Effect of combined *VEGF*₁₆₅/*SDF-1* gene therapy on vascular remodeling and blood perfusion in cerebral ischemia

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OBJECTIVE Therapeutic neovascularization is a promising strategy for treating patients after an ischemic stroke; however, single-factor therapy has limitations. Stromal cell-derived factor 1 (SDF-1) and vascular endothelial growth factor (VEGF) proteins synergistically promote angiogenesis. In this study, the authors assessed the effect of combined gene therapy with *VEGF*₁₆₅ and *SDF-1* in a rat model of cerebral infarction.

METHODS An adenoviral vector expressing *VEGF*₁₆₅ and *SDF-1* connected via an internal ribosome entry site was constructed (Ad-*VEGF*₁₆₅-*SDF-1*). A rat model of middle cerebral artery occlusion (MCAO) was established; either Ad-*VEGF*₁₆₅-*SDF-1* or control adenovirus Ad-*LacZ* was stereotactically microinjected into the lateral ventricle of 80 rats 24 hours after MCAO. Coexpression and distribution of *VEGF*₁₆₅ and *SDF-1* were examined by reverse-transcription polymerase chain reaction, Western blotting, and immunofluorescence. The neurological severity score of each rat was measured on Days 3, 7, 14, 21, and 28 after MCAO. Angiogenesis and vascular remodeling were evaluated via bromodeoxyuridine and CD34 immunofluorescence labeling. Relative cerebral infarction volumes were determined by T2-weighted MRI and triphenyltetrazolium chloride staining. Cerebral blood flow, relative cerebral blood volume, and relative mean transit time were assessed using perfusion-weighted MRI.

RESULTS The Ad-*VEGF*₁₆₅-*SDF-1* vector mediated coexpression of *VEGF*₁₆₅ and *SDF-1* in multiple sites around the ischemic core, including the cortex, corpus striatum, and hippocampal granular layer. Coexpression of *VEGF*₁₆₅ and *SDF-1* improved neural function, reduced cerebral infarction volume, increased microvascular density and promoted angiogenesis in the ischemic penumbra, and improved cerebral blood flow and perfusion.

CONCLUSIONS Combined *VEGF*₁₆₅ and *SDF-1* gene therapy represents a potential strategy for improving vascular remodeling and recovery of neural function after cerebral infarction.

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KEY WORDS angiogenesis; cerebral ischemia; *VEGF*₁₆₅; *SDF-1*; gene therapy; therapeutic neovascularization; rat

AFTER cerebral infarction occurs, angiogenesis and vascular remodeling of brain tissue are required to restore an adequate blood supply and provide the necessary microenvironmental niche for neurogenesis and neural precursor cell survival to enable neural function improvements and patient rehabilitation.^{2,6,9,17} Therefore, neovascularization strategies are important experimental

treatments for patients after cerebral infarction. As a major factor that regulates the development of blood vessels, vascular endothelial growth factor (VEGF) is commonly used to promote angiogenesis in experimental models. However, VEGF alone might not be enough for the formation of a mature vascular structure with circulatory function, because VEGF-induced blood vessels are usually leaky and

ABBREVIATIONS BrdU = bromodeoxyuridine; CBF = cerebral blood flow; CBV = cerebral blood volume; Cy3 = cyanine 3; CXCR4 = chemokine receptor 4; DAPI = 4'-6-diamidino-2-phenylindole; EPC = endothelial progenitor cell; FITC = fluorescein isothiocyanate; MCAO = middle cerebral artery occlusion; mNSS = modified neurological severity score; MTT = mean transit time; MVD = microvascular density; PWI = perfusion-weighted imaging; r = relative; RT-PCR = reverse-transcription polymerase chain reaction; SDF-1 = stromal cell-derived factor 1; TTC = triphenyltetrazolium chloride; T2WI = T2-weighted imaging; VEGF = vascular endothelial growth factor; VEGFR1 = VEGF receptor 1.

