Optimal Dose of Plasmid Vascular Endothelial Growth Factor for Enhancement of Angiogenesis in the Rat Brain Ischemia Model

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Abstract

Vascular endothelial growth factor (VEGF) administration has recently been assessed as a therapeutic strategy for ischemic diseases including brain ischemia because of its angiogenic effect. However, VEGF also causes detrimental adverse effects by increasing vascular permeability. This study examined whether plasmid human VEGF (phVEGF) administration induced angiogenic effects in the rat brain ischemia model caused by permanent ligation of both common carotid arteries, and investigated the occurrence of adverse effects. Administration of various doses (0–200 μg) of phVEGF in the temporal muscle was followed by encephalo-myo-synangiosis. Thirty days after treatment, the numbers and areas of capillaries per field in the extracted brains were analyzed with the National Institutes of Health Image software program. The maximal angiogenic effect occurred with a 100 μg dose of phVEGF in the numbers and areas of capillaries in the VEGF-treated brains. Histological examination showed no apparent adverse effects in the brain parenchyma even at the highest administration dose (200 μg) of phVEGF. The maximal angiogenic effect at the optimal dose of phVEGF can be considered under the threshold to cause serious adverse effects in the rat brain.

Key words: vascular endothelial growth factor, brain ischemia, angiogenic effect, rat brain model, indirect vasoreconstruction

Introduction

Vascular endothelial growth factor (VEGF), an endothelial cell-specific mitogen, is one of the most widely examined promoters of neovascularization.6,18) VEGF induces angiogenesis, but also causes detrimental adverse effects by increasing vascular permeability.4,5,7,8,14) Recently, clinical studies have demonstrated the therapeutic benefits of VEGF-gene administration to induce collateral vessel formation in patients with some types of ischemic diseases.3,11,12,16,23) Many experimental studies on VEGF administration have shown effectiveness against brain ischemia in various models.9,10,15,17,19,21,22,24–26) VEGF was administered by various methods, including VEGF protein,24) plasmid-containing VEGF complementary deoxyribonucleic acid (cDNA),13) adeno-associated viral vector-mediated VEGF cDNA,15,21) VEGF gene-transformed bone marrow stromal cells engineered with a herpes simplex virus,17) encapsulated transformed kidney cells-producing VEGF,25) and others. We previously reported that administration of plasmid human VEGF (phVEGF) combined with indirect vasoreconstructive surgery significantly increased angiogenesis in the rat ischemia model.13) VEGF also increases vascular permeability leading to brain edema,9,15,20,25,26) so might cause adverse effects including brain edema, resulting in complications related to the clinical use of phVEGF.15,20,25) Therefore, evaluation of the most effective but safe dose of phVEGF is critical point for clinical application of phVEGF in human patients with brain ischemia. However, no in vivo studies have examined the angiogenic efficacy and adverse effects including
brain edema depending on the administration dose of phVEGF.

This study examined whether phVEGF administration induced angiogenic effects in the rat ischemic brain, and the incidence of severe adverse effects.

**Materials and Methods**

Four different isoforms (VEGF_{206}, VEGF_{189}, VEGF_{165}, and VEGF_{121}) of VEGF exist in vivo as a result of alternative splicing. VEGF_{165} is the most abundant form and VEGF_{121} is the second most abundant in the brain. VEGF_{206} and VEGF_{189} are rarely expressed in the brain, and do not produce mitogenic activity. Therefore, VEGF_{165} cDNA was employed for this rat brain model. Expression plasmid was schematically illustrated in our previous study. In brief, the plasmid (named phVEGF) contains the full length of human VEGF_{165} cDNA derived from cytomegalovirus promoter, ampicillin-resistant gene, bovine growth hormone polyA, simian virus 40, and Escherichia coli origin. Plasmids from cultures of phVEGF-transformed Escherichia coli were utilized. Purification and quantification of the plasmids were conducted by the column method using a commercial kit (Qiagen Mega Kit; Qiagen, Inc., Valencia, Calif., U.S.A.).

All experimental procedures in this study were governed by the institutional guidelines of Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences. Male Wistar rats (Japan Clea, Inc., Tokyo) aged 9 to 11 weeks were anesthetized with intraperitoneal pentobarbital sodium (45 mg/kg). Following ventriculocervical incision, both common carotid arteries were carefully separated from the sympathetic and vagal nerves and doubly ligated with 3-0 silk sutures. Body temperature was maintained near 37°C throughout the procedure with a heating pad.

