VEGF-mediated angiogenesis stimulates neural stem cell proliferation and differentiation in the premature brain

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ARTICLE INFO

Article history:
Received 12 February 2010
Available online 24 February 2010

Keywords:
Vascular endothelial growth factor
Angiogenesis
Neurogenesis
Neural stem cell
Premature

ABSTRACT

This study investigated the effects of angiogenesis on the proliferation and differentiation of neural stem cells in the premature brain. We observed the changes in neurogenesis that followed the stimulation and inhibition of angiogenesis by altering vascular endothelial growth factor (VEGF) expression in a 3-day-old rat model. VEGF expression was overexpressed by adenovirus transfection and down-regulated by siRNA interference. Using immunofluorescence assays, Western blot analysis, and real-time PCR methods, we observed angiogenesis and the proliferation and differentiation of neural stem cells. Immunofluorescence assays showed that the number of vWF-positive areas peaked at day 7, and they were highest in the VEGF up-regulation group and lowest in the VEGF down-regulation group at every time point. The number of neural stem cells, neurons, astrocytes, and oligodendrocytes in the subventricular zone gradually increased over time in the VEGF up-regulation group. Among the three groups, the number of these cells was highest in the VEGF up-regulation group and lowest in the VEGF down-regulation group at the same time point. Western blot analysis and real-time PCR confirmed these results. These data suggest that angiogenesis may stimulate the proliferation of neural stem cells and differentiation into neurons, astrocytes, and oligodendrocytes in the premature brain.

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1. Introduction

Ischemic brain injury in premature infants is a problem of enormous importance [1]. Despite a large number of studies with promising neuroprotective agents, no clinically successful strategy for neuroprotection has emerged [2]. Previous studies have confirmed that cellular interactions play an important role in the stem cell niche [3]. In adults, neurogenesis occurs near blood vessels, and neural stem cells are closely associated with endothelial cells in both the hippocampus and in the subventricular zone [4,5]. Endothelial cells are known to synthesize numerous basal lamina elements and growth factors involved in neurogenesis, such as brain-derived neurotrophic factor and vascular endothelial growth factor (VEGF) [6–8]. Several factors that can stimulate neurogenesis, such as basic fibroblast growth factor, VEGF, insulin-like growth factor-1, and transforming growth factor-β, can also promote endothelial cell survival, division, and differentiation [9–12]. Coordinated interactions between endothelial cells and neural cell proliferation have been observed in the developing brain and during testosterone-induced neurogenesis in the adult songbird [13]. In 2004, it was suggested that endothelial cells play an essential role in the proliferation of neural stem cells and their differentiation into neurons [14]. These studies have shown that angiogenesis and neurogenesis may have an intrinsic relationship. However, little is known about the relationship between angiogenesis and neurogenesis in the premature brain. Based on the characteristics of VEGF, which are associated with angiogenesis and neurogenesis, we observed the effects of angiogenesis on neurogenesis by up-regulation and down-regulation of VEGF in this study. The results may have value in treating brain injury in premature infants.

2. Materials and methods

2.1. General preparation

We obtained 108 postnatal day 3 (P3) Sprague–Dawley rats weighing between 8.2 and 10.5 g from the Experimental Animal Center, Shanghai Medical College, Fudan University, China. To minimize the effect of the litter-specific response, rats in the same litter were divided randomly into three groups. In group 1, VEGF mRNA expression was up-regulated by adenovirus transfection through injection into the lateral ventricles; in group 2, VEGF mRNA expression was down-regulated by short interfering RNA
(siRNA) interference; and in group 3 (normal control), normal saline was administered. From each group, 12 animals were sacrificed at each of three time points, 4, 7, and 14 days after operation. All efforts were made to minimize both the suffering and the number of animals used. All procedures were conducted in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals, and all procedures have been approved by the Institutional Care of Experimental Animals Committee of Fudan University.

