Scale bar: 100 μm.
and Supplementary Figure III). LacZ expression in the brain was predominantly in the peri-infarct region of AAV9-CMV LacZ–injected mice and was very weak in other brain regions (Supplementary Figure VII). No LacZ expression was detected in the brain of AAV1-CMV LacZ–injected mice, including the peri-infarct region (Figure 1C). LacZ expression was detected only in the peri-infarct region 5 days later (Figure 1B and 1C). No significant gene expression was detected in other brain regions or other organs (Supplementary Figures I and VII).

The infarct size and the number of CD68+ cells at the peri-infarct region were comparable among nonvectors and vector-injected mice (Supplementary Figures IV and V), suggesting that IV-delivered AAV vector did not increase local inflammation and neuronal injury.

**Angiogenic Model**

Brain focal angiogenesis was induced by stereotactic injection of AAV1-VEGF into the basal ganglia. AAV9-CMV LacZ or AAV1-CMV LacZ was injected into the jugular vein 24 days later. LacZ-positive spots were found predominantly in the angiogenic foci 5 days later in the AAV9-CMV LacZ group, but not in the AAV1-CMV LacZ group (Figure 2B and 2C). LacZ expression also was detected in the heart and liver of all mice (Supplementary Figure II).

**Discussion**

We demonstrated that IV injection of ssAAV9 mediates significant transgene expression in the peri-infarct region of focal ischemic injury and brain angiogenic foci, and that HRE restricted transgene expression in the peri-infarct region. Therefore, ssAAV9 combined with regulated elements can mediate targeted therapeutic gene expression in the brain lesion through noninvasive IV injection.

Active transport mechanism has been suggested in facilitating AAV9 crossing the BBB. In our study, however, higher LacZ expression was detected in the peri-infarct region and angiogenic foci than in other brain regions, suggesting that increased BBB permeability plays an important role. The BBB permeability is increased within 10 minutes after permanent MCAO, and the increase lasts at least 24 hours (Supplementary Figure VI). However, we do not know if the expression pattern persists when the vectors are injected at a later stage of MCAO.

Although IV injection of ssAAV9 (5 × 10¹¹ gc) infects some cells in the normal brain, the efficiency is much lower than that of scAAV9. We showed that after IV injection, gene expression in the brain is predominantly at the peri-infarct area and angiogenic foci. A few LacZ-positive cells in the contralateral brain of mice received 8 × 10¹¹ gc and 1 × 10¹² gc of AAV9-CMV LacZ (Supplementary Figure VII), which is similar to what Gary et al found in their study with 5 × 10¹¹ gc ssAAV9-green fluorescent protein.8

In summary, we have demonstrated in this study that IV injection of ssAAV9 can deliver therapeutic genes into the brain regions of adult mice where the BBB permeability is increased. More importantly, to reduce systemic side effects, the therapeutic gene expression can be further restricted to the brain lesion by incorporating regulatory elements. ssAAV9, in combination with regulator elements, can be used to design safe and effective gene-based therapies for the treatment of ischemic stroke and brain vascular diseases.

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**Disclosures**

None.

**References**

ONLINE SUPPLEMENT

Intravenous Delivery of AAV9 Vector Mediates Effective Gene Expression in Ischemic Stroke Lesion and Brain Angiogenic Foci
Supplemental Methods

AAV Vector Production

AAV9-H9LacZ was packaged by Vector Biolabs (Philadelphia, PA). Other vectors were made as previously described\textsuperscript{1,2} using the three plasmid co-transfection system.\textsuperscript{3} 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\textsubscript{2} centrifugation. Viral titers were determined by dot blot analysis of DNA content and expressed as genome copies (gcs).

Distal Permanent Middle Cerebral Artery Occlusion

Mice were anesthetized via isoflurane inhalation. Under a surgical microscope, a 1-cm incision was made between the right orbit and tragus. A piece of 2 mm\textsuperscript{2} skull was removed. The arachnoid was opened, and the middle cerebral artery (MCA) was permanently occluded with electrocoagulation. The temporal muscle was repositioned, and the skin closed. Body temperature was maintained at 37±0.5°C by using a thermal blanket throughout the surgical procedure, and system blood pressure was monitored using tail-cuff system from Visitech Systems (Apex). Surface cerebral blood flow (sCBF) was monitored during MCAO procedure as described below. Mice were excluded from the experiment if sCBF in the ischemic core region measured more than 15% of the baseline.

Stereotactic Injection of AAV Vectors into the Basal Ganglia

Following induction of anesthesia with isoflurane inhalation, the mice were placed in a stereotactic frame with a holder (David Kopf Instruments, Tujunga, CA), and a burr hole was drilled in the pericranium 2 mm lateral to the sagittal suture, 1 mm posterior to the coronal suture, and 3 mm under the cortex. Two µl viral suspension containing 2X10\textsuperscript{9} genome copies (gcs) of AAV-VEGF were stereotactically injected into the right basal ganglia at a rate of 0.2 µl per minute using a Hamilton syringe. The needle was withdrawn after 10 min and the wound was closed with a suture.

Intravenous Injection of AAV Vectors

Following induction of anesthesia with isoflurane inhalation, the right jugular vein was exposed and AAV vectors (2 x10\textsuperscript{11}, 8 x10\textsuperscript{11}, and 1x10\textsuperscript{12} gcs) in 200 µL of PBS were injected. The wound was closed with sutures after the injection.