Encephalo-myo-synangiosis (EMS) and phVEGF administration in the temporal muscle were performed as described previously. In brief, 7 days after induction of cerebral hypoperfusion, the rat was placed in a stereotactic apparatus in which the top of the skull was fixed in a horizontal position under intraperitoneal pentobarbital sodium anesthesia (45 mg/kg). The right temporal muscle was detached from the temporal bone through a linear incision. Cranietomy was performed in the temporo-parietal region with a dental drill, and the dura mater was carefully opened and removed without disruption of the brain surface. phVEGF was injected slowly into the temporal muscle at three sites in a line at 5-mm intervals with a 50-μl microsyringe (Hamilton, Inc., Reno, N.Y., U.S.A.). A total volume of 0.1 ml of 0.9% saline containing phVEGF (25, 50, 75, 100, 125, 150, 175, and 200 μg) was delivered to each of 12 rats. A volume of 0.1 ml of 0.9% saline was administered to 16 control rats. The treated temporal muscle was directly attached to the brain surface by primary su- ture of the temporal muscle and skin. The rats were then maintained for 30 days to allow angiogenesis in the ischemic brain.

The effect of VEGF on collateral vessel formation was evaluated by histological examination. Thirty days after phVEGF administration, animals were deeply anesthetized with sodium pentobarbital and perfused through the ascending aorta with 200 ml of phosphate-buffered saline followed by 200 ml of 4% paraformaldehyde containing Indiana dye which only stains vessels including capillaries. The rat whole brain and temporal muscle tissues were removed and further fixed for 24 hours with 4% paraformaldehyde, dehydrated in a graded ethanol series, and embedded in paraffin. Ten coronal sections were prepared for each animal, mounted on glass slides. Assessment of capillary density filled with dye solution used computerized analysis. Photomicrographs were analyzed using commercially available software (Photoshop version 5.0; Adobe Systems Inc., San Jose, Calif., U.S.A.). The number and the area of capillaries per field were calculated from the images with the National Institutes of Health Image software program (version 1.55; Bethesda, Md., U.S.A.). Sections were also stained with hematoxylin and eosin (HE), and examined for abnormalities.

Calculated data are expressed as the mean ± standard deviation. Statistical significance was evaluated with a statistical software using analysis of variance and the Steel-Dwass (non-parametric) method for the multiple comparison test. A probability value of less than 0.01 indicated statistical significance.

**Results**

The numbers and areas of capillaries in the temporal muscles treated with phVEGF were increased compared with the control groups (Fig. 1). For example, the number of capillaries in the temporal muscle of the 100 μg phVEGF group was 2.7 times greater than that in the control group (623.3 ± 109.4 vs. 232.7 ± 61.6 counts/area, p < 0.01) (Figs. 1 and 2). The capillary area of the 100 μg phVEGF group also significantly increased compared to the control group (71647 ± 7520.1 vs. 14756 ± 3461.4 pixels/area, p < 0.01). All phVEGF groups displayed significantly higher capillary numbers and areas in the temporal muscles compared to the control group. However, phVEGF administration at doses higher than 100 μg demonstrated less angiogenic effect, showing that
Fig. 1 Photomicrographs of the rat temporal muscles perfused with paraformaldehyde and Indian dye solution at 30 days after plasmid human vascular endothelial growth factor (phVEGF) administration. ×100. A: Control group without operation. B: Control group with encephalo-myo-synangiosis. C: Group treated with 100 μg of phVEGF. D: Group treated with 200 μg of phVEGF. Bar = 120 μm.

the maximal angiogenic effect was obtained with 100 μg of phVEGF in the temporal muscles.

The capillary density in the ipsilateral brain was increased by EMS compared to contralateral side (Fig. 3). phVEGF administration further increased the numbers and areas of capillaries in the brain compared to the control group (only EMS treatment group) (Fig. 3). For example, the number of capillaries calculated in the brains of the 100 μg phVEGF group was 3.0 times greater than that in the control group (515.2 ± 85.2 vs. 170 ± 42.4 counts/area, p < 0.01) (Figs. 2 and 3). The capillary area of the 100 μg phVEGF group also significantly increased compared to the control group (39585 ± 4661.7 vs. 7287 ± 2015.4 pixels/area, p < 0.001). All phVEGF groups displayed significantly higher capillary numbers and areas in the brain compared to the control group. However, phVEGF administration at doses higher than 100 μg demonstrated less angiogenic effect, so that the maximal angiogenic effect was obtained with 100 μg of phVEGF in the brain, as observed in the temporal muscle.

The rats maintained for 30 days after treatment retained body weight, looked healthy, and lived until sacrifice without neurological deficits. The operated areas of their heads were not infected and no ulceration or alopecia occurred. These findings suggested that the combined therapy of EMS and phVEGF administration did not affect general or local conditions. Examination of HE-stained sections found newly formed capillaries mainly in the areas near the brain surface attached to the temporal muscles. Our preliminary study found EMS caused no side effects on the rat brain (data not shown). Histological examination searched for edema caused no side effects in the brain surface caused by the initial operation in some rats, but no adverse effects in the brain parenchyma caused by phVEGF or VEGF protein (Fig. 4). The brain sections from the 200 μg phVEGF groups demonstrated the same histological findings as those of the control group.