2.2. Animal model

2.2.1. Gene up-regulated assay by adenovirus vector-mediated VEGF gene transfection

Delivery of VEGF was achieved using an adenovirus vector containing cDNA of the 165 splice variant of human VEGF (ad.VEGF) [15]. Virus containing the green fluorescent protein gene (ad.GFP) was used as a control. Production of VEGF by ad.VEGF-transfected HEK293 cells was assessed by ELISA (R&D Systems). Control cells were infected with ad.GFP. The biological activity of VEGF produced by ad.VEGF transfection into HEK293 cells was assessed by measuring endothelial cell (ATCC, CRL#2299) proliferation. The certified adenovirus vector was used in the following experiment.

To determine the optimal doses of adenovirus vector, 1 × 10⁶, 5 × 10⁶, 1 × 10⁷ pfu ad.VEGF-A165 were injected into the subventricular zone at one spot in the normal rat brain. The methods of injection into the subventricular zone followed published protocols [16]. There were five animals in each group. Brains were harvested and dissected into HEK293 cells was assessed by measuring endothelial cell (ATCC, CRL#2299) proliferation. The certified adenovirus vector was used in the following experiment.

2.2.2. Gene down-regulated assay by siRNA

Four pairs of double-stranded short interfering RNA (siRNA) of VEGF were chemically synthesized by Shanghai GenePharma Co., Ltd., China. GAPDH was used as a positive, irrelevant control. All the sense and anti-sense sequences of siRNA are listed in Table 1. VEGF gene expression in endothelial cells (ATCC, CRL#2299) and VEGF protein presence in the endothelial cell culture medium were measured by real-time PCR and ELISAs, respectively, 48 h after transfection of each siRNA pair. Sequence 1 of the four VEGF siRNA sequences was selected to knock-down VEGF gene expression in the following experiment. The transfection of siRNA was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. In brief, 2 μL of the siRNA–Lipofectamine mixture was injected into the subventricular zone.

2.3. Histology

Fifteen minutes before they were killed at the above-mentioned time points, the rats were used for immunofluorescence assays.

### Table 1

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Sense</th>
<th>Anti-sense</th>
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<tr>
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<td>2</td>
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<td>5'-UAAACCGGAUUCUUUGCCGT-3'</td>
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<td>5'-CCUCUGUACAGCCGAGAATT-3'</td>
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<td>Negative control</td>
<td>5'-UCUCGCGACCGUUGACAGATT-3'</td>
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</tr>
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</table>

2.4. Immunofluorescence assays

Using antibodies for Nestin, Tuj1, glial fibrillary acidic protein (GFAP), and chondroitin sulfate proteoglycan 4 (NG2), we assessed the proliferation and differentiation of neural stem cells in the SVZs with immunofluorescence assays. Cells were labeled with antibodies for Nestin to identify neural stem cells, Tuj1 antibodies to identify neurons, GFAP antibodies to identify astrocytes, and NG2 antibodies to identify oligodendrocytes. Likewise, an antibody for Von Willebrand factor (vWF) was used to assess angiogenesis. All methods followed published protocols [18,19].

For the immunofluorescence assays, SVZ sections were washed (0.1 M Tris, pH 7.6, 15 min), denatured (2 N HCl, 37 °C, 30 min), rinsed (0.1 M PBS, 10 min), incubated with 1% H₂O₂ (0.1 M Tris, 30 min), rinsed, blocked (10% normal goat serum, 37 °C, 30 min), and finally incubated with the mouse anti-rat monoclonal 1° antibody (4 °C overnight, anti-Nestin (1:200, R&D, USA), anti-Tuj1 (1:200, Chemicon, USA), anti-GFAP (1:100, Neuroimm, UK), anti-NG2 (1:200, Chemicon, USA), and anti-vWF (1:200, LABVISION, USA), respectively). All sections were washed and incubated with 2° antibodies, rinsed, mounted, dried, and coverslipped using DAPI. All 2° antibodies were tetraethyl rhodamine isothiocyanate (TRITC)-conjugated rabbit anti-mouse IgG (1:100, room temperature, 2 h). Jackson ImmuResearch Laboratories Inc., USA. When double-labeled Nestin and vWF, the 2° antibodies for Nestin was fluorescein isothiocyanate (FITC)-conjugated.

