Combined gene therapy with vascular endothelial growth factor plus apelin in a chronic cerebral hypoperfusion model in rats

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OBJECTIVE The aim of this study was to evaluate whether combined gene therapy with vascular endothelial growth factor (VEGF) plus apelin during indirect vasoreconstructive surgery enhances brain angiogenesis in a chronic cerebral hypoperfusion model in rats.

METHODS A chronic cerebral hypoperfusion model induced by the permanent ligation of bilateral common carotid arteries (CCAs; a procedure herein referred to as “CCA occlusion” [CCAO]) in rats was employed in this study. Seven days after the CCAO procedure, the authors performed encephalo-myo-synangiosis (EMS) and injected plasmid(s) into each rat’s temporal muscle. Rats were divided into 4 groups based on which plasmid was received (i.e., LacZ group, VEGF group, apelin group, and VEGF+apelin group). Protein levels in the cortex and attached muscle were assessed with enzyme-linked immunosorbent assay (ELISA) on Day 7 after EMS, while immunofluorescent analysis of cortical vessels was performed on Day 14 after EMS.

RESULTS The total number of blood vessels in the cortex on Day 14 after EMS was significantly larger in the VEGF group and the VEGF+apelin group than in the LacZ group ($p < 0.05$, respectively). Larger vessels appeared in the VEGF+apelin group than in the other groups ($p < 0.05$, respectively). Apelin protein on Day 7 after EMS was not detected in the cortex for any of the groups. In the attached muscle, apelin protein was detected only in the apelin group and the VEGF+apelin group. Immunofluorescent analysis revealed that apelin and its receptor, APJ, were expressed on endothelial cells (ECs) 7 days after the CCAO.

CONCLUSIONS Combined gene therapy (VEGF plus apelin) during EMS in a chronic cerebral hypoperfusion model can enhance angiogenesis in rats. This treatment has the potential to be a feasible option in a clinical setting for patients with moyamoya disease.

<https://thejns.org/doi/abs/10.3171/2016.8.JNS16366>

KEY WORDS apelin; gene therapy; moyamoya disease; revascularization; vascular endothelial growth factor

MOYAMOYA disease (MMD) is a chronic, progressive cerebrovascular disease characterized by stenosis or occlusion of the bilateral supraclinoid internal carotid arteries and the development of an abnormal vascular network, called “moyamoya vessels,” at the base of the brain that blocks cerebral flow.²¹ Indirect bypass surgeries such as encephalo-myo-synangiosis (EMS)

are mostly performed in pediatric patients with MMD. This is because indirect bypass surgery often results in insufficient collateral circulation for most adult and some pediatric patients.^{20,25,27,32,39}

With the aim of improving collateral circulation after indirect bypass surgery, we previously investigated the effect of EMS combined with vascular endothelial growth

ABBREVIATIONS Ang-1 = angiopoietin-1; CBF = cerebral blood flow; CCA = common carotid artery; CCAO = CCA occlusion; EC = endothelial cells; ELISA = enzyme-linked immunosorbent assay; EMS = encephalo-myo-synangiosis; MMD = moyamoya disease; PBS = phosphate-buffered saline; PFA = paraformaldehyde; RECA-1 = mouse monoclonal anti-EC antibody; VEGF = vascular endothelial growth factor.

SUBMITTED February 17, 2016. **ACCEPTED** August 24, 2016.

INCLUDE WHEN CITING Published online December 23, 2016; DOI: 10.3171/2016.8.JNS16366.

factor (VEGF) gene administration into the temporal muscle in a chronic ischemia model in rats.^{15,22} Indirect bypass surgery for the treatment of patients with MMD was simulated by adding the EMS surgery to bilateral common carotid arteries (CCAs) ligation. The data demonstrated that EMS plus injection of human VEGF plasmid, compared with EMS alone, significantly increased angiogenesis in the cerebral cortex.^{15,22} However, VEGF overexpression can introduce a risk of immature vessel formation that results in plasma leakage and angioma formation.^{4,11,24,35}

Apelin has been identified as the endogenous ligand for APJ, an orphan G protein-coupled receptor that is expressed in the cardiovascular and central nervous systems.²⁹ The apelin/APJ system is involved in a wide range of physiological activities, such as heart contractility and blood pressure regulation,⁶ appetite and drinking behavior,²³ neuroprotection,³⁰ and angiogenesis.^{7,14,17,18,26} It has been reported that apelin plus VEGF effectively induced the formation of functional vessels that were larger than those induced with VEGF alone in a hindlimb ischemia model.¹⁷

In this study, we evaluated whether combined gene therapy with VEGF plus apelin during indirect vasoreconstructive surgery enhances brain angiogenesis in a chronic cerebral hypoperfusion model in rats.

