Transplantation of hypoxia-inducible factor-1α gene modified neural stem cells increases cell survival and angiogenesis after cerebral ischemia

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Previous studies have indicated the beneficial effects of transplanted neural stem cells (NSCs) on cerebral ischemia. Hypoxia-inducible factor-1α (HIF-1α) is a master transcription factor of cellular hypoxic gene expression, and its signal pathway might be the primary mechanism through which hypoxia promotes the growth of NSCs. To test the hypothesis that HIF-1α contributes to the therapeutic effect of NSCs transplantation in cerebral ischemia, we compared the efficacy of transplanting PBS, NSCs infected with recombinant adenovirus, HIF-1α gene, and NSCs infected with a recombinant adenovirus vector with HIF-1α gene (HIF-1α-NSCs). A transient middle cerebral artery occlusion (tMCAO) was used in this study. PBS, HIF-1α gene, NSCs and HIF-1α-NSCs were respectively injected into cortical peri-infarction of the rat brain at 24 h after MCAO. Neurological deficits were assessed using the modified neurological severity score (NSS). Immunohistochemistry for BrdU, VEGF, Von Willebrand Factor and Nissl staining were performed. Compared with other groups HIF-1α-NSCs showed better behavioral recovery at 7, 14, 21 and 28 days, and lesser degree of brain atrophy in cortex and hemisphere. More BrdU-positive cells in HIF-1α-NSCs group than those in NSCs group. Expression of VEGF and Von Willebrand Factor were both higher in HIF-1α-NSCs than those in HIF-1α or in NSCs group. Thus, we concluded that during the early period after transplantation HIF-1α infected NSCs expressed gene products, which reduced brain injury by improving the survival of NSCs and protecting the vascular system.

Keywords: Neural stem cells; Focal ischemia; Transplantation; Hypoxia-inducible factor-1α; VEGF

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Introduction

HIF-1 is a basic helix-loop-helix heterodimeric transcription factor, comprised of the HIF-1α and HIF-1β subunits [1]. It is well documented that HIF-1 activity is determined primarily by the protein stability of the HIF-1α subunits. HIF-1 is generally considered to play an important role in the cellular response to hypoxia [2]. HIF-1α subunits are degraded rapidly by 26s proteasomes in normoxia. However, they are stabilized and transactivated to the nucleus during hypoxia. HIF-1α target genes include vascular endothelial growth factor (VEGF); erythropoietin (EPO), and several glycolytic enzymes, etc., which play crucial roles for neurogenesis and neuroprotection in the brain [3, 4]. HIF-1α is necessary for the early development of brain and many other mammalian organs [5]. Mice lacking HIF-1α show severe cardiac and vascular malformations, leading to embryonic lethality at embryonic day 10. Overexpression of HIF-1α increased the number of TH-positive cells and the dopamine content in culture medium under normal conditions.

Inhibition of endogenous HIF-1α inactivation using dimethylxalylglycine (DMOG) promoted angiogenesis in ischemic skeletal muscles of mice [6]. HIF-1α gene therapy enhanced capillarization in rabbit hindlimb ischemic and rat myocardial infarction model, increasing regional blood flow and reducing the infract size. Inoculated HIF-1α DNA into the brain surface or the temporal muscle promoted significant angiogenesis development in a rat encephalo-myo-synangiosis (EMS) model [7]. Stabilized HIF-1α markedly increased capillary sprouting and proliferation and overexpression of HIF-1α did not increase vascular leakiness in the transduced muscle [8].