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fail to connect with the original vascular network.²⁵ Recent studies have shown that the protein stromal cell-derived factor 1 (SDF-1) and VEGF synergistically promote angiogenesis and enhance the maturity of the resulting vascular structure. SDF-1 recruits endothelial progenitor cells (EPCs) for angiogenesis and recruits perithelial cells and smooth muscle cells to enhance the stability and maturity of neovessels.²¹

In this study, we constructed an adenoviral vector that expressed both VEGF₁₆₅ and SDF-1 genes (Ad-VEGF₁₆₅-SDF-1).¹⁸ A rat model of suture-occluded middle cerebral artery occlusion (MCAO) was established. Twenty-four hours after MCAO, a stereotactic intracerebroventricular method was used to microinject Ad-VEGF₁₆₅-SDF-1 or a control adenovirus into the lateral ventricle of the rats. We found that combined VEGF₁₆₅/SDF-1 gene therapy promoted angiogenesis and the restoration of a functional vascular structure to achieve an appropriate therapeutic effect.

Methods

Rat MCAO Model and Gene Delivery

Male Sprague-Dawley rats (8–10 weeks old; 280–320 g) were purchased from Beijing Vital River Laboratory Animals Co., Ltd. (Certificate No. SCXK [Jing] 2012-0001) and housed under specific-pathogen-free conditions. All animal procedures were performed according to a protocol approved by the Institutional Animal Care and Use Committee of Qingdao University. The method used in this study adhered to the following criteria of the Stroke Therapy Academic Industry Roundtable³: 1) animals were randomly assigned to the treatment or control group, 2) the researchers were blinded to treatment and outcome evaluation, and 3) the temperature of the animals was controlled effectively during ischemia. A right MCAO model was established using a suture method.¹¹ The animals had free access to water and standard rat chow after recovery. Twenty-four hours after MCAO, the rats were divided randomly into 2 groups (40 per group); 10 μ l of phosphate-buffered saline containing 1×10^{10} Ad-VEGF₁₆₅-SDF-1 (treatment group) or Ad-LacZ (control group) was stereotactically microinjected into the right cerebral ventricle¹² of each rat using a stereotactic instrument designed for use on the brain (Reiwoode Life Science Co.).

Neurological Severity Scores

The rats were assessed using a modified neurological severity score (mNSS)¹³ on Days 3, 7, 14, 21, and 28 after MCAO using a single-blinded method. With the mNSS method, motor and sensory functions, reflex, and balance were assessed and assigned scores ranging from 0 to 18 points; the score positively correlates with the severity of neurological injury (0, no impairment; 1–6, mild impairment; 7–12, moderate impairment; 13–18, severe impairment).

Analysis of VEGF₁₆₅ and SDF-1 Expression

Twenty-one days after MCAO, 6 animals in each group were terminally anesthetized with chloral hydrate, and their brain tissues within 0–2 mm of the coronal bregma

(1 mm around the injection target) were harvested and stored at -80°C .

For reverse-transcription polymerase chain reaction (RT-PCR), total RNA was isolated using TRIzol reagent (Invitrogen), reverse transcribed to cDNA using a SYBR Premix Ex Taq kit (TaKaRa), and subjected to PCR to assess VEGF₁₆₅ and SDF-1 gene expression. We used the primers β -actin (5'-GAACCTAAGGCCAAC-3' [sense] and 5'-TGTCACGCACGATTTC-3' [antisense]; product size 305 bp), VEGF₁₆₅ (5'-AGAGCAACATCACC ATGCAG-3' [sense] and 5'-CAGTGAACGCTCCAGG ATTT-3' [antisense]; product size 205 bp), and SDF-1 (5'-GCTCTGCATCAGTGACGGTA-3' [sense] and 5'-TA ATTTCCGGTCAATGCACA-3' [antisense]; product size 184 bp). PCR products were analyzed by agarose gel electrophoresis.

