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Original Research

Roles of Sema3A and VEGF165 in cortical neurons and vascular endothelial cells during oxygen glucose deprivation stimulation

Ying Yang[1], Chen Li[1], Ruili Wang[1], Hui Wang[1], Qiaoya Ma[1], Ya He[1], Pengbo Zhang[2*]

[1] Department of Geriatrics Neurology, the Second Affiliated Hospital of Xi'an Jiaotong University, 710004, Xi'an, Shaanxi, PR. China
[2] Department of Anesthesiology, the Second Affiliated Hospital of Xi'an Jiaotong University, No. 157 Xiwu Road, 710004, Xi'an, Shaanxi, PR. China

Correspondence to: knnyp9@163.com

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Abstract: To investigate the expressions and roles of semaphorin3A (Sema3A) and vascular endothelial growth factor 165 (VEGF165) in cultured rat cortical neurons and vascular endothelial cells after oxygen glucose deprivation (OGD) stimulation. Cultured cortical neurons (NC) and vascular endothelial cells (VEC) of Sprague Dawley (SD) rats (SPF grade) were randomly divided into control group and OGD treatment group. Western blot assay, immunofluorescent staining and immunohistochemical methods were used to determine the expressions of VEGF165, Sema3A and neuropilin-1 (Nrp-1) protein. Cell migration was determined by Transwell, while TUNEL assay was used to measure apoptosis. The expressions of Sema3A, Nrp-1 and VEGF165 in NC and VEC cells after OGD treatment were up-regulated, when compared with the control group. With transfection of Sema3A shRNA, apoptosis of neurons decreased significantly after 2 h of OGD treatment, but the apoptosis of VEC cells was not obvious. The migration rate of VEC cells in the treatment group was significantly increased, relative to that of the control group. Stimulation with OGD induces neuronal expression of VEGF165 and regulates the migration of vascular endothelial cells, thereby enhancing their participation in angiogenesis, which may involve Sema3A.

Key words: Cortical neurons; Vascular endothelial cells; Oxygen glucose deprivation; Sema3A; Nrp-1; VEGF165.

Introduction

Ischemic cerebrovascular diseases are caused by vascular endothelial damage. The injured neurons can be saved through the repair of damaged vessels and endothelium in ischemic penumbra (1). Ischemia reperfusion injury can lead to oxidative stress or even death of nerve cells (2). The cerebral endothelial cells form a blood brain barrier (BBB) which maintains the microenvironment of the cerebral nervous system and participates in oxygen delivery (3). It has been demonstrated that oxygen glucose deprivation (OGD) treatment of neurons may contribute to disruption of the endothelial barrier (4). Promotion of vascular endothelial cell regeneration and improvement of endothelial function are vital in the prevention and treatment of vascular diseases.

Semaphorin3A (Sema3A) belongs to the semaphorin family which binds to the endothelial receptor neuropilin-1 (Nrp1) and participates in cell migration, vascular patterning and nervous system development (5, 6). The VEGF family is a highly efficient kinin which is a dimeric glycoprotein linked by disulfide bond in VECs. The gene that encodes VEGF forms a variety of polypeptide products due to different shearing modes. These products are divided into five types i.e. VEGF121, VEGF145, VEGF165, VEGF189 and VEGF206, based on amino acid content. It is known that VEGF165 is the most abundant and active form in cells, especially in the brain. It has a strong ability to promote endothelial cell division and proliferation, making it widely useful in animal experiments and clinical research (7). The proteins of VEGF family are required for angiogenesis, and they contribute to immune regulation, neuronal growth and hematopoiesis (8, 9). These proteins promote endothelial cell adhesion, migration, proliferation, and micro-vessel outgrowth, thereby antagonizing Sema3A-induced apoptosis due to competition with Sema3A for binding to NRP1 (10, 11).

In this study, the possible roles of Sema3A and VEGF165 in the regulatory effect of OGD on neurons and vascular endothelial cells in endothelial barrier disruption were investigated. Rat cortical neurons and vascular endothelial cells cultured *in vitro* were used as models to determine changes in VEGF165, Nrp1 and Sema3A protein expressions after OGD treatment. The effect of OGD in VEC migration and function of VEGF165 protein in NC and VEC cells in the presence and absence of Sema3A was studied.