Discussion

Initially, we had hypothesized that higher phVEGF administration dose would cause greater angiogenic effect in a dose dependent manner. On the contrary, our findings suggested that excessive doses of phVEGF diminished the angiogenic effect in the rat brain. Since fresh tissues of temporal muscle and brain were not obtained (tissues were fixed with paraformaldehyde), we could not perform quantitative analyses of expressed VEGF messenger ribonucleic acid or protein using Northern blotting or Western blotting, respectively. Therefore, we could not confirm the reasons for this phenomenon. Some previous reports mentioned the same tendency. Medium (not low and not high) doses of intranasal VEGF administration were the most effective for reducing infarct volume, improving behavioral recovery, and enhancing angiogenesis in stroke brain.24) A therapeutic window was identified in one rat brain model, because chronic intraventricular infusion of VEGF at a concentration of 5 μg/ml (among concentrations of 1 to 25 μg/ml) resulted in a significant increase in vessel density with minimal associated brain edema and no ventriculomegaly.9) Intracerebral administration of low-dose (not high-dose) VEGF through encapsulated grafts of VEGF-producing cells produced neuroprotective and angiogenic effects.25) These findings suggest that there is an optimum dose of VEGF or VEGF gene to obtain the highest angiogenic effect for brain ischemia. Because the ischemic models were different in each study, adequate doses of administration were different in each model. In our model, administration of 100 μg phVEGF provided the highest angiogenic effect for the ischemic rat brain. The mechanism causing the reduced effect of excessive VEGF for ischemic brains has not been identified. Further studies are needed at the molecu-
Fig. 2 Relationship between plasmid human vascular endothelial growth factor (phVEGF) administration dose and density of capillaries in the temporal muscles (A, B) and brains (C, D). Capillary numbers (A, C) and areas (B, D) were calculated with computer software. *p < 0.01, **p < 0.001.

lar level.

The long-term expression of VEGF is also important to obtain higher angiogenic effect in the brain. Various methods have been used to obtain a long-lasting effect of VEGF, including continuous infusion into the ventricle with osmotic minipumps,9) VEGF-producing cells,17,25) and gene transduction with some viral vectors15,21) or plasmids.13) Long-term continuous delivery of VEGF, maintained below a threshold microenvironmental level, can lead to normal angiogenesis in mice.19) Therefore, the total dose of administration is not important, but the duration of VEGF expression is crucial. Consequently, delivery systems using VEGF gene or gene-expressing cells are considered more beneficial than the administration of VEGF protein. A delivery system with a regulating system for VEGF expression is considered the best for angiogenesis and for avoiding adverse effects.

Adverse effects of VEGF result from the effect of increasing the vascular permeability. Ischemic brain in the acute stage produces VEGF which is responsible for vascular leakage.1,20) In previous VEGF treatment models for brain ischemia have shown adverse effects of VEGF. VEGF overexpression in rat brains using adeno-associated virus vectors caused significant increases in intracranial pressure, brain water content, and cerebral edema volume.15) Significant enlargement of the lateral ventricles occurred with the highest doses but not with other doses of human recombinant VEGF infused into the lateral ventricles of rats.9) Early posts ischemic (1 hour) administration of human recombinant VEGF to ischemic rats significantly increased blood-brain barrier leakage, hemorrhagic formation, and ischemic lesions.20) Almost all adverse effects of VEGF were related to vascular permeability, that is, brain edema, hemorrhage, increased intracranial pressure, and enlargement of ventricles. The important point is that excessive doses of VEGF cause severe adverse effects on ischemic brains, but doses under the threshold do not.

Macroscopic and microscopic examination of the rat brains observed no adverse effects in the brain.
Fig. 3 Photomicrographs of the rat brains perfused with paraformaldehyde and Indian dye solution at 30 days after plasmid human vascular endothelial growth factor (phVEGF) administration. ×100. A: Control group without operation. B: Control group with encephalo-myo-synangiosis. C: Group treated with 100 μg of phVEGF. D: Group treated with 200 μg of phVEGF. Bar = 120 μm.