Methods

Animals and Surgical Procedures

All animal procedures performed in this study were specifically approved by the Institutional Animal Care and Use Committee of Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences.

Adult male Wistar rats (9–11 weeks old, weighing 250–350 g) were used for the experiments. With the rats under general anesthesia via 2.0% halothane in a mixture of 40% oxygen and 60% nitrous oxide gas, the CCAs were carefully separated from the sympathetic and vagus nerves using a ventrocervical incision. Bilateral CCAs were ligated with 3-0 silk sutures (herein referred to as “CCA occlusion” [CCAO]). The rats’ body temperature was maintained close to 37°C throughout the procedure by using a heating pad. Sham operations involved a skin incision and exteriorization of the bilateral CCAs without ligation. An interval of 7 days was allowed for postoperative recovery (Fig. 1A).

Seven days after the CCAO procedure, some of the rats underwent EMS surgery (Fig. 1B). This 7-day waiting period between CCAO and EMS surgery was incorporated into the experimental design because CCAO reduces cerebral blood flow (CBF) to 35%–50% of control levels and because CBF starts to recover at 1 week.^{8,37} We thought that delaying EMS surgery beyond the initial CBF recovery period could have a negative effect on angiogenesis. This 7-day waiting period was also used in previous reports from other institutions developing similar models. While under general anesthesia via intraperitoneal administration of pentobarbital sodium (45 mg/kg), the rats were placed in a stereotactic apparatus with the top of the skull positioned horizontally. After a mid-

line linear incision was made, the right temporal muscle was detached from the temporal bone. Craniotomy was then performed in the temporoparietal region using a dental drill. The dura mater was carefully opened and removed with no disruption of the brain surface (Fig. 1C). The exposed brain surface was covered with the muscle flap (Fig. 1D). Transfection of plasmid into the temporal muscle was performed using GenomOne-Neo transfection reagent (Ishihara Sangyo) according to the manufacturer’s protocol. Rats were divided into 4 groups based on which plasmid(s) they received (i.e., LacZ group, VEGF group, apelin group, and VEGF+apelin group). Each rat received 25 µg of plasmid. In a previous study, we found a significant increase in capillary density simply by injecting 50 µg of naked VEGF plasmid (without use of transfection reagent) into the temporal muscle.²² Moreover, we performed an optimal dose analysis that demonstrated a maximal angiogenic effect occurring with a 100-µg dose of VEGF plasmid.¹⁵ As a reference, we used a previous study from another group comparing transfection efficacy between the injection of naked plasmids versus the use of GenomOne-Neo reagent. That study found a 4 times higher transfection efficiency using the GenomOne-Neo method.³⁸

Immunofluorescence

CCAO and sham rats were killed with an overdose of pentobarbital (100 mg/kg) 1 week after the CCAO or sham procedure (Fig. 1A); CCAO+EMS rats were killed 2 or 3 weeks after the CCAO procedure (1–2 weeks after EMS; Fig. 1B). The rats were perfused transcardially with 200 ml of cold phosphate-buffered saline (PBS) and 100 ml of 4% paraformaldehyde (PFA) in PBS. The brain and transfected temporal muscle were removed and postfixed in the same fixative overnight at 4°C and subsequently stored in 30% sucrose in PBS until completely submerged. Frozen coronal sections (17 µm thick) were cut from each specimen on a cryostat, and the sections were thaw mounted on slides. Slides from the CCAO or sham operation without EMS surgery were evaluated by immunofluorescent analysis of endothelial cells (ECs) and apelin/APJ protein. Slides from the CCAO+EMS rat tissue were evaluated with immunofluorescent analysis of ECs. There were 8 slides from each group. Sections that included cortical surfaces were photographed at 10× magnification. The total number of vessels per field, percentage of vessel area per field, and number of large (> 10 µm) vessels per field were calculated for each image using ImageJ software (National Institutes of Health).

