Adeno-Associated Viral Vector-Mediated Hypoxia-Inducible Vascular Endothelial Growth Factor Gene Expression Attenuates Ischemic Brain Injury After Focal Cerebral Ischemia in Mice

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Background and Purpose—Exogenous delivery of vascular endothelial growth factor gene (VEGF) may provide a useful approach to the treatment of brain ischemia. We investigated the use of a hypoxia-responsive element to control VEGF expression given for neuroprotection.

Methods—Three groups (n=36) of mice received AAVH9-VEGF, AAVH9-lacZ, or saline injection. Five days after gene transfer, the mice underwent 45 minutes of transient middle cerebral artery occlusion (tMCAO) followed by 1 to 7 days of reperfusion. Infarct volume was determined using cresyl violet staining; neuronal injury was examined using TUNEL, cleaved caspase-3, and fluoro-Jade B staining.

Results—Hypoxia-inducible factor-1 (HIF-1) was overexpressed after tMCAO in the ischemic hemisphere in the brain. Expression of lacZ, mediated by AAV-lacZ, was seen before and after tMCAO; however, AAVH9-lacZ-mediated lacZ expression was detected only after tMCAO. Infarct volume was smaller in the AAVH9-VEGF-transduced group compared with AAVH9-lacZ and saline groups (55% reduction, P<0.05) with reduced TUNEL (29±5% and 30±7% versus 12±3%, P<0.05), cleaved caspase-3 (20±3% and 21±5% versus 13±4%, P<0.05) and fluoro-Jade B (23±3% and 24±5% versus 12±5%, P<0.05) -positive neurons, respectively.

Conclusion—Exogenous expression of VEGF through AAVH9-VEGF gene transfer 5 days before the onset of ischemia provides neuroprotection. Hypoxia-responsive element is a viable strategy of restricting VEGF expression to areas of ischemia to minimize adverse effects of therapy on adjacent normal parenchyma. (Stroke. 2006;37:2601-2606.)

Key Words: adeno-associated virus ■ brain ■ hypoxia-responsive element ■ ischemia ■ neuroprotection ■ VEGF

Vascular endothelial growth factor (VEGF), an angiogenic growth factor, is considered a potentially useful therapeutic agent to attenuate ischemic brain injury. Mechanisms for protective effects include improved perfusion, increased neurogenesis,1-3 and neurite outgrowth.4,5 VEGF expression is increased in ischemic regions after hours to days of ischemia.6 Endogenous VEGF secretion, however, is inadequate to fully protect the brain from injury. It may be possible to augment the native response therapeutically. Because VEGF has a short half-life, multiple injections might cause further injury. Gene transfer has the potential to maintain a sufficient concentration of VEGF protein over a long period of time from a single administration. However, VEGF expression mediated by adenovirus in the brain lasted only a few weeks.7

Adeno-associated virus (AAV), a single-stranded DNA virus, belongs to the nonpathogenic, helper-dependent member of the parvovirus family. It has several advantages over other viral vectors such as low immunogenicity and the ability to mediate long-term transgene and infect both dividing and non-dividing cells.8 AAV-delivered VEGF gene has shown therapeutic potential for many ischemic diseases; however, long-term uncontrolled VEGF expression may cause side effects such as hemangioma formation in the heart9 or limb.10

Hypoxia-inducible factor-1α (HIF-1α) plays a role during ischemia. In normoxia, HIF-1α subunits are degraded rapidly by 26s proteasomes, whereas during hypoxia, HIF-1α is stabilized and transactivated to the nucleus.11 HIF-1α is an essential regulator in response to hypoxia and upregulates VEGF expression.12 Hypoxia-responsive element (HRE) has been reported in three or five flanking regions of VEGF,13 Epo,14 and some other genes. The core consensus sequence is (A/G)CGT(G/C). HRE was first found and used to regulate several genes such as suicide gene15 and apoptosis gene16 expression in hypoxic tumors to enhance tumor...
killing; results indicate that HRE can effectively trigger the VEGF expression.