Materials and Methods

Main reagents and experimental animals

Sprague Dawley rats (SPF grade), aged 3 - 5 days, and pregnant SD rats of SPF grade on E18 were used. Type II collagenase and DMEM were purchased from Gibco (US). Collagenase and dispase were purchased from Roche (US). Deoxyribonuclease I (DNase I) was

product of TaKaRa (China), while Percoll was purchased from GE Company (US). Bovine serum albumin (BSA) was obtained from Sigma (US). Phosphate buffered saline (PBS) and 4 % paraformaldehyde were products of Dongao Bioscience (China). Anti-Semaphorin 3A and anti-Neuropilin 1 antibodies were purchased from Abcam (UK), while anti-VEGF 165b antibody was from Millipore (US). Tris and Tween-20 were products of Amresco (US), while BCA Protein Assay Kit was produced by Thermo (US).

Culture of Rat neuron cells (NC)

Pregnant SD rats of SPF grade on E18 were used. After disinfecting the abdomen with 75 % alcohol, the abdominal cavity was opened. The uterus was taken out and the fetal mice were removed and placed in a 10-cm petri dish containing cold PBS buffer. The fetal rat skull and dura mater were isolated, and the cerebral cortex was fully exposed and placed in a culture dish containing DMEM/HIGH medium on ice. The meninges and vascular tissues were adequately removed from the cortex. The cortex was digested with 0.05 % trypsin (Amresco, US) for 20 min in 5 % CO_2 incubator at 37 ℃. The digestion was terminated with serum-containing medium. The digested tissue was transferred to a centrifuge tube, with 3 to 5mL DMEM/HIGH medium added each time. Then, the supernatant was removed, and the washing was repeated 2 to 3 times. The cell suspensions were filtered through 200 mesh screen, and the filtered cell suspensions were counted. The suspensions were inoculated on lysine-coated confocal at appropriate density and placed in 5 % CO_2 incubator at 37 ℃ for 4 h. Then, the medium was replaced with neurobasal+B27+L-glutamine (Gibco, US), and changed in half volume every 2 to 3 days.

Primary culture of rat brain vascular endothelial cells (VEC)

Sprague Dawley rats (SPF grade) (aged 3 - 5 days) were sacrificed by cervical dislocation and disinfected in 75 % ethanol for 5 min. The brain of each rat was removed in aseptic condition and placed in a petri dish containing cold PBS solution. Then, the cortex was taken out with adequate removal of meninges and vascular tissue. The collected cerebral cortex was washed thrice with cold PBS buffer, and then cut into about 1-mm^3 portions. Then, 5 ml of 0.1 % type II collagenase (containing 0.005% D-Nas I) was added, and blended well 30 times. After digestion at 37 ℃ for 40 min, 0.1% collagenase and dispersase were added to digest for 40 min. Then, the mixture was centrifuged at 800 ×g for 5 min at 4 ℃. The supernatant was discarded, and appropriate amount of DMEM culture medium was added, with mixing. Then, the filtrate was collected through a 100-mesh screen and centrifuged at 800 ×g for 5 min. Appropriate amount of 20 % BSA was added to the precipitate, and the mixture was centrifuged at 1000 g for 20 min at 4 ℃. The precipitate was rinsed with DMEM culture solution once, and centrifuged at 800 ×g for 5 min at 4 ℃. The supernatant was discarded and laid on the surface of continuous percoll centrifuge solution with 44 % mass fraction. After centrifugation at 1000 ×g for 10 min at 4 ℃, a yellowish white part near the bottom of red precipitate was obtained. This was rinsed once in DMEM and centrifuged to obtain microvascular segments with high purity (all these operations were carried out on ice). Then, 7 ml of high-sugar DMEM (containing 20 % FBS, 2 μg/ L^{-1} b-FGF, 100 $mg{\cdot}L^{-1}$ heparin sodium, and 1 % penicillin-streptomycin) was added to the culture dish coated with mouse-tail glue, and then cultured in a 5 % CO_2 incubator at 37 ℃. The solution was changed after 12 h, and thereafter changed once every 2 to 3 days.