For Western blotting, the protein concentration of each sample was determined using the bicinchoninic acid assay method, and then the samples were diluted 1:4 with sodium dodecyl sulfate (SDS) loading buffer, separated by SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes. Membranes were incubated with mouse anti-VEGF monoclonal antibody (1:800; Santa Cruz Biotechnology) or rabbit anti-SDF-1 polyclonal antibody (1:200; Santa Cruz Biotechnology) at 4°C overnight, followed by incubation with horseradish peroxidase-conjugated secondary goat anti-mouse (1:8000) and goat anti-rabbit (1:2000) (Zhongshan Biotechnology) antibodies, and bands were visualized using an enhanced chemiluminescence substrate. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal loading control (mouse anti-GAPDH monoclonal antibody [1:1000]; Zhongshan Biotechnology). Quantification of the PCR and Western blot bands was carried out using Quantity One 4.4 densitometric analysis software (Bio-Rad); results are expressed here as the ratio of the optical densities of PCR and Western blot bands relative to those of the internal controls.

Magnetic Resonance Imaging

Brain scans were acquired 28 days after MCAO (6 per group) using an HDx 3.0-T superconducting MRI scanner (General Electric) and a 3.0-T 5-cm 4-channel phased-array coil (Chenguang Medical Technologies Co., Ltd.). A 3D positioning scan and then coronal T1-weighted imaging, T2-weighted imaging (T2WI), perfusion-weighted imaging (PWI), and T1-enhanced imaging were performed in sequence. An echo planar imaging sequence was used for PWI (20 scanning layers; 50 scans per layer; total imaging time 80 seconds; bolus of 0.1 mmol/kg gadobutrol injected into the femoral vein via a 24-gauge indwelling needle after the fourth scan). Immediately after PWI, a T1-enhanced examination with the same number of layers and the same layer thickness was performed. Images were transferred to an ADW4.4 workstation (General Electric) and fused with the perfusion images to analyze perfusion; obtain the cerebral blood flow (CBF), cerebral blood volume (CBV), and mean transit time (MTT) values of the ischemic penumbra; and calculate the relative values (rCBF, rCBV, and rMTT) (relative values are equal to the value of the affected side divided by the value of the

unaffected side). Image-Pro Plus 6.0 (Media Cybernetics) was used to calculate the relative volume of the abnormal signal area on T2WI.

Triphenyltetrazolium Chloride Staining

On Day 28 after MCAO, rats (6 per group) were terminally anesthetized with 2% pentobarbital sodium (40 mg/kg) and perfused with saline via cardiac perfusion, and brain tissues were collected rapidly. Triphenyltetrazolium chloride (TTC) staining and image acquisition of brain slices were performed as described previously.¹¹ Cerebral infarction volume fractions were calculated using Image-Pro Plus 6.0 software using the formula $\%I = 100 \times (VC - VL)/VC$, where $\%I$ is infarction volume, VC is the contralateral hemisphere volume, and VL is the noninfarcted volume in the affected hemisphere.²⁰

Bromodeoxyuridine Accumulation and Frozen-Section Preparation

Six days after MCAO, bromodeoxyuridine (BrdU) (Sigma-Aldrich) incorporation was initiated using a cumulative method.¹⁰ Rats (8 per group) were injected intraperitoneally with 50 mg/kg (6 mg/ml) BrdU every 12 hours for 3 days. On Day 28 after MCAO, the animals were terminally anesthetized with 2% sodium pentobarbital (40 mg/kg), perfused with normal saline, and fixed with 4% paraformaldehyde via cardiac perfusion. Their brain tissues were harvested, fixed with 4% paraformaldehyde at 4°C overnight, and dehydrated in 15% and 30% sucrose. Coronal sections (25 µm thick) were prepared using a cryostat microtome (Leica) from 1.5 to 2.5 mm around the bregma at 100-µm intervals (8 sections per brain), placed in cryoprotectant in 24-well plates, and stored at -20°C.