The aim of the present study was to investigate whether conditional expression of VEGF, using tissue hypoxia as a trigger, would reduce ischemic brain injury in a mouse transient middle cerebral artery occlusion (tMCAO) model.

Materials and Methods

Construction and Production of AAVH9-VEGF and AAVH9-lacZ Vectors

Two AAV vectors with HRE controlling VEGF or lacZ gene expression were constructed and described previously. Briefly, nine copies of HRE were inserted into the PGal-promoter plasmid (Clontech, Inc), which contains the SV40 minimal promoter upstream of the lacZ gene. We cloned the expression cassette (nine copies of HRE, SV40 minimal promoter, lacZ gene, and SV40 polyadenylation signal) to an AAV vector between two inverted terminal repeats (ITRs) to generate the AAVH9-LacZ vector. To construct the AAVH9-VEGF vector, we replaced the lacZ gene with human VEGF165 cDNA. Both AAVH9-VEGF and AAVH9-lacZ vectors were generated by using a three-plasmid cotransfection system. Viral vectors were purification and concentration by CsCl gradient centrifuge and dialyses against HEPES buffer.

Experimental Groups

The Institutional Animal Use and Care Committee approved all experimental procedures for the use of laboratory animals. Experiment 1 examined transduction efficiency, in which two groups of adult male CD-1 mice weighing 30 to 35 g received AAVH9-lacZ and AAV-lacZ injections (n=12 per group). Experiment 2 determined the effect of AAVH9-VEGF in stroke protection; here, three groups of CD-1 mice received AAVH9-VEGF, AAVH9-lacZ, or saline injections (n=12 per group). Five days after AAV vector injection, these mice underwent 45 minutes of tMCAO followed by 1 to 7 days of reperfusion. To examine the potential neuroprotective effect of VEGF against ischemia-induced brain damage, infarct volume was measured in the AAVH9-VEGF, AAVH9-lacZ, and saline-injected brain after tMCAO; neuronal injury was examined using TUNEL, cleaved caspase-3, and fluoro-Jade B staining.

Adeno-Associated Virus Vector Transduction in the Mouse Brain

The mice were anesthetized using 100/10 mg/kg ketamine/xylazine body weight intraperitoneally and then placed in a stereotactic frame with a mouth holder (Kopf Instruments). A burr hole was drilled in the pericranium 2 mm lateral to the sagittal suture and 1 mm posterior to the coronal suture. A 10-μL Hamilton syringe was stereotactically inserted into the lateral ventricle and caudate approximate 3.0 mm under the cortex. Five-microliter viral suspensions (AAVH9-VEGF, AAVH9-lacZ, AAV-VEGF) containing 5×10⁹ genome copies of virus were injected into the left ventricle and caudate putamen at a rate of 0.2 μL per minute based on prior experience. The needle was withdrawn after 15 minutes of injection. Control animals received the same amount of saline injection.

Transient Middle Cerebral Artery Occlusion

Animals were anesthetized with 1.5% isoflurane in 70%/30% N₂O/O₂. Body temperature was maintained at 37.0±0.5°C. The tMCAO method was described in our previous studies. Briefly, the internal carotid artery and external carotid artery were isolated, and a 2-cm length of 5-0 rounded Dermalon suture was gently advanced from the external carotid artery to the beginning of middle cerebral artery for a distance of 10.0±0.5 mm. Reperfusion was performed by partially withdrawing the suture. The occlusion lasted for 45 minutes and reperfusion was maintained up to 7 days.

Infarct Volume

A series of 20-μm thick coronal sections 2 mm distal from the frontal pole were cut and mounted on slides. The sections were dried and stained using cresyl violet. Sections were digitized, and the border between infarct and noninfarct tissue was outlined using image analysis system (National Institutes of Health, Image J). The ischemic lesion area was calculated as the difference between the area of the nonischemic hemisphere and the normal area of the ischemic hemisphere. The infarct volume was calculated by multiplying the infarct areas by the thickness of sections.