Protein extraction and western blot assay

The degree of fusion of cerebral microvascular endothelial cells reached more than 80 %, and on the 6th day of neuron culture, correlation analysis was performed after OGD for 2 h, and re-oxygenation for 24 h. Adherent cell protein was extracted from the following groups: NC group, NC+OGD treatment group, VEC group, VEC+OGD treatment group, NC+OGD treatment+Sema3A shRNA group, and VEC+OGD treatment+Sema3A shRNA group. After draining the cell culture medium, the remaining culture medium was removed by rinsing twice with pre-cooled PBS. Pre-cooled cell lysis buffer was added, and the cells were scraped off with a clean cell scraper and put into a new 1.5-mL centrifuge tube. The cells were lysed fully on ice for 10 min. After centrifugation at 12000 rpm for 10 min at 4 ℃, the supernatant was transferred to a pre-cooled centrifuge tube. Bicinchoninic acid (BCA) protein quantitative kit was used to quantify the extracted protein.

Detection of Sema3A, VEGF165 and Nrp1 expressions by western blot assay

Protein samples (30 μg) were taken from each group; 5× protein sample buffer solution was added, and the mixture was boiled in water bath for 5 min, and then subjected to SDS-PAGE. The resultant protein bands were transferred to polyvinylidene difluoride (PVDF) membrane and the PVDF membrane was stained in Ponceau staining solution for 2-5 min. The upper right corner of the PVDF membrane was labeled and the membrane was transferred to TBST blocking buffer containing 5 % BSA (0.05% Tween-20) for 1.5 h at room temperature. The PVDF membrane was put into a dish, and the primary antibodies anti-Sema3A (diluted 1:800), anti-VEGF165 (diluted 1:500) and anti-Nrp1 (diluted 1:1000) were added and incubated overnight at 4 ℃. The membrane was washed 4 times with TBST (each wash for 10 min). Then, the PVDF membrane was placed in a dish containing diluted second antibody and incubated in a shaking bed for 1 h. After incubation, the membrane was rinsed thrice with TBST, and thereafter placed for 2 to 3 min in an enhanced chemiluminescence reaction mixture.

Immunofluorescent staining for detection of Sema3A and Nrp-1 expression

The cultured cells were fixed in 4 % paraformaldehyde at room temperature for 30 min. Then, 0.3 % TritonX-100 was used as permeability agent for 15 min. The cells were incubated with goat anti-Sema3A (diluted 1:100) and anti-Nrp1 antibodies (diluted 1:200) at 4 ℃ overnight. After rinsing 3 times with PBS, they were incubated with Alexa Fluor 594 donkey-rabbit

IgG (diluted 1:400) for 30 min in the dark at 37 ℃, and mounted in Vectashield mounting medium with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI, Vector). The immune-stained samples were observed and analyzed with a fluorescent inverted microscope system (OLYMPUS IX5).

Immunohistochemical detection of VEGF165 expression

Cultured cells were fixed with 4 % paraformaldehyde at room temperature for 30 min, and then rinsed thrice with PBS. Peroxidase blocker in streptavidin-peroxidase kit (SP kit, BIOSS, US) was added and reaction was allowed to proceed for 10 min at room temperature. Normal non-immune animal serum blocking buffer was added. After 1h, 100 μL of primary anti-VEGF165b (diluted 1: 100) was added per well, and incubated overnight at 4 ℃ in a wet box. After rinsing thrice with PBS, biotinylated secondary antibody working buffer was added and, reaction was allowed for 20 min at 37 ℃. The HRP-labeled *Streptomyces* ovalbumin working buffer was added and left for 10 min at room temperature. Then, DAB developing solution was added to the tissue, and the developing time was controlled under the microscope. The positive color was brown, and color development was terminated by flushing the section with tap water. The nucleus was re-stained with Hematoxylin (Sigma, US) for 1 min, and 1 % HCl alcohol was used for differentiation for 1 second, followed with flushing in tap water, returning to blue color with ammonia water, and rinsing in running water. The tissues were dehydrated in xylene and sealed with neutral gum. Then, microscopic examination and image acquisition were carried out.

Determination of cell migration with Transwell assay

In order to study changes in VEC cells during the OGD treatment on NC cells, cell morphology and migration of VEC were measured. After the cells adhered, the neuronal supernatant medium treated with OGD and glucose-free medium were cultured at 37 ℃ for 24 h, and morphological changes in the cells were observed microscopically. When the cell confluence was greater than 80 %, the cells were digested, and single cell suspension was made with PBS buffer. The cell concentration was adjusted to 1×10^6/mL. Then, 100 μL of cell suspension was added to the upper chamber of the Transwell migration system. The upper chamber was placed in a sugar-free medium and in culture well of the supernatant of OGD-treated neurons, followed by incubation at 37 ℃ for 24 h. The cells were rinsed thrice in PBS, each rinse for 5 min, and fixed with 4 % formaldehyde for 30 min, then washed thrice with PBS buffer, and stained with crystal violet for 15 to 20 min. After removing the un-migrated cells from the upper well side, the cells below the bottom of the migration cup were observed. Five fields of vision were randomly selected under the microscope to calculate the average number of cells in each field.