VEGF₁₆₅/SDF-1 and CD34/BrdU Double Immunostaining

Free-floating sections were subjected to double-immunofluorescent labeling for BrdU/CD34 and VEGF₁₆₅/SDF-1. For BrdU/CD34 immunofluorescence, frozen brain sections were incubated with 50% formamide in 2× saline-sodium citrate solution at 65°C for 2 hours, followed by 2 mol/L hydrochloric acid for 30 minutes at 37°C to denature DNA, and then neutralized in 0.1 mol/L boric acid (pH 8.5) for 20 minutes. Sections were treated with 1× antigen-repair solution at 37°C for 5 minutes, blocked with 10% donkey serum, and permeabilized for 1 hour in 0.3% Triton X-100. Sections were then incubated with primary rat anti-BrdU monoclonal antibody (1:800; Cell Signaling Technology) and mouse anti-CD34 polyclonal antibody (1:100; Santa Cruz Biotechnology) at 4°C for 14 hours, followed by the secondary antibody cyanine 3 (Cy3)-labeled donkey anti-rat IgG (1:400; Chemicon) and fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse (1:200; Jackson Laboratory) at room temperature for 2 hours. With the exception of the blocking and primary antibody incubation steps, sections were washed with phosphate-buffered saline 3 times for 5 minutes after each step. The tissue sections were loaded on glass slides and mounted in 50% glycerol, and images were acquired using an upright fluorescence microscope (Olympus). Sections were also immunostained using primary rabbit polyclonal antibody

to SDF-1 (1:400; Abcam), mouse monoclonal antibody to VEGF (1:200; Abcam), and the corresponding FITC-conjugated goat anti-mouse (1:200; Jackson Laboratory) and Cy3-conjugated donkey anti-rabbit secondary antibodies (1:400; Jackson Laboratory) following the method described above, except the DNA denaturation and neutralization steps were omitted; nuclei were counterstained with 10 µg/ml 4'-6-diamidino-2-phenylindole (DAPI) (Roche).

Image Analysis and Assessment of Microvascular Density

Four serial frozen brain sections in the rostrum-to-caudal direction were sampled at intervals (spaced 100 µm apart, 1.5–2.5 mm from the bregma) for each animal. The numbers of BrdU- and CD34-positive cells in 3 areas of interest around the infarct were counted at ×400 magnification in each slide using a single-blinded method. The positive cell number for each rat was calculated as the sum of all positive cell numbers. CD34-positive vascular structures or CD34-positive cell populations with or without tubelike structures were classified as microvessels; the counting method described above was used to calculate microvascular density (MVD).

Statistical Analysis

SPSS 17.0 statistical software (SPSS, Inc.) was used for statistical analysis. Data are presented as means ± SE of the resulting data. One-way ANOVA was used to determine the significance of the differences between groups; $p < 0.05$ was considered statistically significant.

Results

Adenovirus-Mediated Coexpression of VEGF₁₆₅ and SDF-1 in the Marginal Ischemic Zone

In the treatment group, 5 rats suffered epileptic seizures within 72 hours of receiving the adenovirus; these animals were excluded from the study to avoid other issues (such as injection-induced cerebral hemorrhage). RT-PCR revealed that the relative expression levels of both VEGF₁₆₅ and SDF-1 were significantly higher in the treatment group than in the control group (0.5951 ± 0.0671 and 0.6424 ± 0.0939 vs 0.4095 ± 0.0537 and 0 ± 0.0349 , respectively; $p < 0.01$ for both) (Fig. 1A). Western blotting confirmed that the relative protein expression levels of VEGF₁₆₅ and SDF-1 in the marginal ischemic zone were significantly higher in the treatment group than in the control group (3.9844 ± 0.8099 and 1.1655 ± 0.2674 vs 0.0911 ± 0.0814 and 0.2304 ± 0.1426 , respectively; $p < 0.001$ for both) (Fig. 1B).

Immunofluorescence staining of frozen brain sections showed that VEGF₁₆₅ and SDF-1 were widely coexpressed in multiple areas of the rat brains in the treatment group, including the cortex, corpus striatum, and hippocampal granular layer (Fig. 2). In the control group, the expression levels of both VEGF₁₆₅ and SDF-1 were undetectable.