Nuclear Extraction and Western Blot Analysis

The brains were removed and homogenized. The pellet was resuspended in an extraction buffer and used for Western blot analysis. Protein concentrations were determined using the BCA protein assay. Equal amounts of protein were loaded on a 7 to 12% gradient gel for electrophoresis and electroblotted onto a nitrocellulose membrane in a transfer buffer (Invitrogen Corp). The nitrocellulose membrane was immunoprobed overnight with anti-HIF-1α antibody (1:500 dilution; Novus Biologicals) at 4°C. After washing, the membrane was incubated with HRP-conjugated antibody (1:2000 dilution; Amersham) and then reacted with an enhanced ECL (Amersham). The membrane was wrapped in plastic and exposed to Kodak film, which was developed according to the manufacturer’s instructions.

Immunohistochemistry

The sections were incubated with 10% serum for 1 hour to block nonspecific binding, washed with Tris buffer saline TWEEN-20 (TBST), and then incubated with anti-mouse HIF-1α (1:500 dilution; Chemicon) in TBST overnight at 4°C. Biotin-conjugated secondary antibody, avidin–biotin enzyme reagent (Vector Labs), and DAB were used to visualize the positive signal. After counterstaining with hematoxylin, the sections were dehydrated for further microscopic study. For double fluorescent staining of the cleaved caspase-3/NeuN or TUNEL/NeuN, sections were treated as described and incubated overnight with primary antibodies (1:200 dilution, cleaved caspase-3, and 1:500 dilution, NeuN; Chemicon) at 4°C. TUNEL staining was performed according to the manufacturer’s manual (Roche). After washing, fluorescent secondary antibodies (Vector Labs) were applied for 1 hour at 37°C. Double immunostaining sections were evaluated using a fluorescence microscope (Nikon Microphoto-SA). Three microscope fields (20×) of TUNEL-positive cell or cleaved caspase-3-positive cells in two separate brain coronal sections were chosen and imaged. Cell numbers were counted in images captured from these areas. The number of positive cells was calculated as the mean of the numbers obtained from the six pictures.

Fluoro-Jade B Staining

Fluoro-Jade B staining was used to identify nonviable neurons. Briefly, sections were immersed in a 80% Eoth/1%NaOH solution for 5 minutes and 0.06% KMnO₄ for 15 minutes followed by incubation in 0.0004% fluoro-Jade solution (Sigma) for 20 minutes. The slides were rinsed and dried, then cleared by immersion in xylene, and coverslipped with plastic mounting media. The fluoro-Jade B-positive cells were semiquantified using the same procedure as for immunostaining.

Statistical Analysis

Parametric data in different groups were compared using a one-way analysis of variance followed by Fisher protected least significant difference test as appropriate. All data are presented as mean±SD. A probability value of less than 5% was considered to represent statistical significance.

Results

Detection of HIF-1 After Transient Middle Cerebral Artery Occlusion in Brain

To determine whether focal cerebral ischemia induces focal HIF-1 expression, we examined HIF-1 in mice that were subjected to tMCAO. Nuclear HIF-1α expression greatly

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increased after tMCAO compared with the nonischemia control brain (Figure 1A, \( P < 0.05 \)). Immunohistochemistry showed that AAVH9-lacZ and AAVH9-VEGF gene transduction did not influence HIF-1 expression, whereas HIF-1-positive staining increased in the ischemic hemisphere in both the perifocal and core region after 1 day of tMCAO; no HIF-1-positive staining was detected in the nonischemia control brain (Figure 1B). Higher magnification shows that positive staining increased in the ischemic hemisphere in both the ischemic hemisphere in both the perifocal and core region after 1 day of tMCAO; no HIF-1-positive staining was detected in the nonischemia control brain (Figure 1B). Higher magnification shows that increased after tMCAO compared with the nonischemia control brain (Figure 1A, \( P < 0.05 \)). Immunohistochemistry showed that AAVH9-lacZ and AAVH9-VEGF gene transduction did not influence HIF-1 expression, whereas HIF-1-positive staining increased in the ischemic hemisphere in both the perifocal and core region after 1 day of tMCAO; no HIF-1-positive staining was detected in the nonischemia control brain (Figure 1B). Higher magnification shows that positive staining increased in the ischemic hemisphere in both the perifocal and core region after 1 day of tMCAO; no HIF-1-positive staining was detected in the nonischemia control brain (Figure 1B). Higher magnification shows that
HIF-1 distribution was predominantly in the endothelial cell monolayer of microvessels.