Microvascular endothelial cell infection test

A 96-well plate was inoculated with microvascular endothelial cells at a density of 3×10^4 cells/mL at a volume of 90 μL to ensure that the degree of fusion was 20 to 30 % when virus-infected. On the second day, 10 μL of virus diluent was added to each well according to MOI values of 0, 5 and 10. A parallel control group with 5 μg/mL polybrene added was set up. The cells were further cultured after mixing, and the cell status was observed after 8 to 12 h. Then, the cells were replaced with fresh medium and infected for 3 to 4 days, and the fluorescence expression was observed.

Detection of apoptosis by TUNEL

Cultured cells were fixed with 4 % paraformaldehyde at room temperature for 30 min, and then rinsed with PBS buffer 3 times. Then, 0.3 % TritonX-100 was used as permeability agent for 10 min, followed by washing with PBS buffer. The TUNEL detection solution was prepared with TdT enzyme: fluorescent labeling solution in a volume ratio of 1:9; and 50 μL of TUNEL detection solution was added to each well, followed by incubation at 37 ℃ for 60 min in the dark. VECTASHIELD and DAPI were added for staining of nucleus and sealing. Then, the cells were observed under the fluorescence microscope. Red color represented apoptotic cells.

Results

Morphology of rat neuronal cells (NC) and cerebral vascular endothelial cells (VEC)

After culturing, the rat neuronal cell (NC) body was full, and the nucleus was large and obvious. The cytoplasm was clear, and most of the cells were long fusiform, while individual cells were conical. The cell surface was smooth, the boundary was clear, the cell refraction was strong, the membrane and neurites were obvious, and there were two to three neurites. The synapses between the cells were interwoven into a network in the form of tree roots, creating an obvious "neural network". The cerebral vascular endothelial cells (VEC) were long spindle-shaped and irregularly arranged. When cultured for 9 to 10 h, the monolayer fused, and the endothelial cells grew in a "swirl" shape (Figure 1).

Effects of OGD treatment on the expression of Sema3A, Nrp1 and VEGF165 in NC and VEC

As shown in figure 2, it was found that the expressions of Sema3A, Nrp1 and VEGF165 in NC and VEC cells after OGD treatment were up-regulated, when compared with the control group. To further investigate the expressions of Sema3A and Nrp1 in NC and VEC cells during OGD exposure, immunofluorescent staining was performed. As shown in Figure 3, enhanced fluorescent signals representing Sema3A and Nrp1 were observed

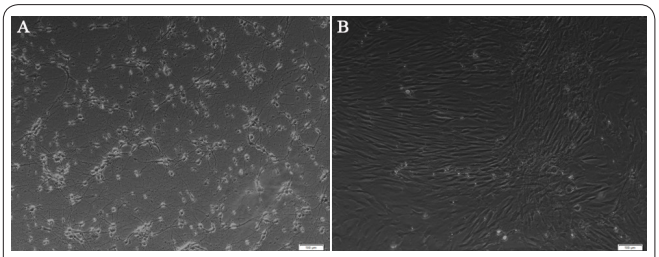

Figure 1. Cellular morphologies of rat neurons and vascular endothelial cells after culturing. A, Neuronal cells; B, Vascular endothelial cells. Scale bar, 100 μm.