Coexpression of VEGF₁₆₅ and SDF-1 Improves Neural Function and Reduces Cerebral Infarct Volume

On Day 3 after MCAO, the mNSSs in the treatment and control groups were not significantly different ($p > 0.05$). However, the mNSSs in the treatment group were signifi-

FIG. 1. Injection of Ad-VEGF₁₆₅-SDF-1 increases VEGF₁₆₅ and SDF-1 expression in the marginal ischemic zone. **A:** RT-PCR analysis of VEGF₁₆₅ (a) and SDF-1 (b) expression and quantification of their expression (c). **B:** Western blot analysis of VEGF₁₆₅ and SDF-1 coexpression (a) and quantification (b). Data are means $\pm$ SD of resulting data. * $p < 0.01$; ** $p < 0.001$.

cantly lower than those in the control group 7 days after MCAO ($p < 0.05$); this difference was more significant when the mNSSs were assessed on Days 14, 21, and 28 ($p < 0.01$) (Fig. 3), which suggests that combined VEGF₁₆₅/SDF-1 gene therapy improved the restoration of neural function after MCAO.

On T2WI, the image intensities in the right cortex and striatum were abnormally high in both groups on Day 28 after MCAO, indicating softening of the focus after cerebral infarction. However, T2WI revealed that combined

VEGF₁₆₅/SDF-1 gene therapy significantly reduced the cerebral infarct volume ($23.65 \pm 1.71\%$ [treatment group] vs $28.99 \pm 2.25\%$ [control group]; $p < 0.05$) (Fig. 4A and C).

Normal brain tissue is pink or crimson after TTC staining. White infarcted foci were observed in the cortex and striatum of the right hemisphere in both groups 28 days after MCAO. Similar to MRI, TTC staining confirmed that the infarct volume ratio was significantly lower in the treatment group than in the control group ($26.97 \pm 3.28\%$ vs $32.23 \pm 4.23\%$, respectively; $p < 0.05$) (Fig. 4B and

FIG. 2. Distribution of VEGF₁₆₅ and SDF-1 expression in the rat brain. Representative images of immunofluorescent staining of frozen brain sections from an animal in the treatment group injected with Ad-VEGF₁₆₅-SDF-1 and subjected to MCAO. Frozen brain sections were prepared on Day 28 after MCAO. **A:** VEGF₁₆₅ and SDF-1 were expressed at high levels in cortical neurons. **B:** Expression of VEGF₁₆₅ and SDF-1 in the cells of the granular layer of the hippocampus. **C:** Expression of VEGF₁₆₅ and SDF-1 in the striatum. Bar = 20 μm. Green indicates FITC-labeled VEGF; red, Cy3-labeled SDF-1; blue, DAPI-stained nuclei; and yellow, merged green and red fluorescence. Original magnification $\times 400$. Figure is available in color online only.

FIG. 3. Combined $VEGF_{165}/SDF-1$ gene therapy improves neural function. Neurological function was assessed using mNSSs on Days 3, 7, 14, 20, and 28 after MCAO. The mNSSs were not significantly different on Day 3 after MCAO; however, the mNSSs of the treatment group were significantly lower on Day 7 after MCAO, and on Days 14, 21, and 28, the differences were even more significant (20 rats per group). * $p < 0.05$; ** $p < 0.01$ compared with control group.

C). Altogether, these results demonstrate that combined $VEGF_{165}/SDF-1$ gene therapy significantly reduced the cerebral infarct volume after MCAO.

Coexpression of $VEGF_{165}$ and $SDF-1$ Promotes Angiogenesis, Increases CBF, and Improves Blood Perfusion

CD34-positive vessels were visible in both the cortex and striatum around the cerebral infarct in both groups 28 days after MCAO (Fig. 5A). However, the mean MVD of the treatment group was significantly higher than that of the control group (380.25 ± 83.43 vs 209.25 ± 49.24 , respectively; $p < 0.01$) (Fig. 5B). In addition, tubelike structures with a regular morphology and good patency were more obvious in the treatment group, whereas the blood vessels in the control group were “beaded” with

a discontinuous structure. In confirmation testing, immunofluorescent double labeling revealed that the BrdU/CD34-positive cell numbers in the area surrounding the ischemic foci were significantly higher in the treatment group than in the control group 28 days after MCAO (Fig. 5C and D). These results demonstrate that the coexpression of $VEGF_{165}$ and $SDF-1$ promoted angiogenesis and improved the integrity of the resulting vascular structure in the infarct zone.

