Enhanced brain angiogenesis in chronic cerebral hypoperfusion after administration of plasmid human vascular endothelial growth factor in combination with indirect vasoreconstructive surgery

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Object. Vascular endothelial growth factor (VEGF) is a secreted mitogen associated with angiogenesis. The conceptual basis for therapeutic angiogenesis after plasmid human VEGF gene (phVEGF) transfer has been established in patients presenting with limb ischemia and myocardial infarction. The authors hypothesized that overexpression of VEGF using a gene transfer method combined with indirect vasoreconstruction might induce effective brain angiogenesis in chronic cerebral hypoperfusion, leading to prevention of ischemic attacks.

Methods. A chronic cerebral hypoperfusion model induced by permanent ligation of both common carotid arteries in rats was used in this investigation. Seven days after induction of cerebral hypoperfusion, encephalomyosynangiosis (EMS) and phVEGF administration in the temporal muscle were performed. Fourteen days after treatment, the VEGF gene therapy group displayed numbers and areas of capillary vessels in temporal muscles that were 2.2 and 2.5 times greater, respectively, in comparison with the control group. In the brain, the number and area of capillary vessels in the group treated with the VEGF gene were 1.5 and 1.8 times greater, respectively, relative to the control group.

Conclusions. In rat models of chronic cerebral hypoperfusion, administration of phVEGF combined with indirect vasoreconstructive surgery significantly increased capillary density in the brain. The authors’ results indicate that administration of phVEGF may be an effective therapy in patients with chronic cerebral hypoperfusion, such as those with moyamoya disease.

Key Words • chronic cerebral hypoperfusion • moyamoya disease • angiogenesis • vascular endothelial growth factor • indirect vasoreconstruction • rat

Moyamoya disease is a rare and chronic cerebral hypoperfusion condition characterized by progressive stenosis of the internal carotid artery and its distal arteries. Various surgical approaches have been suggested; moreover, direct and/or indirect vasoreconstructive surgical protocols are widely accepted as effective treatments for ischemic-type moyamoya disease. Indirect bypass surgery can create transmuscular and/or transdural anastomosis via external arteries to the hypoperfused brain. We originally performed surgery in these patients by using Ribbon encephaloduroarteriomyosynangiosis; many patients were treated successfully. In some patients, however, this treatment failed to provide optimal revascularization from the perspective of collateral formation.

Angiogenesis, the formation of new blood vessels, occurs in a wide range of developmental, physiological, and pathological processes. Among the numerous polypeptide growth factors associated with angiogenesis, VEGF has become the focus of much interest because it is a mitogen specific for endothelial cells. Results of previous investigations have indicated that VEGF administration stimulates development of collateral arteries and reduces tissue injury in animal models of hind limb ischemia and myocardial infarction. Results of several clinical studies have also indicated that administration of phVEGF is therapeutically beneficial through the potentiation of angiogenesis and enhancement of collateral blood flow in patients presenting with myocardial and limb ischemia.

The effect of angiogenesis therapy on chronically hypoperfused brain tissue has not been examined. In the current study we evaluated whether phVEGF administration combined with indirect vasoreconstructive surgery enhances brain angiogenesis in a chronic cerebral hypoperfusion model in rats.

Materials and Methods

Plasmid DNA (phVEGF)

Plasmids from cultures of phVEGF-transformed Escherichia coli...
Angiogenic effect of phVEGF in brains with chronic hypoperfusion

coli were used (Fig. 1). Preparation and purification of the plasmid were conducted using the column method (Quagen Mega Kit; Qiagen, Inc., Valencia, CA).

Chronic Cerebral Hypoperfusion Model

All experimental procedures performed in this investigation were in accordance with the institutional guidelines of Okayama University Graduate School of Medicine and Dentistry. Male Wistar rats (Japan Clea, Inc., Tokyo, Japan) 9 to 11 weeks of age were anesthetized with pentobarbital sodium (45 mg/kg, administered intraperitoneally). Using a ventrocaudal incision, the CCAs were carefully separated from the sympathetic and vagal nerves. Both CCAs were doubly ligated with 3-0 silk sutures; body temperature in the rats was maintained close to 37°C throughout the procedure by using a heating pad. An interval of 7 days was allowed for postoperative recovery.

Operative Procedure of EMS and phVEGF<sub>astro</sub> Administration

Seven days after induction of cerebral hypoperfusion, the rats were placed in a stereotactic apparatus with the top of the skull positioned horizontally following the induction of anesthesia with pentobarbital sodium (45 mg/kg, administered intraperitoneally). The right temporal muscle was detached from the temporal bone through a linear incision. After craniectomy was performed in the temporoparietal region by using a dental drill, the dura mater was carefully opened and removed with no disruption of the brain surface. Fifty micrograms of phVEGF<sub>astro</sub> in 0.1 ml of 0.9% saline (0.5 μg/μl) was injected slowly into the temporal muscle of 19 rats at three different sites by using a 50-μl microsyringe (Hamilton, Inc., Reno, NV). An equal volume of 0.9% saline was administered to 13 rats serving as the control group. After the injections, the exposed brain surface was covered with the muscle flap to imitate surgery for EMS caused by moyamoya disease and the skin was closed with 3-0 silk sutures.