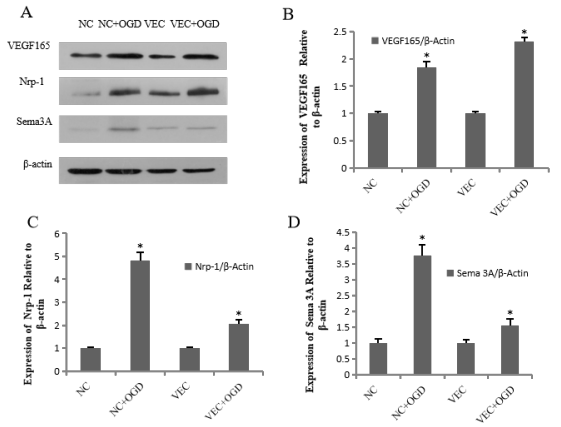

Figure 2. Expressions of VEGF165, Nrp-1 and Sema3A following OGD treatment in NC and VEC cells. (A) Western blot detection of VEGF165, Nrp-1 and Sema3A in the protein extracted from NC and VEC cells with or without OGD treatment. The blots were probed with antibodies against VEGF165, Nrp-1 and Sema3A. β-Actin was used as the endogenous control for protein arrays. (B) Quantitative analyses of VEGF165 protein accumulation in NC and VEC cells. (C) Quantitative analyses of Nrp-1 protein accumulation. (D) Quantitative analyses of Sema3A protein levels. Asterisks (*) indicate significant difference by t test from the NC and VEC cells without OGD treatment (*, P<0.05). The experiment was repeated three times with similar results.

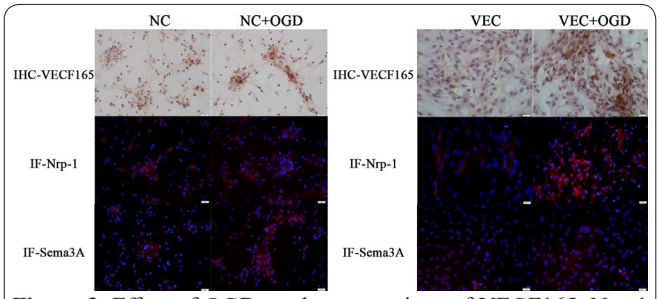

Figure 3. Effect of OGD on the expressions of VEGF165, Nrp-1 and Sema3A (A) The expression of VEGF165 in NC cells with or without OGD treatment was determined with immunohistochemical assay. The Nrp-1 and Sema3A levels in NC cells were measured with immunofluorescent staining. (B) The expression of VEGF165 in VEC cells was determined by immunohistochemical assay, while the Nrp-1 and Sema3A levels in VEC cells were measured using immunofluorescent staining. Scale bar, 20 μm.

in NC and VEC cells after OGD treatment. In addition, immunohistochemical measurements showed that VEGF165 was expressed in the cytoplasm, as indicated by brown-yellow spots. After treatment with OGD, the protein level of VEGF165 was increased significantly in the NC and VEC cells, when compared with the control group (Figure 3). Collectively, these results indicate that OGD treatment induced enhanced expression levels of Sema3A, Nrp1 and VEGF165 in NC and VEC cells.

Effect of OGD treatment of NC supernatant medium on VEC migration

Compared with the control group, VEC cells showed decreased adherent ability, gradual shrinkage of adherent cells, enlargement of intercellular spaces, decreased refractive index, and incomplete structure of some cells (Figure 4A). As shown in Figure 4B, Transwell assay indicated that the migration of VEC cells in

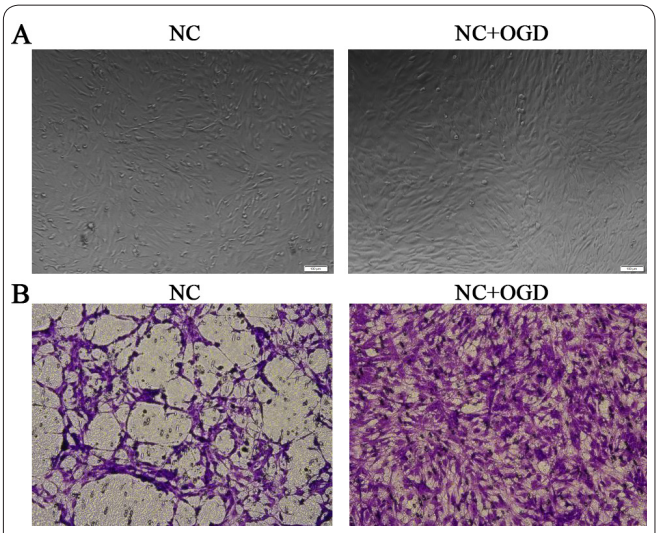

Figure 4. Effect of OGD on neurons and VEC cell migration. (A) Cellular morphology of rat VEC cells after treatment of NC supernatant medium with OGD. (B) Migration of VEC cells as measured using Transwell assay after OGD treatment of NC supernatant medium. Scale bar, 100 μm.