Fig. 4 Macroscopic (A, B) and microscopic findings (C, D) of the rat brain treated with encephalo-myo-synangiosis and 200 μg of plasmid human vascular endothelial growth factor. Photograph of the treated side of the brain (A) showing normal structures compared to the non-treated side of the brain (B). “T” indicates attached temporal muscle. Photomicrographs of the brain portion near the attachment of the temporal muscle (C) showing newly formed vessels, but no apparent adverse effects compared to the opposite side of the brain (D). Hematoxylin and eosin stain, ×100. Bar = 120 μm.

parenchyma, possibly induced by VEGF in the present study. Presumably, no severe adverse effects occurred in the treated rat brains for the following reasons: low dose of phVEGF, indirect administration method (not directly into the brain), and timing of administration. We did not examine doses higher than 200 μg of phVEGF. In this study, examination of more than 200 μg of phVEGF could not be considered meaningful, because we obtained the maximal angiogenic effect at a dose of 100 μg of phVEGF. Administration of excessive doses (over the threshold) of phVEGF might cause adverse effects on the brains. The present findings suggest maximal angiogenic effect can be obtained at the optimal dose of phVEGF, which was under the threshold to cause serious adverse effects for ischemic brains. We employed EMS and phVEGF administration in our rat brain model. As reported in our previous study,13) immunohistochemical staining with monoclonal anti-VEGF antibody observed VEGF expression in the phVEGF-treated temporal muscles, but not in the brains. Therefore, the angiogenic effect was mostly induced by the VEGF expressed in the temporal muscles. This indirect VEGF effect might result in the low but long-lasting effect of VEGF for angiogenesis in the ischemic brain. Possibly, the ischemic rat brain could obtain the ideal VEGF volume and effect from the temporal muscle. We administered phVEGF 7 days after the induction of cerebral hypoperfusion. The vascular permeability of ischemic brain is very high in the acute stage, but decreases with time. The waiting time for 7 days permitted repair of the vascular permeability of the ischemic brain, but avoided the synergistic adverse effects of ischemia and VEGF.

An adequate dose of VEGF or VEGF gene must be administered to obtain the highest angiogenic effect for brain ischemia, as overdose or overexpression of VEGF leads to less angiogenic effect and serious adverse effects because of the increased vascular permeability. The present model of ischemic rat brains identified 100 μg of phVEGF administration into the temporal muscle with EMS in the chronic stage as the optimum dose. However, the optimum dose of VEGF or VEGF gene may be different in primates, so further studies are needed before the clinical application of VEGF therapy for brain ischemia.
References


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Commentary

Vascular endothelial growth factor (VEGF) has been demonstrated both experimentally and clinically to stimulate angiogenesis in a variety of clinical conditions. These investigators have previously demonstrated that the administration of plasmid human VEGF (phVEGF) combined with encephalo-myo-synangiosis surgery significantly increases angiogenesis in a rat brain ischemia model. In the current publication the authors report the results of an elegant experiment utilizing this model to evaluate a dose escalation of phVEGF both for efficacy in angiogenesis and potential risks.

Interestingly, the authors discovered that there appears to be an optimum dose for the therapeutic benefits of VEGF rather than a dose dependent relationship. Although all phVEGF treated rats had significantly higher capillary numbers and areas in the brain compared to the control groups, the number of capillaries and capillary area was enhanced maximally in animals receiving 100 µg of the agent and higher doses of VEGF showed less angiogenic effect.

Importantly, the authors were unable to identify any microscopic or gross evidence of toxic effects of VEGF. These toxic effects have generally been related to increased vascular permeability resulting in edema, hemorrhage, intracranial hypertension and ventriculomegaly. In their model, the rats were treated 7 days after the ischemic insult and sacrificed 30 days after treatment. Although the current study does not rule out subtle or long-term adverse effects of VEGF on the brain it does support the safety of this treatment paradigm in the acute phase of this particular model. These encouraging results should stimulate further research and other animal models.

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Katsumata and colleagues previously published findings that showed administration of plasmid human VEGF gene (phVEGF) combined with indirect vasoreconstructive surgery significantly increased capillary density in the chronic ischemic brain. In this paper, they have clarified the dose response of phVEGF using the same rat ischemic model. Interestingly, excessive doses of phVEGF diminished the angiogenic effects without histological changes, which is in agreement with a previous report that high doses of VEGF induced immature or permeable angiogenic vessels.3

VEGF has been reported to activate the phosphatidylinositol 3'-kinase (PI3-K)/Akt pathway and regulate angiogenesis.3) A number of Akt target proteins have been identified in survival and cell proliferation. Recently, Girdin, an actin-binding protein, was revealed to be phosphorylated by Akt and to promote VEGF-mediated angiogenesis. In this study, even high doses of phVEGF demonstrated the same histological findings without brain edema as those of the control group, suggesting that this methodology of indirect administration of phVEGF into the temporal muscles is useful and might become the basis of a method of treatment for moyamoya disease. Further studies focusing on the mechanism of angiogenesis induced by phVEGF are necessary to elucidate the potential of treatment using phVEGF for focal cerebral ischemia. In the near future, VEGF treatment will be established, however, this study tells us that VEGF is useful for angiogenesis, but the optimal dose might be different in individual patients with cerebral ischemia.

References


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