5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal) Staining

X-gal staining was performed as described. Twenty µm coronal sections were fixed in 0.5 % glutaraldehyde for 10 minutes, incubated overnight 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, and 1 mg/ml X-gal in PBS), and photographed.

**Identifying the Infarct Region and Analysis Infarct Size**

A series of 20-µm thick coronal sections was obtained. One in every 10 sections was stained with cresyl violet and digitized. After binary imaging, the infarct and the ipsilateral hemisphere areas were outlined using Image J software, then measured. The infarct and ipsilateral hemisphere volumes were estimated as the sum of each area multiplied by 200 µm. The percentage of infarct volume was determined as the ratio of the infarct volume divided by the ipsilateral volume multiplied by 100.

**Identifying the Angiogenic Foci**

The angiogenic foci were identified on the lectin-stained sections. The staining was performed using a series of 20-µm-thick coronal sections. Sections were fixed with 100% ethanol at 20°C for 20 minutes, then incubated overnight with fluoresceinlycopersicin esculentum lectin (Vector Lab, Burlingame, CA), 2 g/ml at 4°C.

For X-gal and lectin double staining, we did X-gal staining first, and then lectin staining.

**Immunohistochemistry**

Brain samples were frozen in dry ice. Twenty µm thick coronal cryostat sections were made on a Leica CM1900 Cryostat (Leica). Sections were stained overnight with antibodies specific to NeuN (1:500, Chemicon, Temecula, CA), CD68 (1:50, AbD Serotec, Raleigh, NC) and β-gal (1:500, Abcam Inc., Cambridge, MA). Sections were incubated for 90 min with secondary antibodies Alexa 594 anti-mouse IgG and Alexa 488 anti-rabbit IgG (1:500 dilution; Invitrogen, Carlsbad, CA), and coverslipped with Vectashield mounting medium with 4’-6-diamidino-2-phenylinidole (DAPI) (Vector Laboratory) to label cell nuclei. Sections were imaged with a Leica DMLS fluorescent microscope with Spot Insight Software (Diagnostic Instruments, Inc., Sterling Heights, MI). Two sections 200 µm apart (between bregma 1.2 to 1.4 mm) were selected from each brain for quantification of the CD68 positive cells. Two pictures were taken under 20X objective from each section in the peri-infarct region shown in Supplemental Figure S6. The CD68 positive cells were quantified using image J (NIH, USA).

**Evans Blue Leakage Analysis**

We used a method modified from previously described Evans blue assay. Before the animals were sacrificed, 4 ml/kg of 2% Evans blue (Sigma) in normal saline were injected into the left jugular vein of anesthetized animals. The animals were perfused with saline 60 minutes later, followed by 1% paraformaldehyde in 50 mmol/L citrate buffer. Photos were taken after the brain was removed.

**Statistical Analyses**
Data are presented as mean ± standard deviation (SD). Means were compared using one-way ANOVA with Fisher's comparison tests. A p value < 0.05 was considered statistically significant. Sample sizes were six per group.
Supplemental Figures

Supplemental Figure S1. LacZ expression in the heart and liver of MCAO mice.

The organs were collected from MCAO mice five days after the IV-injection of AAV vectors expressing LacZ. Robust LacZ expression was detected in the heart and liver of mice that received IV-injection of AAV9-CMVLacZ. Minimum LacZ expression was detected in the heart and liver of mice injected with AAV9-H9LacZ. Scale bar: 100 µm.
Supplemental Figure S2. LacZ expression in the heart and liver of mice with brain angiogenesis.

The organs were collected from mice with brain angiogenesis five days after the IV-injection of AAV vectors expressing LacZ. Robust LacZ expression was detected in the heart and liver of mice that received IV-injection of AAV9-CMVLacZ. LacZ expression was also detected in the heart and liver of mice injected with AAV1-CMVLacZ. Scale bar: 100 µm.
Supplemental Figure S3. LacZ expression in the peri-infarct region defined by NeuN positive neurons.

Representative images of a NeuN (red) and LacZ (green) specific antibodies double-stained brain section collected from an AAV9-CMV\LacZ vector-injected MCAO mouse. The white lines delineate the infarct border. The merged picture (right) shows that LacZ expression is located in the peri-infarct region around the NeuN positive neurons. Scale bar: 100 \( \mu \text{m} \).
Supplemental Figure 4. Quantification of infarct volume.

All groups have similar infarct volume (P=0.56). Data are means±SD. n=6.
Supplemental Figure 5. Quantification of CD68\(^+\) cells in the peri-infarct region.

The picture on the left shows the areas (squares) used for CD68\(^+\) cell quantification. Bar graph shows the quantification of CD68\(^+\) cells. There is no difference among the three groups (P=0.96). Data are means±SD. n=6.
Supplemental Figure 6. Evans blue leakage in the infarct region.

The brain was perfused with Evans blue one hour after pMCAO. Evans blue dye had leaked out of the vessels in the infarct area (blue, arrow), which suggests that the BBB integrity in this region is impaired.
Supplemental Figure 7. LacZ expression in the contralateral brain of MCAO mice.

A few LacZ positive cells (arrow) were detected in the brain of the contralateral side of MCAO mice that received IV-injection of AAV9-CMVLacZ. No LacZ expression was detected in the brain of mice that received IV-injection of AAV9-H9LacZ except in the peri-infarct region. Scale bar: 100 µm.
Supplemental References