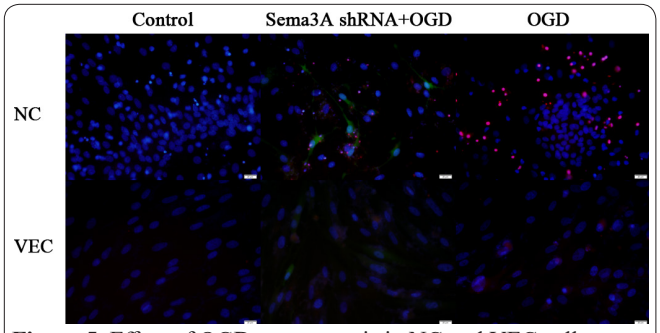

Figure 5. Effect of OGD on apoptosis in NC and VEC cells transfected with Sema3A shRNA. The NC and VEC cells were observed under fluorescence microscope after staining and sealing. Red stains represent the apoptotic cells, while blue stains indicate all cells in the field of view. Scale bar, 20 μm.

the treatment group was significantly increased, when compared with those in the control group. Based on these results, OGD stimulates neurons and in turn regulates the migration of VEC cells in their participation in angiogenesis.

Protective effect of VEC cells in the presence of Sema3A during OGD treatment

The apoptosis of neurons transfected with Sema3A shRNA decreased significantly after 2 h of OGD treatment, but the apoptosis of VEC cells was not obvious after 2 h of OGD treatment (Figure 5). These observations suggest that during OGD treatment, VEC cells were protected from death in the presence of Sema3A. The OGD treatment reduced the protein expressions of Sema3A, Nrp1 and VEGF165 after transfection with Sema3A shRNA in cortical neurons and vascular endothelial cells (Figure 6).

Discussion

The development of the vascular system and angiogenesis are regulated by receptors on endothelial cell surface which are activated by soluble or insoluble mediators (14). Neuropilin-1 (Nrp-1) is one of the im-

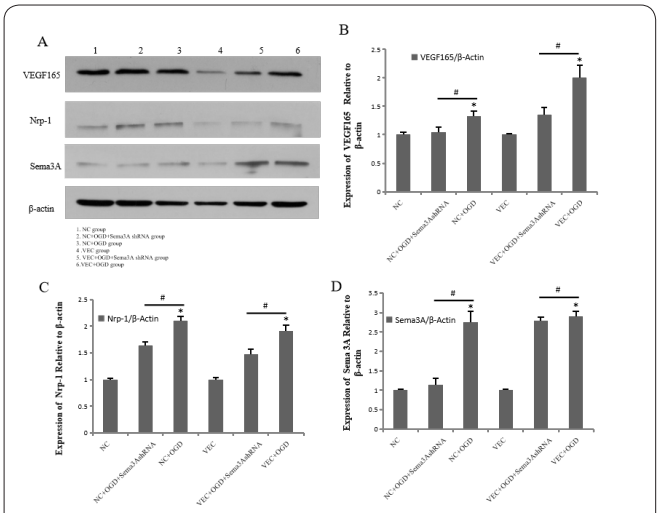

Figure 6. Effect of OGD on protein expressions of VEGF165, Nrp-1 and Sema3A following OGD in NC and VEC cells transfected with Sema3A shRNA were analyzed. (A) Western blot results for VEGF165, Nrp-1 and Sema3A in the protein extracted from NC and VEC cells transfected with Sema3A shRNA. The blots were probed with antibodies against VEGF165, Nrp-1 and Sema3A. β-Actin was used as the endogenous control. (B) Quantitative analyses of VEGF165 protein accumulation. (C) Quantitative analyses of Nrp-1 protein levels. (D) Quantitative analyses of Sema3A protein expression. Asterisks (*) indicate significant difference by t test from the NC and VEC cells without OGD treatment (*, $P<0.05$). The pound sign (#) indicate significant difference by t test from the NC and VEC cells transfected with Sema3A shRNA (#, $P<0.05$). The experiment was repeated three times with similar results.

portant members of the NRP family often expressed in endothelial cells and some tumor cells. It is a co-receptor of Sema3A, VEGF, platelet-derived growth factor (PDGF) and other related factors. Previous research has confirmed that Nrp-1 promotes angiogenesis mediated by VEGF, and promotes tumor development (15). It has been demonstrated that Nrp-1 mediates signal pathways orderly with different ligands through complex mechanisms such as transducing VEGF165 signals using VEGF receptor 2 (VEGFR-2) (16), and transducing Sema3A signals through plexin-A1, plexin-A2 or plexin-A4 (17, 18).