For the immunofluorescent staining of ECs, after several rinses in PBS, the slides were incubated in 10% fetal bovine serum in PBS for 1 hour. The slides were then washed and incubated with an affinity-purified mouse monoclonal anti-EC antibody (RECA-1, Abcam) with 1% fetal bovine serum for 2 hours at room temperature. The slides were washed and then incubated for 1 hour with a Cy3 anti-mouse IgG antibody (1:200 dilution) at room temperature.

For the immunofluorescent staining of APJ and ECs, after several rinses in PBS, slides were incubated in 10% fetal bovine serum in PBS for 1 hour. The slides were then washed and incubated with RECA-1 and an affinity-puri-

FIG. 1. Time course of the experimental design and indirect bypass surgery. **A:** Scheme showing the experimental design for the CCAO model. **B:** Scheme showing the experimental design for the indirect bypass surgery model. **C:** Exposed brain after craniotomy in the right temporoparietal region. **D:** Fascia (*) attached to the remaining parietal bone and brain covered with the temporal muscle (**). IF = immunofluorescence.

fied rabbit polyclonal anti-APJ antibody with 1% fetal bovine serum for 2 hours at room temperature. Anti-APJ antibody was provided by Dr. Kidoya, which was described previously.¹⁸ The slides were washed and then incubated for 1 hour with a Cy3 anti-mouse IgG antibody (1:200 dilution) and Alexa Fluor anti-rabbit IgG antibody (1:200 dilution) at room temperature.

For the immunofluorescent staining of apelin and ECs, after several rinses in PBS, slides were incubated in 10% normal goat serum in PBS for 1 hour. The slides were then washed and incubated with an affinity-purified rabbit polyclonal anti-von Willebrand factor antibody and an affinity-purified mouse monoclonal anti-apelin antibody with 1% normal goat serum for 2 hours at room temperature. Anti-apelin antibody was provided by Dr. Kidoya, which was described previously.¹⁸ The slides were washed and incubated for 1 hour with an FITC anti-rabbit IgG antibody (1:300 dilution) and a Cy3 anti-mouse IgG antibody (1:300 dilution) at room temperature.

Enzyme-Linked Immunosorbent Assay Analyses

One week after EMS surgery, CCAO+EMS rats ($n = 2$ per group) anesthetized with an overdose of pentobarbital (100 mg/kg, intraperitoneal) were decapitated, and

their brains and muscle tissues were quickly harvested. Brain and muscle were sliced at a 2 mm thickness. Brain cortex was obtained using a biopsy punch (3-mm hole, Kai Corp. and Kai Industries Co., Ltd.). The tissues were then homogenized in T-PER Tissue Protein Extraction Reagent (ThermoFisher Scientific Inc.) and processed in a centrifuge at 10,000g for 10 minutes at 4°C, retaining the supernatant. VEGF and apelin protein levels were measured using human VEGF enzyme-linked immunosorbent assay (ELISA; Immuno-Biological Laboratories Co., Ltd) and apelin-12 ELISA kits (Phoenix Pharmaceuticals, Inc.).

Statistical Analyses

The total number of blood vessels and the VEGF, apelin, and LacZ protein levels were evaluated statistically using single ANOVA, with a subsequent post hoc Tukey-Kramer test. Statistical significance was set at $p < 0.05$.

Results

At the capillary level, the total number of blood vessels 2 weeks after EMS was significantly higher in the VEGF and VEGF+apelin groups than in the LacZ group

FIG. 2. Analysis of angiogenesis in an indirect bypass surgery model. **A:** RECA-1 staining of the cortex in the 4 groups. **B:** Quantitative evaluation of the number of vessels ($*p < 0.05$). **C:** Percentage of vessel area per field ($*p < 0.05$). **D:** Quantitative evaluation of the number of large ($> 10 \mu\text{m}$) vessels ($*p < 0.05$).

($p < 0.05$, respectively; Fig. 2A and B). The percentage of vessel area per field was significantly higher in the VEGF+apelin group than in the LacZ group ($p < 0.05$; Fig. 2C). Moreover, the number of large ($> 10 \mu\text{m}$) vessels in the VEGF+apelin group was significantly higher than the number found in the LacZ, VEGF, and apelin groups ($p < 0.05$, respectively; Fig. 2D).