Neural stem cells (NSCs) are immature cells with the ability of renewing themselves and giving rise to neurons, astrocytes and oligodendrocytes. NSCs may be extracted from the nervous system, grown, expanded and propagated in a culture dish and then transplanted into the pediatric and adult nervous system. Previous studies have already demonstrated that transplanted NSCs could differentiate into a range of different cell types and compensate for the neurological deficits following cerebral ischemia [9, 10]. And NSCs carrying potentially therapeutic genes may play a valuable role in novel strategies for repair and regeneration of nervous [11]. In our previous study we have demonstrated that HIF-1α treatment promoted both neurogenesis and angiogenesis, and thus improved neurologic function following cerebral ischemia in rats [12]. In this experiment, we investigated to whether NSCs carrying HIF-1α gene can have a better beneficial outcome in transient middle cerebral artery occlusion (tMCAO) model in rats.

Materials and methods

In this study, we have followed all the guidelines stated in Guide for the Care and Use of Laboratory Animals, prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources Commission on Life Sciences, National Research Council, China (1985).

Cell cultures

Embryonic Spargue-Dawley rats brain (E14) was used to collect the hippocampus. The detailed culture methods were described elsewhere [12]. The tissues were transferred to cold PBS, minced with scissors, and dissociated with pasteurpipette. Then the suspension was centrifuged at 75 × g in a 10-ml centrifuge tube for 10 minutes. At last cells were seeded (final density: 1×10^5 viable cells/ml) in Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1; Gibco) plus B27 supplement (2 ml/100 ml, Gibco), bFGF (20 ng/ml, Sigma) and EGF (20 ng/ml, Sigma) and cultured at a humidified atmosphere of 5% CO2/95% air for 3 to 7 days. After passing four generations, neurospheres were dissociated into single cells by incubation in 0.1% trypsin-ethylenediamine tetra-acetic acid at 37°C for 2 minutes and then centrifugation in 10 ml of DMEM/F12 medium containing 4% bovine serum albumin at 110 × g for 5 minutes. Neurospheres from single cells were transferred to a poly-l-ornithine-coated 96-well dish and cultured in the same medium. After 2 days of culture, neurospheres in NSC culture were differentiated using DMEM/F12 with 20% fetal bovine serum medium.

Adenovirus infection

Recombinant adenovirus carrying HIF-1α gene and green florescent protein (GFP) gene have been prepared using AdEasy system in Chinese academy of science. The titrer of Ad-HIF-1α was between 10^9 and 10^10 PFU/ml. Adenovirus-mediated gene transfection was performed as described previously [13]. After passing four generations, neurospheres were dissociated into single cells and infected by recombinant adenovirus with for 2 hours at 37 °C(at a MOI[multiplicity of infection] of 20), and then the medium was removed, and the cells were washed once with DMEM and then re-cultured with normal medium.

Focal cerebral ischemia rat model

Male Sprague-Dawley rats weighting 230-250 g were used in the experiments. Animals were housed individually and maintained at a room temperature of 25 °C and had free access to food and water under the condition of a 12-hour light-dark cycle before surgery. Rats were initially
anesthetized with 50 mg/kg of ketamine and 10 mg/kg of xylazine administered intraperitoneally in O2 by a face mask. We induced transient MCAO according to the method of intraluminal vascular occlusion. The right common carotid artery, external carotid artery (ECA), and internal carotid artery (ICA) were isolated. A 3 cm length of 4-0 nylon suture with a slightly enlarged and rounded tip was advanced from the ECA into the lumen of the ICA until it blocked the origin of the MCA. The distance from the tip of the suture to the bifurcation of the common carotid artery is 18.5 to 19.5 mm. Reperfusion was performed by withdrawal the suture until the tip cleared the lumen of the ECA after 90 min of MCAO.