**Conditional Gene Expression After AAVH9 Vector Transduction**

To determine whether the nine copies of HRE can conditionally control gene expression, we examined lacZ expression after AAV gene transfer. LacZ gene expression was detected in the AAV-lacZ, but not in AAVH9-lacZ-transduced normal brain (Figure 2A). Interestingly, lacZ-positive staining could be detected in AAVH9-lacZ-transduced mice up to 7 days after tMCAO. LacZ gene was expressed in both lateral ventricle and parenchyma, which covered the tMCAO-induced injured brain region. We further performed Western blot for VEGF expression and found that VEGF expression was greatly increased in the AAVH9-VEGF-transduced mice compared with the AAVH9-lacZ-transduced mice ($P<0.05$, Figure 2B). Overexpression of VEGF sustained for at least 1 week, suggesting that ischemia-induced HIF-1 could stimulate AAVH9-VEGF actively expressing VEGF.

**Attenuation of Ischemia-Induced Brain Injury by AAVH9-VEGF Transduction**

We found that the overall Infarct volumes 7 days after tMCAO in the AAVH9-VEGF and AAVH9-lacZ-transduced and saline-treated group were $9\pm3$ mm$^3$, $20\pm6$ mm$^3$, and $23\pm7$ mm$^3$, respectively (Figure 3). There was no difference between the AAVH9-lacZ-transduced and saline-treated group ($P>0.05$). However, infarct volume in the AAVH9-VEGF-transduced mice was smaller than in the other two groups ($P<0.05$).

**Vascular Endothelial Growth Factor Overexpression Attenuates Neuronal Injury**

TUNEL-positive neurons were greatly reduced in the AAVH9-VEGF-transduced mice 7 days after tMCAO compared with the AAVH9-lacZ and saline-treated mice (Figure 4; $P<0.05$). TUNEL-labeled cells were distributed mainly in the ischemic core areas at the inner boundary zone after tMCAO. These cells were densely labeled in the nuclei and showed morphologic signs of apoptosis. The result of cleaved caspase-3 staining appeared similar to TUNEL assay results. Cleaved caspase-3-positive neurons were also reduced in the AAVH9-VEGF-transduced mice 7 days after tMCAO compared with the AAVH9-lacZ- and saline-treated mice (Figure 4; $P<0.05$). We then performed fluoro-Jade B staining after AAV vector injection in the normal brain. No fluoro-Jade staining was detected 5 days after AAV gene transfer in all three groups (data not shown here); however, fluoro-Jade B-positive staining was detected in the ischemic perifocal regions after tMCAO. The number of fluoro-Jade B-positive cells in the AAVH9-VEGF-transduced mice was much less than in the AAVH9-lacZ- or saline-treated mice (Figure 4; $P<0.05$).

**Discussion**

In this study, we demonstrated that the ischemic brain exhibited an increase of HIF-1 expression, indicating that choosing HIF-1 as a “sensor” to express therapeutic growth factors in endothelial cells is feasible and innovative. It is crucial to control VEGF expression, because it has been shown that excessively high levels of VEGF expression in the rat myocardium may result in hemangioma formation. LacZ-positive staining can only be detected in the AAVH9-lacZ-transduced brain after tMCAO, suggesting that HRE can effectively regulate gene expression only in the ischemic brain. Infarct volume and ischemia-induced apoptotic neuronal injury were attenuated in the AAVH9-VEGF-transduced mice, demonstrating that local injection of AAVH9-VEGF protected the brain against ischemia-induced injury; this neuroprotection is conducted through the antiapoptotic pathway. Our findings suggest that AAVH9 vector is a novel tool that can deliver VEGF gene into the brain.
ischemic brain and diminish the side effects of uncontrolled gene expression. We further verified that exogenous VEGF could produce neuroprotection during cerebral ischemia, although acute upregulation of VEGF is related to blood–brain barrier permeability leakage. A combination of other angiogenic factors to promote microvessel maturation should be studied in the future.