When cerebral ischemia occurs, neurons may act as the initiators of disruption of the blood-brain barrier (BBB). It has been demonstrated that ischemic neurons induce endothelial barrier disruption by activating astrocytes to increase the production of VEGF (3). Vascular endothelial cells (VECs) are the targets of ischemic vascular injury. Damage to VECs has been shown to cause a variety of vascular dysfunctions. Prolonged ischemia results in VEC apoptotic death and vascular dementia, arteriosclerosis and brain oedema (19). In this study, the morphology of rat cerebral VEC cells before and after OGD treatment was investigated. After OGD treatment of NC supernatant medium, VEC cells showed decreased adherent ability, gradually shrinkage of adherent cells, enlargement of intercellular space, decreased refractive index and incomplete structure of partial cells, suggesting that OGD stimulates neurons and in turn regulates the participation of angiogenesis in VEC cells (Fig. 1, 4). In agreement with the previous study, VEC cells act as a important targets of ischemic vascular injury. Cerebral ischemia induces the expression of Nrp-1 which is involved in brain ischemic response (20). Studies have shown that OGD treatment increases the expressions of Sema3A and Nrp-1, which in turn take part in the neurite damage and neuron apoptosis (13, 21). Neuronal axon outgrowth and extension are inhibited remarkably by exogenous Sema3A treatment (21). However, the possible roles of Sema3A and VEGF in VEC during oxygen glucose deprivation is still unclear. Our study on the role of VEGF and its signal pathway in ischemic and hypoxic brain injury may provide a therapeutic method for nervous system injury.

In previous study (12), it was found that Sema3A combined with Nrp1 antagonized angiogenesis of vascular endothelial growth factor 165 (VEGF165). It was suggested that in different endothelial cell angiogenesis, imbalance in endogenous Sema 3A/VEGF165 ratio made Sema3A and VEGF-165 play different roles. In addition, studies have shown that Sema3A and Nrp1 are probably involved in neuronal axonal injury caused by OGD treatment (13). It was found in this study that apoptosis of neurons transfected with Sema3A shRNA decreased significantly after 2 h of OGD treatment, but the apoptosis of VEC cells was not obvious under the same treatment, indicating that VEC cells were protected from death in the presence of Sema3A during OGD stimulation (Fig. 5). The VEGF has been demonstrated as an important angiogenic factor and a potent inducer of blood–brain barrier (BBB) disruption following cerebral ischemia (4). Not only does VEGF promote the proliferation of vascular endothelial cells, participate in angiogenesis, and enhance vascular permeability, it is also involved in nerve protection and nerve regeneration in central nervous system injury. Previous studies showed that the balance between repellent signal Sema3A and growth factor VEGF165 determines the proliferation, migration and apoptosis of neural progenitor cells (11, 22). Our results based on western blot and immunohistochemical analyses, reveal that the protein expressions of Sema3A, Nrp1 and VEGF165 in NC and VEC cells after OGD treatment were up-regulated, when compared with the control group (Fig. 2, 3); and Sema3A, Nrp1 and VEGF165 levels were reduced after transfection with Sema3A shRNA (Fig. 6). These results suggest that VEGF165 antagonizes apoptosis in VEC cells in the presence of Sema3A. Furthermore, the Transwell assay showed that the migration of VEC cells in the treatment group was significantly increased, when compared with those in the control group (Fig. 4). These results suggest that OGD stimulates neurons and promotes VEC cell migration, thereby enhancing their participation in angiogenesis which involves a balance between Sema3A and VEGF165. It is necessary to elucidate the particular regulatory mechanism involved in the balance between Sema3A and VEGF165 after OGD induction in vascular endothelial cells, so as to provide new ideas on angiogenesis and neuroprotection after stroke.

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Conflict of Interest

There are no conflicts of interest in this study.

Author's contribution

All work was done by the author named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Pengbo Zhang; Ying Yang, Chen Li, Ruili Wang, Hui Wang, Qiaoya Ma, Ya He and Pengbo Zhang collected and analysed the data; Ying Yang wrote the text and all authors have read and approved the text prior to publication.

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