Furthermore, dynamic susceptibility contrast-enhanced PWI indicated that coexpression of $VEGF_{165}$ and $SDF-1$ significantly improved rCBF, increased the rCBV, and reduced the rMTT in the ischemic penumbra by 28 days after MCAO ($p < 0.01$ for all) (Fig. 6A). The mean rCBF, rCBV, and rMTT values in the treatment group were $65.74 \pm 9.15\%$, $114.17 \pm 17.27\%$, and $153.33 \pm 31.49\%$, and those for the control group were $47.17 \pm 8.22\%$, $83.33 \pm 13.87\%$, and $222.00 \pm 52.72\%$, respectively ($p < 0.01$ for all) (Fig. 6B).

Discussion

Combined gene therapy might represent an appropriate approach for proangiogenic gene therapy. Here, we demonstrate that coexpression of $VEGF_{165}$ and $SDF-1$ promoted angiogenesis and the formation of an effective vascular network in the area surrounding the ischemic foci and improved restoration of neuronal function in a rat model of cerebral infarction.

Adenovirus-Mediated Coexpression of $VEGF_{165}$ and $SDF-1$ in the Ischemic Brain

The adenoviral vector constructed in this study mediated coexpression of $VEGF_{165}$ and $SDF-1$ in the brain surrounding the infarcted foci. This vector contains an internal ribosome entry site (IRES), which allows the genes connected to both ends of the IRES to be transcribed under control of an upstream promoter and translated

FIG. 4. Combined $VEGF_{165}/SDF-1$ gene therapy reduces cerebral infarct volume. **A:** T2-weighted MR images of animals in the control (a) and treatment (b) groups 28 days after MCAO. T2WI signals were abnormally high in the right cortex and striatum, indicating the softening of focus after cerebral infarction. **B:** Representative images of TTC staining of brain slices prepared from animals in the control (a) and treatment (b) groups 28 days after MCAO. **C:** TTC staining revealed that the volume ratio of the infarcted foci in the treatment group ($26.97 \pm 3.28\%$) was significantly lower than that in the control group ($32.23 \pm 4.23\%$). * $p < 0.05$ (6 rats per group). T2WI revealed that combined $VEGF_{165}/SDF-1$ gene therapy significantly reduced cerebral infarct volume ($23.65 \pm 1.71\%$ [treatment group] vs $28.99 \pm 2.25\%$ [control group]). * $p < 0.05$ (6 rats per group). Figure is available in color online only.

FIG. 5. Combined VEGF₁₆₅/SDF-1 gene therapy promotes angiogenesis in a rat model of MCAO. **A:** Representative images of FITC-labeled CD34-positive microvessels surrounding the infarcted foci in the cortex and striatum on Day 28 after MCAO. Bar = 500 μ m. **B:** Assessment of MVD in the peri-infarct areas of the brain on Day 28 after MCAO. * $p < 0.01$ (6 rats per group). **C:** Representative images of CD34/BrdU immunofluorescent double labeling in the peri-infarct areas in the brain of an animal in the treatment group 28 days after MCAO. Bar = 50 μ m. **D:** Quantification of BrdU/CD34-positive cells in the affected hemisphere around the cerebral infarct 5 weeks after MCAO. BrdU/CD34-positive cell numbers were significantly higher in the treatment group than in the control group. * $p < 0.01$ (8 rats per group). Data in B and D are means $\pm$ SD of resulting data. Figure is available in color online only.

and expressed at the same time.¹⁶ Adenoviral genomic sequences can now be deleted from adenoviral vector systems, leaving only the inverted terminal repeat at both ends of its sequence and the packaging signal. So-called empty-shell carriers have greatly reduced immunogenicity and improved loading capacity for exogenous DNA and are increasingly widely used in basic research and clinical trials.¹⁶