Protein levels of VEGF and apelin in the attached muscle and cortex 1 week after EMS were evaluated in all 4 groups. VEGF protein levels in the attached muscle tended to be higher in the VEGF and VEGF+apelin groups than in the other groups, but the differences were not statistically significant ($p = 0.095$, single ANOVA). VEGF protein was not detected in the cortex for any of the groups (Fig. 3A). Apelin protein was detected in the attached muscle only in the apelin group and the VEGF+apelin group. Apelin protein was not detected in the cortex for any of the groups (Fig. 3B).

Immunohistochemical staining of brain tissue harvested 7 days after CCAO with monoclonal anti-apelin antibody and polyclonal anti-APJ antibody revealed that ECs stained by RECA-1 or von Willenbrand factor antibody in the cortex express apelin or APJ (Fig. 4).

Discussion

Indirect Bypass Surgery for Moyamoya Disease

Direct and/or indirect bypass surgery is often performed as a treatment for patients with MMD. Although direct bypass surgeries such as superficial temporal artery–middle cerebral artery anastomosis are frequently performed in adult and pediatric patients, they are sometimes difficult, especially in young children. Given that indirect bypass surgery is easy and simple to perform, it is a commonly used method to increase CBF in patients with MMD. Although indirect bypass surgeries are effective for pediatric and young adult patients with MMD, direct bypass surgery remains the main treatment option for most adults with MMD.²⁷ This is because indirect bypass surgery often results in insufficient collateral circulation for most adult and some pediatric patients.^{20,25,27,32,39} An endogenous angiogenic factor may be involved in the induction of blood vessel growth associated with collateral circulation. Park et al. demonstrated that the genotype of the VEGF allele was related to better collateral vessel formation after bypass surgery in patients with MMD.³³ These data indicate that the addition of an exogenous an-

FIG. 3. ELISA results for human VEGF and apelin. **A:** Human VEGF levels in the cortex and attached muscle 1 week after indirect bypass surgery and injection of plasmids. **B:** Apelin levels in the cortex and attached muscle 1 week after indirect bypass surgery and injection of plasmids. N.D. = not detected.

giogenic factor to indirect bypass surgery could enhance the number and growth of collateral vessels.

Gene Therapy in a Chronic Cerebral Hypoperfusion Model

In this report, we confirmed that the total number of blood vessels in the cortex was significantly larger in

the VEGF group and the VEGF+apelin group than in the LacZ group. Moreover, larger vessels were induced in the VEGF+apelin group than in the other groups. In the hypoperfusion model, indirect bypass surgery without angiogenic factors could increase the number of vessels, and the presence of additional angiogenic factors could

FIG. 4. Immunohistochemical analysis of cortex tissue from CCAO and sham-operated (sham) rats. **A:** Immunohistochemical staining using anti-apelin antibody. **B:** Immunohistochemical staining using anti-APJ antibody. vWF = anti-von Willebrand factor antibody.

lead to a further increase.^{1,2,12,15,19,22,31} Hecht et al. showed that combining EMS with VEGF-expressing myoblasts in mice that had undergone unilateral internal carotid artery ligation improved not only the vessel density of the cortex but also the cerebrovascular reserve capacity.¹² However, similar reports investigating vessel caliber size are limited. Ohmori et al. reported that combining encephaloleosynangiosis (EGS) with the intramuscular injection of recombinant granulocyte–colony stimulating factor (G-CSF) in hypoperfusion rats increased the number of smaller vessels.³¹ We thought that the increase in the number of larger vessels was an important factor in mature angiogenic development. Kidoya et al. reported that the apelin/APJ system spatially and temporally modulates enlargement of vessel diameter during embryogenesis.¹⁸ Several reports suggest that apelin expression is induced in ECs during hypoxia.^{7,14,17,18} The mechanism by which enlarged blood vessels are produced by the apelin/APJ system has been described by Takakura and Kidoya.³⁶ VEGF and angiopoietin-1 (Ang-1) induce expression of APJ and apelin on ECs. Under the expression of VEGF, apelin promotes the aggregation and proliferation of ECs, which lead to mature angiogenesis, such as caliber size enlargement. When caliber size becomes sufficient to supply oxygen to the tissue, angiogenesis factors will be downregulated and the regulation of caliber size is completed.