Immunohistochemical Analysis

Animals were deeply anesthetized and perfused with 100 to 200 ml of PBS with a pH of 7.4, followed by 200 ml of 4% paraformaldehyde 4 days after administration of the plasmid. Brain and transfected temporal muscle tissue were removed and fixed with 4% paraformaldehyde; subsequently, the tissues were bathed sequentially in 10, 15, 20, and 30% sucrose. Tissues were embedded in optimal cutting temperature compound and snap frozen in liquid nitrogen. Frozen coronal sections (10 μm thick) were cut from each specimen on a cryostat. The sections were thaw-mounted on slides and stored at −80°C. Next, the sections were rehydrated and washed three times in 1 × PBS (pH 7.4). Slides were exposed to 0.3% H<sub>2</sub>O<sub>2</sub> and 70% methanol for 30 minutes to quench endogenous peroxidase activity. Three additional washes in 1 × PBS were performed prior to incubation in 10% blocking serum in 1 × PBS for 20 minutes. The slides were incubated with an affinity-purified monoclonal rabbit anti-human VEGF antibody (1:50; Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. Slides were washed and incubated for 30 minutes with a biotinylated anti–rabbit antibody at 1:200 dilution. Subsequently, slides were incubated with avidin–horseradish peroxidase–linked anti–rabbit antibody (Amersham Biosciences Corp., Piscataway, NJ). Protein bands were detected by using an enhanced chemiluminescence method (ECL Plus kit; Amersham). Protein levels on Western blots were quantified by densitometry.

Capillary Density and Histological Study

The effect of VEGF on collateral vessel formation was evaluated with a perfusion technique in which India ink was used, as described previously (with some modifications). Fourteen days after administration of the phVEGF<sub>astro</sub> animals were deeply anesthetized with sodium pentobarbital and perfused through the ascending aorta with 200 ml of PBS followed by 200 ml of India ink mixed with 4% paraformaldehyde (1:1). (There were eight control animals and 12 VEGF gene–treated animals.) Brain and temporal muscle tissue on the treated side were removed and fixed for 24 hours with 4% paraformaldehyde, dehydrated in a graded ethanol series, and embedded in paraffin. Ten coronal sections were prepared from each animal. Sections were mounted on glass slides and examined by light microscopy. A total of two different fields (each brain and muscle) from one section were randomly photographed under magnification (×100) and the images were analyzed using commercially available software (Photoshop version 5.0, Palo Alto, CA). Computerized analysis permitted the assessment of capillary density in a selected field occupied by microvessels filled with India ink. The area and number of capillaries per field were calculated from the images with the National Institutes of Health Image software program (version 1.55; Bethesda, MD). Some coronal sections were stained with H & E, and their microscopic histological views were observed.

Statistical Analysis

Results are expressed as the means ± standard deviations. Statistical significance was evaluated with the Student t-test. A probability value less than 0.05 indicated statistical significance.

Results

Expression of VEGF Protein in Brain and Muscle

Immunohistochemical staining of brain and temporal muscle with monoclonal anti-VEGF antibody at 4 days following administration of phVEGF<sub>astro</sub> revealed significant immunoreactivity in VEGF gene–treated muscles (Fig. 2). On the other hand, immunoreactive VEGF was not ob-

Fig. 1. Schematic illustrating phVEGF<sub>astro</sub>. Amp = ampicillin-resistant gene; BGH polyA = bovine growth hormone polyA; CMV = cytomegalovirus promoter; ori = Escherichia coli origin; SV40 = simian virus 40.
served in either VEGF gene–treated or control brain tissue. Western blotting with monoclonal anti-VEGF antibody demonstrated induction of VEGF protein on administration of phVEGF165 in the temporal muscle; in contrast, VEGF expression was not induced in the brain (Fig. 3). As shown in Fig. 3, elevated VEGF expression is evident in VEGF gene–treated muscles when compared with muscles of control samples. On the other hand, no apparent changes in the level of VEGF expression were detected between VEGF gene–treated and control brains. Our results indicated that
induction and expression of VEGF protein after administration of phVEGF<sub>165</sub> was limited to transfected temporal muscle.