VEGF₁₆₅ and SDF-1 Synergistically Promote Angiogenesis

Accumulating evidence indicates that angiogenesis requires VEGF and SDF-1. VEGF is a key proangiogenic molecule; however, Zentilin et al.²⁵ reported that overexpression of VEGF induced leaky neovessels that failed to connect correctly with existing vessels. In our study, expression of VEGF₁₆₅ and SDF-1 increased the number of

CD34- and BrdU-positive cells in the ischemic penumbra, which suggests higher levels of endothelial cell division and angiogenesis. This effect might be a result of both in situ division and proliferation of preexisting endothelial cells and/or increased migration of CD34-positive cells that can form vascular structures or EPCs from peripheral blood and bone marrow. Such migrating cells could be integrated into the vascular lumen and undergo cell division to enable angiogenesis and vascular remodeling around infarcted foci. In fact, in addition to EPCs, a variety of more primitive cell types, such as recruited bone marrow-derived circulating cells,⁵ might also be involved in angiogenesis, and hematopoietic progenitors⁷ might also be involved in the revascularization process.

Grunewald et al.⁵ confirmed that SDF-1 recruits EPCs expressing the SDF-1 receptor chemokine receptor 4

FIG. 6. Combined $VEGF_{165}/SDF-1$ gene therapy improves CBF and CBV and shortens rMTT in the ischemic penumbra. **A:** Perfusion-weighted MRI showing that CBF and CBV in the ischemic penumbra improved in the treatment group over those in the control group 28 days after MCAO. Black circles indicate mirror-image areas in the other hemisphere. **B:** Quantification of rCBF, rCBV, and rMTT 28 days after MCAO (6 rats per group) showing that both rCBF and rCBV improved significantly and rMTT was reduced significantly in the ischemic penumbra 28 days after MCAO. Data are means $\pm$ SD of resulting data. * $p < 0.05$; ** $p < 0.01$. Figure is available in color online only.

(CXCR4) to sites of damage and recruits perivascular cells and smooth muscle cells to surround endothelial cells; therefore, SDF-1 plays a supplementary role in angiogenesis and increases neovessel stability. SDF-1 acts downstream of VEGF; VEGF recruits EPCs by upregulating SDF-1, whereas CXCR4 might be necessary to keep angiogenic cells in the ischemic region. Tang et al.²² found that bone mesenchymal stem cells modified with VEGF promoted release of SDF-1, upregulated CXCR4 on cardiac stem cells, and consequently enhanced the repair of myocardial infarction. It is interesting to note that, in turn, SDF-1 upregulates endogenous VEGF,²⁴ and VEGF can also promote CXCR4 expression on bone marrow macrophages via VEGF receptor 1 (VEGFR1) and recruits CXCR4-positive cells from the bone marrow via the VEGFR1 kinase pathway, which suggests that SDF-1-induced angiogenesis depends on the VEGFR1 pathway.¹

Activation of endothelial tip cells is a vital process in vascular sprouting. CXCR4 is enriched on tip cells and promotes the formation and bifurcation of capillaries independent of VEGF.¹⁹ Unoki et al.²³ found that VEGF promotes neovascular sprouting in the retina, and the SDF-1/CXCR4 signaling pathways directly promote the extension and bifurcation of newly sprouted vessels.

Combined $VEGF_{165}/SDF-1$ Gene Therapy Increases MVD and Blood Perfusion

Perfusion-weighted MRI was used to illustrate the perfusion of the new blood vessels. The rCBF and rCBV values were significantly higher in the treatment group than in the control group, whereas rMTT values were significantly lower. These results demonstrate that the expression of $VEGF_{165}$ and $SDF-1$ enhanced neovessel formation and promoted appropriate vascular remodeling around the infarct, which in turn enhanced vascular perfusion. These processes might explain why the relative cerebral infarct volumes were significantly lower in the treatment group

than in the control group. It was surprising to note that CBV values were even higher in the peri-infarct areas of the brain in the treatment group than in the unaffected hemisphere of the same animals; we suggest that the increased density of neovessels on the affected side enlarged the microvascular bed area. When assessing CBF, CBV, and MTT, we compared the mirror-image ratios of the affected and unaffected hemispheres instead of comparing the absolute values for the affected side to rule out individual differences between the rats and exclude other external confounding factors such as enhancer dose, bolus injection rate, and data processing.