Knowing that endogenous VEGF is insufficient to induce angiogenesis, we wanted to determine whether, for a given level of endogenous VEGF that accompanies injury, neuronal death could be attenuated by supplementing exogenous VEGF through the transgene approach. Figure 2 shows that VEGF protein was significantly increased in the AAVH9-VEGF-transduced mice compared with AAVH9-lacZ mice after 1 day to 1 week of tMCAO. Because no VEGF protein was detected in the nonischemic brain, we considered the VEGF level in the lacZ-transduced mice ischemia-induced VEGF. The difference in VEGF levels between the AAVH9-VEGF and AAVH9-lacZ groups was AAVH9-VEGF-induced VEGF.

Nine copies of HRE in AAV vector can trigger lacZ expression after binding HIF-1 in the ischemic brain, which suggests that this is the key to regulate gene expression in response to hypoxia. Nine copies of HRE were more effective than three or six copies. The binding sites of other transcription factors such as AP1, p300/CREB, and the binding sites of orphan receptors hepatocyte nuclear factor 4 and EAR3.COUPTF-1 may be needed for higher downstream gene expression. However, many experiments also show that multiple copies of HIF-1 consensus sequence of human VEGF and human EPO can also mediate transgene expression.

Figure 4. A, Photomicrographs show TUNEL/NeuN (upper row), caspase-3/NeuN (middle row), and fluoro-Jade B (bottom row) staining after tMCAO in the control (a, e, i), saline (b, f, j), AAVH9-lacZ (c, g, k), and AAVH9-VEGF (d, h, l) -transduced mouse brain, respectively. Red-positive cells (NeuN) indicate neuronal cells. Green color represents TUNEL and cleaved caspase-3-positive staining. We detected apoptotic neurons (yellow color) in the ischemic core areas at the inner boundary zone after tMCAO. There were much less apoptotic neurons in the AAVH9-VEGF-transduced mice than in the AAVH9-lacZ-transduced and saline-treated mice. Similarly, damaged neurons were attenuated in the AAVH9-VEGF-transduced mice. Bar=20 μm. B, Bar graphs indicate quantitative TUNEL-positive cells (left), cleaved caspase-3-positive cells (middle), and fluoro-Jade B cells (right) after 1 day and 1 week of tMCAO. Data are mean±SD. N=6 per groups. *P<0.05, AAVH9-VEGF vs AAVH9-lacZ or saline group.
in response to hypoxia/ischemia. We demonstrated that AAVH9-lacZ produced lacZ expression only when ischemia occurs. Therefore, abnormal microvasculature induced by uncontrolled and constantly expressed VEGF can be avoided by HRE-controlled VEGF expression.

The advantage of using an AAVH9-VEGF is that VEGF is a secreted protein. We injected the AAVH9-VEGF into the left parenchyma and left ventricle rather than the ischemic region alone. We did not choose to inject into the core alone because the cells in the ischemic core were seriously injured or died a few hours after ischemia and thus did not have the ability to transduce VEGF protein. VEGF protein spreads through diffusion and cerebrospinal fluid circulation to adjacent cells and remote brain regions. We found lacZ gene expression in both parenchyma and ependymal tissues after tMCAO in the AAVH9-lacZ-transduced mice. Our previous studies showed the distribution of the lacZ gene in the adenoviral lacZ-transduced rodent brain.19,26

The timing of gene production is another important issue. The ischemic core experiences irreversible injury within a few hours; however, the perifocal region may have substantially wider timeframes for rescue. We injected AAVH9-VEGF before tMCAO because we expected that AAV vector could effectively transduce to the brain cells, producing VEGF during ischemic stimulation. Although exogenously delivered VEGF attenuates ischemic neuronal cell death even if the protein is given after the onset of cerebral ischemia,27,28 future studies are needed to refine the time window of VEGF expression after cerebral ischemia. We demonstrated that AAVH9-VEGF’s effectiveness both before and after the onset of cerebral ischemia,27,28 future studies are needed to refine the time window of VEGF expression even if the protein is given after the onset of cerebral ischemia.

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