Newly formed vessels induced by overexpression of VEGF alone can be immature and are at risk for tissue edema¹¹ or hemangioma.³⁵ Therefore, several groups have reported on the use of combined gene therapy, namely VEGF plus Ang-1 for ischemic hindlimb models, myocardial infarction models, or acute cerebral ischemia models and VEGF plus apelin for ischemic hindlimb models.^{3,5,17,34,40,41} The merits of VEGF plus apelin combined gene therapy were reported to include increases in vessel density and the maturation of newly developed vessels, such as larger vessel formation and low permeability.³⁶ Some studies have reported that VEGF-mediated permeability occurs through the disorganization of endothelial junction proteins, such as vascular endothelial (VE)-cadherin.^{10,16} It was also reported that apelin inhibited the down-modulation of VE-cadherin by VEGF, resulting in the suppression of hyperpermeability.¹⁷ Although we observed the formation of larger vessels, we cannot confirm the suppression of hyperpermeability in the VEGF+apelin group. We think that when we add gene therapy to the indirect bypass method, the development of mature, newly formed vessels due to the bypass surgery itself may lead to a reduction in adverse effects and an increase in CBF.

In the current study, secreted proteins were detected only in muscles, and this has also been described in a previous report by our group.²² We thought that the injection of VEGF plus apelin plasmids into the muscle would increase angiogenesis not only in the muscle but also in the muscle-cortex interface. Then, the transpial vessel sprouted into the cortex. Similar models have demonstrated this mechanism.^{12,28} In particular, Nakamura et al. showed the mechanism of revascularization in an experimental model using EMS in pigs.²⁸ During cerebral ischemia, the infiltration of inflammatory cells between the temporal muscle

and the arachnoid membrane induced angiogenesis and led to revascularization between the external cerebral artery and the cerebral cortex artery.

Study Limitations

This study has several limitations. First, the observational periods of the immunohistochemical analyses and protein assay were short. Our previous studies and similar studies from other institutions have reported results from evaluations between 2 and 4 weeks.^{1,12,15,19,22,31} In general, angiogenesis occurs over several months after indirect bypass surgery in patients with MMD. Future studies need to analyze collateral formation and protein levels in this model over a longer period.

Second, we could not conduct behavioral assessments in our model. Cognitive impairment can occur in patients with MMD.^{9,13} Assessing the correlation between development of collateral formation and change in cognitive function in this model is desirable.

Third, we did not measure vessel dysfunction, such as breakdown of the blood-brain barrier, vessel leakiness, tight junction protein assessment, or brain edema.

Fourth, we could not conduct blood flow measurements. In previous reports by other groups, CCAO in rats reduced CBF to 35%–50% of control levels.^{8,37} In the future, we need to measure blood flow to assess the effect of enhanced angiogenesis.

Therefore, further analyses such as those described above are needed before applying the described technique in the clinical setting.

Conclusions

Combining EMS with gene therapy (VEGF plus apelin) in a chronic cerebral hypoperfusion model in rats can enhance mature angiogenesis. It could be a feasible treatment option in the clinical setting for patients with MMD.

Acknowledgments

We thank Dr. H. Kidoya for supplying VEGF, apelin, and LacZ plasmids and Dr. H. Michiue, Ms. A. Ueda, Dr. T. Ohashi, Ms. A. Machara, Ms. M. Arao, and Ms. N. Uemori for technical assistance.

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Disclosures

The authors report no conflict of interest concerning the materi-

als or methods used in this study or the findings specified in this paper.

Author Contributions

Conception and design: Hiramatsu, Tokunaga, Sugiu, Date. Acquisition of data: Hiramatsu, Nishihiro. Analysis and interpretation of data: Hiramatsu, Nishihiro, Haruma, Shimizu, Takasugi, Shinji. Drafting the article: Hiramatsu, Hishikawa, Date. Critically revising the article: Hiramatsu, Hishikawa. Reviewed submitted version of manuscript: all authors. Approved the final version of the manuscript on behalf of all authors: Hiramatsu. Statistical analysis: Hiramatsu. Administrative/technical/material support: Kidoya, Takakura. Study supervision: Hishikawa, Tokunaga, Kidoya, Sugiu, Takakura.

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