MRI examination and TTC staining confirmed that the cerebral infarction volume was significantly lower in the treatment group than in the control group, which is consistent with the neural function improvements indicated by the mNSSs. In our opinion, the reduced cerebral infarction volume and neural function improvements induced by $VEGF_{165}$ and $SDF-1$ were related to increased formation of neovessels and enhanced blood perfusion, which in turn increased the survival of brain cells in the ischemic penumbra region after MCAO. In addition, it is possible that the expression of $VEGF_{165}$ and $SDF-1$ promoted neurogenesis; this hypothesis will form the basis of our future studies.

Problems Encountered in This Study

Five experimental rats in the treatment group had epileptic seizures in the early stages (within the first 72 hours after the Ad- $VEGF_{165}-SDF-1$ vector was injected) after MCAO, which might have been associated with increased permeability of the blood-brain barrier (BBB); these rats were excluded from this study. Li et al.¹⁴ suggested that administering exogenous VEGF during the acute phase after cerebral infarction can destroy BBB integrity and increase exudation; Friedman⁴ indicated that increased exudation from the BBB can induce a variety of neurological

disorders, including epilepsy. In addition, SDF-1 has been reported to participate in brain remodeling and therefore can also cause epileptic seizures.⁸

Limitations of This Study

Young male rats were used for this MCAO model. In the clinic, the risk of cerebral ischemia increases with age. However, it is more difficult to establish MCAO models in older rats, because they are heavier (in weight) and have a lower tolerance to anesthesia. Moreover, sex differences need to be considered. Some studies have reported that the procedure used in the MCAO model leads to a significantly lower infarct volume in female rats than in male rats and that the infarct size and volume in ovariectomized female rats are similar to those of male rats, which suggest that estrogen might play a regulatory role in this model.¹⁵ Therefore, adult male rats are commonly used for animal models of stroke. Despite these limitations, additional animal studies should be performed, following Stroke Therapy Academic Industry Roundtable recommendations, to assess translational applicability; after an initial assessment of young, healthy male animals, additional studies should be conducted in female and older animals and even animals with comorbidities such as hypertension, diabetes, and hypercholesterolemia.³ Another limitation of this study is the fact that expression quantification was based on Western blots, which only reflect the relative amounts (ratios) of each protein band, not the absolute values. Enzyme-linked immunoassays could be used in the future to determine the actual protein expression levels. Similar to previous studies of other neuroprotective agents,^{1,5,24} VEGF₁₆₅/SDF-1 provided a therapeutic effect in this animal model of stroke. However, these laboratory successes should not be equated with an actual clinical effect, and further intensive research is required to assess the clinical value of this experimental protocol.

Conclusions

Combined adenovirus-mediated expression of exogenous VEGF₁₆₅ and SDF-1 exerted a desirable treatment effect after cerebral infarction in a rat model of MCAO; coexpression of VEGF₁₆₅ and SDF-1 promoted angiogenesis and vascular remodeling, increased CBF and perfusion, and improved neural function after cerebral ischemia. We believe that there is a certain similarity between multigene therapy and multidrug combination therapy; treatment based on a single gene might not result in a satisfactory therapeutic effect, whereas combined gene therapy might exert a synergistic and complementary therapeutic effect, especially with complicated conditions such as stroke. As far as we are aware, this study is the first investigation of the ability of double-gene therapy (based on VEGF₁₆₅ and SDF-1) to promote angiogenesis and vascular remodeling for the treatment of cerebral ischemia.

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Disclosures

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author Contributions

Conception and design: SF Li, Hu. Acquisition of data: SF Li, Lu. Analysis and interpretation of data: SF Li, Lu, Xie. Drafting the article: Hu. Reviewed submitted version of manuscript: SF Li, HT Li. Approved the final version of the manuscript on behalf of all authors: SF Li. Statistical analysis: HT Li. Administrative/technical/material support: Xie. Study supervision: Feng.

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