Immunofluorescence staining was visualized by fluorescence microscopy (Leica, Germany). The changes in angiogenesis were reflected by vWF-positive areas, and neural stem cell proliferation and differentiation was quantified using stereologic counting of positive cells in the SVZs at the above-mentioned time points in each group (n = 6). Every 4th section in a series of 10-μm-thick sections through the SVZ was sampled, following the methods of Plane et al. [20]. All counts were performed using a 200× objective lens on a Leica fluorescence microscope and the Stereoinvestigator v.5.0 computer program (MicroBrightField, Colchester, VT). The total number of cells/region was estimated using the Optical Fractionator technique [21].

2.5. Western blot analysis

Frozen SVZs were cut into small pieces and homogenized in 0.5 mL of RIPA buffer (150 mMol/L NaCl, 1% N-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 50 mMol/L Tris–hydrochloric acid, 2 mMol/L phenylmethylsulfonyl fluoride, pH 7.4) and then transferred into small tubes and rotated at 4 °C overnight. Solubilized protein was collected after centrifugation at 10,000g for 30 min. The supernatant from each culture was collected, and pro-
tein concentrations were quantified with the Enhanced BCA Protein Assay Kit (Beyotime Biotechnology, Haimen, China).

To detect VEGF, vWF, Nestin, GFAP, Tuj1, and NG2 protein levels, tissue protein lysates from each group of rats were subjected to SDS–PAGE analysis using a 10% (wt/vol) acrylamide gel and electrotransferred onto a PVDF membrane (Millipore Corporation). The nonspecific proteins on the membranes were blocked in skim milk powder (5% in PBS, 0.1% Tween 20, 2 h at room temperature). Immunoblotting was performed using 1 µg/mL of mouse anti-rat VEGF mAbs, 1 µg/mL of mouse anti-rat vWF mAbs, 2 µg/mL of mouse anti-rat Nestin monoclonal antibody (R&D, USA), 1 µg/mL of mouse anti-rat Tuj1 mAbs (Chemicon, USA), 10 µg/mL of mouse anti-rat GFAP mAbs (Neuromics, England), or 1 µg/mL of mouse anti-rat NG2 mAbs (Chemicon, USA). The membranes were then incubated with the appropriate secondary horseradish peroxidase-conjugated anti-mouse IgG antibodies at a 1:2,500 dilution (Jackson Immunoresearch Laboratories Inc., USA). Immunoreactive proteins were then visualized using ECL plus a Western blotting detection system (Beyotime Biotechnology, Haimen, China).

2.6. Reverse transcription–real-time quantitative PCR assays

We extracted total RNA from the frozen SVZs using the TRIzol reagent following the manufacturer’s suggested protocol (Invitrogen, USA). RNA concentrations were measured spectrophotometrically in a GenQuant RNA/DNA calculator (Pharmacia Biotech, UK). For reverse transcription (RT), we prepared 2 µg of total RNA and 1 µL random primer for each sample and then added RNase-free water to a total volume of 15 µL. The mixture was kept at 70 °C for 5 min before cooling on ice. We then added 5 µL of 5× MMLV RT buffer, 1.25 µL dNTPs, 25 U (0.6 µL) RNase inhibitor (TOYOBO Co., Ltd., Osaka, Japan), 200 U (1 µL) MMLV Reverse transcriptase (Promega, USA), and 2.15 µL RNase-free water. Reverse transcription of 2 µg RNA to cDNA was performed in a total volume of 25 µL. The reverse transcription reaction was carried out in a Thermal Cycler PTC-150 (MJ Research, USA) using a program with 40 RT cycles at 25 °C for 10 min followed by 60 min at 37 °C and 5 min at 95 °C, after which the reaction was quenched at 4 °C. After the reaction was completed, samples were stored at −20 °C until real-time PCR analysis. Real-time quantitative PCR was conducted as described in the instruction manual for the Real-time PCR Master Mix (TOYOBO Co., Ltd., Osaka, Japan). cDNA samples (1 µL) and gene-specific primers (1 µL sense primer, 1 µL anti-sense primer) were added to 25 µL SYBR Green PCR Master Mix and then ddH2O was added to reach a 50 µL total volume. PCR amplification (1 cycle at 95 °C for 20 min, 40 cycles at 95 °C for 30 s, 52 °C for 30 s, 72 °C for 40 s) was performed in the Eppendorf Mastercycler ep realplex (Eppendorf, Germany). For each RT, real-time PCR was conducted three times in duplicate using each of the cDNA samples. The amplified transcripts were quantified with the comparative CT method using standard curves. The primers were designed using the Primer Express software (Primer Premier 5.0) based on the GenBank accession numbers, and the sequences are shown in Table 2. β-Actin was used as the internal control.