**Capillary Density of Brain and Muscle**

Fourteen days after phVEGF<sub>165</sub> administration, favorable effects of phVEGF<sub>165</sub> gene transfer on angiogenesis were apparent at the capillary level. The capillary density of phVEGF<sub>165</sub>-transfected rats displayed an obvious increase in temporal muscle and brain tissue (Fig. 4). In the temporal muscles of the VEGF gene–treated rats, the number of capillaries was 2.2 times greater than those in the control group (451 ± 22.3 compared with 208 ± 64.6 per area; p < 0.01). Capillary area increased in the VEGF gene–treated group compared with controls (VEGF = 25,835 ± 11,239.4, control = 10,045.3 ± 3976.8; p < 0.01). Analysis of capillary density in brain samples yielded similar results (1.5 times in number of capillaries, VEGF = 256 ± 78.9, control = 164 ± 46.7, p < 0.05; area of capillaries: VEGF = 9887.6 ± 2309, control = 5518.4 ± 2173.8; p < 0.05).

**Histological Changes in Brain and Muscle**

Light microscopy revealed significant angiogenic effects in VEGF gene–treated muscle and brain tissue at 14 days following phVEGF<sub>165</sub> administration, which induced an apparent increase in capillaries in comparison with controls (Fig. 5). Theoretically, it is possible that VEGF may increase vascular permeability and cause brain edema; however, H & E staining demonstrated no apparent brain edema (Fig. 6).

**Discussion**

Moyamoya disease involves chronic cerebral hypoperfusion characterized by progressive narrowing or obstruction of major intracranial cerebral arteries. Vasoreconstructive surgery is an accepted treatment for this disease. Several surgical approaches have been proposed: direct bypass (STA–MCA), indirect bypass (EMS), encephaloduroarteriovenous graft, and a combination of direct and indirect bypass modalities. Indirect bypass techniques have been shown to foster satisfactory neovascularization through the external arteries to the hypoperfused brain and to reduce the risk of cerebral ischemia. Data from previous studies also demonstrated that the combination of STA–MCA bypass and indirect bypass surgery was superior to the sole use of indirect bypass surgery both in the development of collateral circulation and in postoperative clinical improvement. It is important to note that the STA–MCA bypass procedure is occasionally difficult to perform and is particularly challenging in pediatric patients. In addition, the procedure may increase the risk of perioperative ischemia. Many patients have been treated successfully by using only indirect bypass. In some patients, however, indirect bypass failed to obtain optimal effects in collateral vessel formation. Pre-
vention of mental deterioration and achievement of an overall good outcome in patients receiving only indirect bypass surgery depend on the collateral blood flow from the external arteries. In patients undergoing only indirect bypass surgery, administration of substances responsible for angiogenesis, such as VEGF, may have a beneficial effect on postoperative clinical improvement arising from the development of collateral vessels.

The secreted mitogen VEGF plays important roles in angiogenesis, the maintenance of cerebral vasculature, and permeability in many organ systems. As a result of alternative splicing, four different isoforms (VEGF_{165}, VEGF_{189}, VEGF_{121}, and VEGF_{206}) exist in vivo. Data from previous studies revealed that VEGF_{165} is the most abundant form in the brains of healthy rats and VEGF_{121} is the second most abundant. Isoforms VEGF_{165} and VEGF_{121} were also induced by focal ischemia in rat brains. On the other hand, VEGF_{206} and VEGF_{189} are relatively rare in rat brain and do not produce mitogenic activity in vascular endothelial cells. Therefore, VEGF_{165} and VEGF_{121} may function in a more important capacity in an ischemic brain. The VEGF receptors are associated exclusively with the early development of vascular patterns and are significantly downregulated in the adult brain. The potential role of VEGF in postnatal brain development is unclear; however, it has been suggested that after its initial activity in early

![Image](152x310 to 522x753)

**Fig. 5.** Light photomicrographs of rat muscle and brain tissue perfused with India ink 14 days after phVEGF_{165} administration. A and B: Control group, muscle and brain tissue, respectively. C and D: The VEGF gene–treated group, muscle and brain tissue, respectively. An apparent increase in capillary number is observed in the VEGF gene–treated group. The upper portion of the photographs in panels B and D corresponds to the cerebral cortex. Original magnification × 100.
vasculogenesis. VEGF and/or its receptors may be produced in response to tissue insults that require wound healing and angiogenesis. For example, the results of a study involving adult mouse brains demonstrated that glial cells respond to hypoxia by elevating production of VEGF. Significant increases in vascular density have been noted throughout postnatal development of the cortex in hypoxic-reared rats in comparison with normoxic animals. The areas of the brain in which neurons tend to survive longer are identical to those areas shown to be highly angiogenic. Angiogenesis may promote ischemic brain plasticity, thereby improving functional neurological outcome.