Transplantation procedures

At day 1 after MCAO, animals with NSS scores of 7 to 12 were randomly divided into four groups (n=12, respectively): ischemia+PBS (group 1), ischemia+HIF-1α (group 2), ischemia+NSCs (group 3), and ischemia+ HIF-1α-NSCs (group 4). Rats were anesthetized with equithesin (3 ml/kg administered intraperitoneally) and transferred to a stereotaxic apparatus. Using aseptic technique a 2 to 5 mm incision was made in the scalp 1.5 mm lateral to the bregma. A burr hole was made in the bone 3 mm lateral to bregma with a dental drill, and about 10 μl of PBS (group 1), HIF-1α (1×10^6 cells), NSCs, and HIF-1α-NSCs (1×10^6 cells ) were slowly injected during 20 min into the ischemic area at a depth of 2.0 mm from the surface of the brain, respectively. The needle was retained in the brain for an additional 5 min before retraction. All NSCs had been labeled with 5 mmol/L BrdU for 3 days before transplantation.

Behavioral testing

In a blind test of all the 4 groups, behavioral testing was performed weekly using the modified neurological severity score (NSS) [14]. The NSS is a composite of motor (muscle status, abnormal movement), sensory (visual, tactile, proprioceptive), reflex, and balance tests. Neurological function was graded on a scale of 0 to 18 (normal score, 0; maximal deficit score, 18), the higher score, the more severe the injury.

![Figure 1. Behavioral tests.](http://www.smartscitech.com/index.php/scti)
Histological analysis

At day 7 and day 35 after ischemia, rats were sacrificed under deep anesthesia and transcardially perfused for histological analysis. After 24 h fixation in 4% paraformaldehyde, the brains were cryoprotected with 30% sucrose for 24 h and cut into 30 μm sections. To compare the hemispheric atrophy of the four experimental groups, we measured the hemispheric areas (n=3 per group) by examining Nissl stained slides. The brain sections were traced with the use of an analysis system (Image-pro Plus, Media Cybernetics). Because 30% sucrose solution can make tissue shrink, the ipsilateral atrophy was compared with the contralateral side and expressed as the ratio (%). Serial 10μm-thick coronal sections were processed for immunostaining with Mouse Anti-BrdU (1:200, Boster) to identify transplanted cells. The number of BrdU-positive cells was determined by direct counting of one tenth of whole sections. BrdU (1:200)/NF-200 (1:100, Boster) and BrdU(1:200)/GFAP(1:400, Abcam) double labeled immunofluorescence were used to identify the differentiation of transplanted cells. To evaluate the expression of VEGF, immunofluorescence for VEGF(1:200, Santa Cruz) was performed. Immunohistochemistry for endothelial cells were performed with anti-vWF antibody (1:400, Abcam, USA).

Statistical analysis

Data were showed as means ± standard deviation. Data were analyzed by repeated measures of analysis of variance (ANOVA), and unpaired Student’s t-test, if they were normally distributed (Kolmogorov-Smirnov test, p>0.05). Otherwise, we used Mann–Whitney U-test and specified the test used. A probability value of less than 5% was considered statistically significant.

Results

HIF-1α-NSCs treatment showed the best functional recovery

To determine if the transplanted HIF-1α-NSCs could improve sensorimotor deficit, we compared NSS scores (n=12 for each group). There were no significant differences among the groups in NSS scores either before MCAO or on day 1 after MCAO. Behavioral deficits evaluated by the NSS demonstrated a progressive recovery in each group from week 1 to week 4 after MCAO. HIF1-α treatment showed a significant improvement in NSS scores compared with group 1 on post ischemia day 7 (P7), P14, P21 and P28 (Fig. 1). NSCs treatment had a more significant functional recovery compared with groups 1 on P21 and P28 (Fig. 1). HIF-1α-NSCs treatment showed the best functional recovery compared with groups 2 and group 3 on P7, P14, P21 and P28 (Fig. 1). Body weights among the 4 groups had no significant difference during the 4 weeks.

HIF-1α-NSC transplantation reduced hemispheric atrophy

The brain atrophy in group 2 and group 3 was significantly different from that in group 1 (Fig. 2). When compared with other groups, Group 4 showed a significantly lesser atrophy in cortex and total hemisphere (Fig. 2).
result demonstrates that there is less cell death or more transplanted cell survival in rats treated by HIF-1α-NSCs.