2.7. Statistical methods

All values are presented as means and standard deviations. One-way analysis of variance (ANOVA) was used to examine differences between the control group and the treatment group at each time point. The LSD procedure was used for pairwise comparisons. Alpha was set at 0.05, and all tests were two-tailed. All analyses were performed using SPSS 12.0.

3. Results

3.1. Changes in VEGF expression at the mRNA and protein level

VEGF mRNA and protein expression in the subventricular zone were measured by real-time PCR and Western blot assays, respectively, at each time point. VEGF mRNA and protein expression peaked 7 days after injection. Compared to the control group, VEGF mRNA and protein expression was reduced in the siRNA group and increased in the adenovirus vector-transfected group at all three time points (Fig. 1).

3.2. Effects of up-regulation and down-regulation of VEGF on angiogenesis

To detect the effects of up-regulation and down-regulation of VEGF on angiogenesis, we measured the changes in vWF expression by immunofluorescence assays, real-time PCR, and Western blot assays. The vWF-positive area peaked at day 7 in all three groups. Up-regulation of VEGF expression increased the vWF-positive area at all three time points. Moreover, down-regulation of VEGF expression decreased the vWF-positive area. There was a significant difference among the three groups at each time point (Fig. 2A, p < 0.01). One particularly exciting finding was that the vWF-positive cells were close in proximity to the Nestin-positive cells in the double-labeled immunofluorescence assays. This suggests that there is an intrinsic relationship between angiogenesis

<table>
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<th>Table 2</th>
<th>Primer sequence of 7 genes used for quantitative real-time PCR.</th>
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<td>Gene name</td>
<td>Accession No. (GeneBank)</td>
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<tr>
<td>Vascular endothelial growth factor A (VEGF-A)</td>
<td>NM_031836</td>
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<tr>
<td>Von Willebrand factor (vWF)</td>
<td>BC_081983</td>
</tr>
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<td>Actin, β (Actb)</td>
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and neural stem cells (Fig. 2B). The expression of vWF was highest in the VEGF up-regulation group and lowest in the VEGF down-regulation group at both the mRNA and protein levels, as shown by real-time PCR and Western blot assays, respectively (Fig. 2C–D, \( p < 0.01 \)).

3.3. Effects of up-regulation and down-regulation of VEGF on neural stem cell proliferation and differentiation

Immunofluorescence assays were used to determine changes in the number of different neural cells over time in each group. To determine whether the cells expressed phenotypic neuronal features, we labeled frozen sections with antibodies against Nestin, a marker for neural stem cells; Tuj1, a marker for mature neurons; GFAP, a marker for astrocytes; and NG2, a marker for oligodendrocytes. The number of Nestin-positive cells was highest in the VEGF up-regulation group at all three time points and peaked on the 7th day. The number of Nestin-positive cells was lowest in the VEGF down-regulation group at all three time points. There was a significant difference among the three groups at each time point. Up-regulation of VEGF expression also increased the number of neurons, astrocytes and oligodendrocytes, which peaked at day 7 and day 14, respectively. The number of neurons, astrocytes and oligodendrocytes in the VEGF down-regulation group was lowest when compared to the VEGF up-regulation group and the control group. As before, there was a significant difference among the three groups at each time point (Fig. 3).

Likewise, we also observed changes in neural stem cell proliferation and differentiation at both the mRNA and protein levels. Consistent with the immunofluorescence results, the gene and protein expression of Nestin, Tuj1, GFAP, and NG2 were highest in the VEGF up-regulation group and lowest in the down-regulation group at all three time points. There was a significant difference among the three groups at each time point (Fig. 4).

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**Fig. 1.** Changes in VEGF expression. (A) At the mRNA level; (B) at the protein level. Values are means and SDs. Values were compared with ANOVA, *\( p < 0.05 \) vs control.