Angiogenic effect of phVEGF in brains with chronic hypoperfusion

![Image](https://example.com/image.png)

**Fig. 6.** Light photomicrographs of rat brain tissue at 14 days after phVEGF administration. A and B: Control group, muscle and brain tissue, respectively. H & E. C and D: The VEGF gene–treated group, muscle and brain tissue, respectively. Dots stained dark blue indicate capillaries (counterstained with H & E). No apparent brain edema was observed. Original magnification ×400.
to its effect on angiogenesis, VEGF may have a direct effect on neuronal plasticity consequent to its neurotrophic activity and through stimulation of axonal outgrowth.12,36,38

The conceptual basis for therapeutic angiogenesis after phVEGF<sub>165</sub> gene transfer has been established previously.44,45,46 Other investigations have disclosed convincing evidence of transgene expression after direct injection of a nonviral, covalently closed plasmid DNA into skeletal muscle.3,7,23,39,42,48 In animal models of hind limb ischemia and myocardial infarction, the application of VEGF<sub>165</sub> improved blood flow and achieved a therapeutic modulation of vascular insufficiency.2-4,43,52 Subsequently, other clinical studies have also demonstrated that the administration of phVEGF<sub>165</sub> is therapeutically beneficial in terms of potentiation of angiogenesis and the enhancement of collateral blood flow in patients presenting with myocardial and limb ischemia.18,42 For the sake of safety and efficacy, predominant local production of VEGF resulting from the transgene may be preferable to the administration of a single large dose of recombinant protein.3

The angiogenic effect of VEGF on the ischemic brain is poorly understood. At the acute stage, local application of VEGF<sub>165</sub> in transient ischemic rat brains subjected to 90 minutes of MCA occlusion resulted in the significant reduction of infarct volume.21 Zhang et al.53 reported that late (48 hours posts ischemic incident) intravenous infusion of VEGF<sub>165</sub> enhanced angiogenesis in a rat model of focal cerebral embolic ischemia, thus improving neurological recovery. In contrast, early (1 hour after the ischemic incident) administration of VEGF<sub>165</sub> exacerbated blood–brain barrier leakage and induced brain edema. Whether administration of VEGF enhances angiogenesis in a chronically hypoperfused brain has not been examined; furthermore, no appropriate animal model for moyamoya disease exists at present. Therefore, we simulated EMS surgery by using rats in a chronic cerebral hypoperfusion model.34-36,44,46,48,52 Permanent ligation of both CCAs led to a significant reduction in cerebral blood flow, even at 12 weeks postsurgery.35,47-49 Because chronic cerebral hypoperfusion is a distinguishing feature of moyamoya disease, this animal model may be useful for further investigation of this disease.

Despite the effect of VEGF in enhancing vascular permeability, VEGF application significantly reduced edema formation in rat brains following transient ischemia.21 Numerous differences may exist between brain edema associated with brain tumors and that associated with brain infarctions; moreover, VEGF may not be primarily involved in ischemic brain edema formation.6,9,13 The fact that VEGF increases vascular permeability might exacerbate brain edema and present complications related to its therapeutic use in the brain. Indeed, a preliminary study in monkeys revealed that the direct application of encapsulated phVEGF<sub>165</sub> in a normal monkey brain induced severe brain edema (data not shown); however, the findings in the present study demonstrated no apparent brain edema caused by local application of this factor to the temporal muscle. The indirect effect of phVEGF<sub>165</sub> on the brain is an advantage of this methodology; local application of VEGF at the bordering muscle might be safer than direct administration in the brain.

Using immunohistochemical analysis, induction and expression of VEGF protein by the site-specific administration of phVEGF was not observed in the brain and was limited to the transfected temporal muscle. Nevertheless, a significant increase in capillary density was observed in the muscle as well as in the brain. These results of the present study indicate that intramuscular administration of phVEGF<sub>165</sub> can significantly increase newly formed capillaries in transfected muscle, and that transmuscular anastomosis develops in the bordering, chronically hypoperfused brain through the temporal muscle.

Several topics related to this study must be investigated further. First, a more detailed study of cerebral blood flow conducted using single-photon emission computerized tomography is warranted. Second, a comparison of indirect and direct application of phVEGF<sub>165</sub> in the brain should be evaluated. Third, functional recovery of the brain after the use of the plasmid and its influence on neurons should be examined. Fourth, these findings are preliminary and the long-term safety of phVEGF action on the brain still needs to be investigated. Fifth, dosing strategies and/or alternative vector systems, such as adenovirus, must be evaluated. Our phVEGF regimen is simple, effective, and may be suitable for clinical application.

Conclusions

In a chronic cerebral hypoperfusion model in rats, the administration of phVEGF<sub>165</sub>, combined with indirect vasoreconstructive surgery significantly increased capillary density in the brain. In the future, the indirect administration of phVEGF<sub>165</sub> in the brain may prove useful in the treatment of selected patients presenting with chronic brain ischemia, such as those with moyamoya disease.

References

Angiogenic effect of phVEGF in brains with chronic hypoperfusion


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