HIF-1α-NSCs improved survival of transplanted cells

At week 5 after ischemia, donor cells in the brains of recipients were detected by immunofluorescence for BrdU. BrdU-positive cells were found in cortex and striatum. The number of BrdU-positive cells was significantly higher in group 4 than that in group 3 (Fig. 3). To identify if
transplanted NSCs differentiate to be neuron or glia cells, we did BrdU/NF-200 and BrdU/GFAP double staining. The results showed that some BrdU-positive cells were NF-200-positive too, suggesting that transplanted NSCs could differentiate into neuronal cells (Fig. 4). Some BrdU/GFAP double positive cells demonstrated that transplanted cells can also differentiate to be astrocytes (Fig. 4).

HIF-1α-NSCs treatment increased VEGF expression

VEGF was found in endothelial cells in the four groups. On day 7 after ischemia in PBS group, VEGF was decreased obviously. It indicated cerebral ischemia destroyed endothelial cells. Expression of VEGF in HIF-1α and NSCs group was higher than that in PBS group. In HIF-1α-NSCs group, expression of VEGF was significantly higher than that in PBS group p<0.01. There was no significant difference between HIF-1α and HIF-1α-NSCs group (Fig. 5).

HIF-1α-NSCs increased vWF-positive endothelial cell number

To determine if transplanted HIF-1α-NSCs promoted proliferation of host endothelial cells in the lesion sites, we calculated the number of endothelial cells using vWF immunostaining at week 5 after MCAO. Results indicated that the number of factor vWF-positive cells in the HIF-1α-NSCs group was significantly increased compared with that in other groups (Fig. 6).

**Discussion**

The purpose of this study was to test the hypothesis that HIF-1α contributes to the therapeutic effect of NSCs transplantation in cerebral ischemia. Our results showed HIF-1α-NSCs transplantation increased the cell survival and angiogenesis, and decreased hemispheric atrophy, leading to the better functional recovery. HIF-1α-NSCs treatment showed more benefits than both NSCs and HIF-1α. These results suggest that HIF-1α gene modified NSCs transplantation could provide more benefits for neurological recovery after cerebral ischemia. HIF-1α gene modified NSCs may be a potential therapeutic agents for cerebral ischemia.

In adult brain, ischemia can induce NSCs proliferation in the rostral subventricular zone of the hippocampal dentate gyrus and the subgranular zone (SGZ). It has been proved that these endogenic NSCs can migrate, differentiate, and integrate into the injured tissue, replace the damaged neuronal cells, and reconstruct neuronal circuitry 

![Figure 4. Differentiation of transplanted NSCs in the peri-ischemic area. At week 5 after ischemia, HIF-1α-NSCs (BrdU-positive, A, D) differentiated into astrocytes (BrdU/GFAP-positive, C) (white arrows) or neurons (BrdU/NF-positive, E) (with arrows), Bar=50µm.](image)
Figure 5. Immunohistochemical detection of VEGF in ischemic penumbra tissue at day 7 after tMCAO. PBS (A), HIF-1α (B), NSCs (C) and HIF-1α-NSCs group (D). Bar=50µm. (E) Quantitative analysis of HIF-1α-positive cells in ischemic penumbra tissue. Compared with PBS group (#: p < 0.01).
Figure 6. Analysis of vWF immunoreactivities in the peri-ischemic area at week 5 after ischemia. vWF staining for endothelial cells in the PBS(A), HIF-1α(B), NSCs(C) and HIF-1α-NSC group(D). Bar=50µm. (E) Quantitative analysis showed an increase of vWF immunoreactivities in the HIF-1α-NSCs group. Bars represent mean±SD. compared with PBS (#: p < 0.01) or NSCs and HIF-1α(*:p < 0.05)group, respectively.
(GFAP-positive). The NSS scores in group 2 were not significantly different from those in group 1 until P21. This result confirmed the fact that a certain length of time is required for NSCs to differentiate into neural cells and to integrate with the host cells after transplantation [17].