**Fig. 2.** Changes in vWF expression. (A) vWF-positive area at the day 7 in the three groups after intervention. (B) Double-labeled vWF and Nestin at the day 7 in the adenovirus group. (C) vWF mRNA expression at the three time points in the three groups. (D) vWF protein expression at the three time points in the three groups. Values are means and SDs. Values were compared with ANOVA, *\( p < 0.05 \) vs control.
4. Discussion

Mammalian brain development studies have shown that angiogenesis and the neural cell differentiation and maturation phases are almost synchronized. Factors that are currently known to stimulate neurogenesis, such as basic fibroblast growth factor, VEGF, insulin-like growth factor-1, and transforming growth factor-β, can also promote endothelial cell survival, division, and differentiation [9–12]. Previous studies have also shown that most of the proliferating cells in the hippocampal dentate gyrus are concentrated around small blood vessels. This indicates that microvascular and secreted factors may provide favorable conditions for neurogenesis [22]. Therefore, angiogenesis and neurogenesis may have an intrinsic relationship.

Many factors can affect angiogenesis; VEGF especially plays a central regulatory role in the process [23]. Recent evidence indicates that VEGF can act as a neurotrophic factor and produce neurogenic effects on neuronal progenitors [8]. Zhang et al. showed that VEGF is a powerful activator of immature neural progenitor cells and can guide blood vessel growth and neural stem cell migration in the developing mammalian brain [24]. As VEGF not only stimulates angiogenesis but also promotes neurogenesis, we were able to observe changes in neurogenesis by promoting and inhibiting angiogenesis via the up- and down-regulation of VEGF.

First, we observed VEGF mRNA and protein expression following adenovirus transfection and siRNA interference. VEGF mRNA and protein expression decreased in the siRNA group and increased in the adenovirus vector-transfected group at each time point. These data confirmed that VEGF was successfully up-regulated and down-regulated. By observing the expression of vWF, a marker for blood vessels, we found that angiogenesis was promoted by up-regulation of VEGF and inhibited by down-regulation of VEGF in the subventricular zone of the rat brain. These data confirm that VEGF regulates angiogenesis in the premature brain.

Furthermore, we also observed changes in neural stem cell proliferation and differentiation. Immunofluorescence results showed that the number of neural stem cells in the VEGF up-regulation group was higher than in the control group, which peaked at day 7. When VEGF was down-regulated, the number of neural stem cells was significantly reduced. To detect the spatial relationship between blood vessels and neural stem cells, we double-labeled vWF and Nestin in the same tissue sections and observed protein localization using immunofluorescence. We found vWF-positive cells close in proximity to Nestin-positive cells. Consistent with previously published studies, this also indicates that angiogenesis can affect neurogenesis. By the 14th day, the number of neural stem cells in the brain slices had decreased, and the number of neurons, astrocytes, and oligodendrocytes had increased in the VEGF up-regulation group. Likewise, vWF expression also decreased between the 14th and 7th day in the VEGF up-regulation group. The real-time PCR and Western blot assays confirmed these results. The relationship between angiogenesis and neurogenesis in both time and space indicates that angiogenesis can regulate neurogenesis. Thus by promoting angiogenesis, we can stimulate neurogenesis.

Fig. 3. The proliferation and differentiation of neural stem cells in the SVZ in the three groups. (A–D) Immunofluorescence staining in the SVZ on frozen sections in rats at the day 7 after intervention. (A) Cells staining positive for Nestin, a marker for neural stem cells; (B) cells staining positive for TuJ1, a marker for immature neurons; (C) cells staining positive for GFAP, a marker for astrocytes; (D) cells staining positive for NG2, a marker for oligodendrocytes. (E–H) Quantitative analysis of different cells in the SVZ after intervention. Values are means and SDs. Values were compared with ANOVA, *P < 0.05 vs control.
In summary, this study showed that angiogenesis and neurogenesis have an intrinsic relationship. Angiogenesis may stimulate the proliferation of neural stem cells and their differentiation into neurons, astrocytes, and oligodendrocytes in the premature brain. These results suggest the possibility of treating premature brain injury by regulating angiogenesis.

**Acknowledgments**

This study was supported by the National Natural Science Foundation of China (No. 30571963), the Program for New Century Excellent Talents in University (NCET-07-0211), the National 973
Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.02.132.

References