This study also demonstrated that NSCs over-expressing HIF-1α improved the cell survival. HIF-1α-NSCs expressed HIF-1α after being transplanted. The activation of HIF-1α could increase the expression of several key targeted genes such as VEGF, erythropoietin, tyrosine hydroxylase and OCT4, which act as regulators of cellular proliferation and differentiation [19, 20]. Recently it has been reported that relief of hypoxia in the developing cerebral cortex by ingrowth of blood vessels temporo-spatially coincided with NSCs differentiation [21]. To better understand the exact role of HIF-1α in maintaining stem cells, more studies should be done.

It has been identified that newly formed microvessels improved microperfusion of the tissue surrounding the ischemia, and that increased angiogenesis promoted neurologic functional recovery after stroke [22]. Enhancement of angiogenesis can be a valuable therapeutic option for the cerebral ischemia. This study demonstrated that HIF-1α-NSCs enhanced angiogenesis, supporting previous reports which showed that HIF-1α DNA could form new vessels and protects the ischemic injury [23-25]. Angiogenesis is a complex process including increase of vascular permeability, degradation of surrounding matrix, proliferation and migration of endothelial cells, and stabilization of newly formed microvessels [26]. It involves both angiogenic and anti-angiogenic factors expressed by different cell types. Promotion of angiogenesis in ischemic tissue is currently the subject of intensive research. Since the activation of HIF-1α was observed in the rat cerebral cortex after ischemia, it is generally accepted that HIF-1α exerts benefits over neuronal survival. HIF-1α, a universal molecular master switch, controls cellular survival, glucose metabolism and transport, and metabolic adaptation. Approximately 30 target genes are known, including VEGF, EPO, glycolytic enzymes and glucose transporter 1, insulin-like growth factor 2 etc. Among them, VEGF is the most well-known angiogenic factor in each step of angiogenesis [27]. It participates in angiogenesis by recruiting endothelial cells into hypoxic and avascular area and stimulates their proliferation. Therefore, the induction of VEGF and various other proangiogenic factors in the ischemic region leads to an increase in the vascular density and hence a decrease in the oxygen diffusion distance. In this study, we demonstrated both HIF-1α and HIF-1α-NSCs promoted VEGF expression after ischemia. HIF-1α may activate the expression of additional genes promoting angiogenesis besides VEGF and EPO. HIF-1α upregulates the expression of the urokinase receptor to enhance cellular migration and invasion. And hypoxic induction of the expression of Flt-1, one of two VEGF receptors, is mediated by an HIF-1α binding site found upstream of the gene. In addition, some uncharacterized factors may be involved in angiogenesis regulated by HIF-1α and therefore possibly activated by HIF-1α.

Conclusions

This study provides evidence that in the MCAO rat brain, transplantation of HIF-1α-NSCs improves functional recovery in cerebral ischemia animals, produces neuroprotection against ischemic injury, and stimulates angiogenesis after ischemia. These results suggest that a possible application of genetically modified NSCs to overexpress HIF-1α might be a therapeutic agent for ischemic stroke.

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Conflicting interests

The authors have declared that no conflict of interests exist.

Abbreviations

HIF-1α: hypoxia-inducible factor-1α; NSCs: neural stem cells; tMCAO: transient middle cerebral artery occlusion; PBS: phosphate buffered solution; NSS: neurological severity score; BrdU: 5-bromodeoxyuridine; VEGF: vascular endothelial growth factor; EPO: erythropoietin; GFP: green fluorescent protein; NF: neurofilament; GFAP: glial fibrillary acidic protein; BDNF: brain-derived neurotrophic factor, CNTF: cholinergic neurotrophic factor, GDNF: glial cell derived neurotrophic factor; NT-3: neurotrophin-3.

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