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Research report

Growth differentiation factor 11 improves neurobehavioral recovery and stimulates angiogenesis in rats subjected to cerebral ischemia/reperfusion

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ABSTRACT

The recent suggestion that growth differentiation factor 11 (GDF11) acts as a rejuvenation factor has remained controversial. However, in addition to its role in aging, the relationship between GDF11 and cerebral ischemia is still an important area that needs more investigation. Here we examined effects of GDF11 on angiogenesis and recovery of neurological function in a rat model of stroke.

Exogenous recombinant GDF11 (rGDF11) at different doses were directly injected into the tail vein in rats subjected to cerebral ischemia/reperfusion (I/R). Neurobehavioral tests were performed, the proliferation of endothelial cells (ECs) and GDF11 downstream signal activin-like kinase 5 (ALK5) were assessed, and functional microvessels were measured.

Results showed that rGDF11 at a dosage of 0.1 mg/kg/day could effectively activate cerebral angiogenesis *in vivo*. In addition, rGDF11 improved the modified neurological severity scores and the adhesive removal somatosensory test, promoted proliferation of ECs, induced ALK5 and increased vascular surface area and the number of vascular branch points in the peri-infarct cerebral cortex after cerebral I/R. These effects were suppressed by blocking ALK5.

Our novel findings shed new light on the role of GDF11. Our results strongly suggest that GDF11 improves neurofunctional recovery from cerebral I/R injury and that this effect is mediated partly through its proangiogenic effect in the peri-infarct cerebral cortex, which is associated with ALK5. Thus, GDF11/ALK5 may represent new therapeutic targets for aiding recovery from stroke.

1. Introduction

Cerebral angiogenesis, which is a major mechanism for sprouting new microvessels and remodeling existing vasculature (Carmeliet, 2000), is most prominent in the peri-infarct zone after ischemia and plays an important role in recovery of neural function in animal models of stroke (Liu et al., 2014) and human stroke patients (Krupinski et al., 1994). A better understanding of the mechanisms that underlie angiogenesis may contribute to finding a promising therapeutic target for restoring neurological function following ischemic stroke.

Growth differentiation factor 11 (GDF11), also known as bone morphogenetic protein 11, is a secreted member of the transforming growth factor-β (TGF-β) superfamily (Nakashima et al., 1999). Recently, several studies showed that GDF11 could partly duplicate rejuvenating effects of heterochronic parabiosis and reverse age-related dysfunction in the heart (Loffredo et al., 2013; Poggioli et al., 2016), skeletal muscle (Sinha et al., 2014), and brain (Katsimpardi et al., 2014) by rejuvenating stem cells. GDF11 can increase proliferation of primary brain capillary endothelial cells (ECs), participate in vascular remodeling, increase the volume of blood vessels, and improve vascular and neurogenic rejuvenation (Katsimpardi et al., 2014) of the disease-free aging mouse brain. Moreover, GDF11 supports migration and

Abbreviations: ALK5, activin-like kinase 5; d, days; ECs, endothelial cells; EPCs, endothelial progenitor cells; FITC, fluorescein isothiocyanate; GDF11, growth differentiation factor 11; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; I/R, ischemia/reperfusion; MCA, middle cerebral artery; MCAO/R, middle cerebral artery occlusion/reperfusion; mNSS, modified neurological severity scores; pSmad2/3, phosphorylated Smad2/3; rGDF11, recombinant GDF11; rCBF, regional cerebral blood flow; s, seconds; TGF-β, transforming growth factor-β

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sprouting (Finkenzeller et al., 2015) of endothelial progenitor cells (EPCs) *in vitro*. However, the proangiogenic effects of GDF11 were questioned by another study (Zhang et al., 2016b), which reported that GDF11 showed no significant effect on proliferation and migration of ECs *in vitro*. Therefore, the suggestion that GDF11 improves vasculature of the neurogenic niche as an angiogenic factor in the aged brain remains controversial.

However, the potential proangiogenic effects suggest a new and unexpected role for GDF11. In addition to aging, the relationship between GDF11 and cerebral ischemia is an interesting area that needs more investigation. Our recent work (Ma et al., 2016) has strongly suggested that GDF11, which first caught our attention as a circulating factor (Katsimpardi et al., 2014) in the vasculature, is also expressed in the cerebral cortex. Moreover, after cerebral ischemia, levels of GDF11 are significantly enhanced in blood circulation as well as the peri-infarct cerebral cortex where angiogenesis is remarkable. Expression of activin-like kinase 5 (ALK5), a receptor for GDF11, increases in ECs in the peri-infarct cerebral cortex where active vessel remodeling occurs after stroke. However, the function of GDF11 in post-apoplectic vascular remodeling *in vivo* is unclear. These findings prompted us to determine whether systemic administration of GDF11 could exert proangiogenic effects following cerebral ischemia and contribute to recovery of neurological function in a rat cerebral ischemia/reperfusion (I/R) model.

In the present study, we examined whether direct administration of the optimum dose of exogenous recombinant GDF11 (rGDF11) could activate downstream signals, ALK5 and phosphorylated Smad2/3 (pSmad2/3), induce cerebral angiogenesis and improve recovery of neurological function, and whether SB431542 (an inhibitor of ALK5) could block these protective effects, in a rat model of stroke.

2. Experimental procedures

2.1. Animals and grouping

All animal protocols were performed in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Chongqing Medical University, Chongqing, China. Male Sprague-Dawley rats (220–240 g) were purchased from the Experimental Animal Center of Chongqing Medical University. All rats were housed and fed under specific pathogen-free and controlled conditions (12/12 h light/dark cycle, humidity = 60%, temperature = 22 °C) with free access to food and water.

There were 2 steps in our experiment, in which rats were randomly assigned to the experimental groups prepared for each step. To ensure reliability of results and minimize interference of age, we limited the experiment to rats at 42–48 days (d) of age. There were no significant differences in weight and age among the groups.

Six groups of animals were prepared for the first step: (1) sham I/R group (control group), (2) I/R group, (3) I/R + 0.01 mg/kg/d rGDF11 group (0.01 rGDF11 group), (4) I/R + 0.03 mg/kg/d rGDF11 group (0.03 rGDF11 group), (5) I/R + 0.1 mg/kg/d rGDF11 group (0.1 rGDF11 group), and (6) I/R + 0.2 mg/kg/d rGDF11 group (0.2 rGDF11 group). We assessed expression of ECs in the peri-infarct cerebral cortex, performed neurobehavioral tests after 7 d of rGDF11 administration, and characterized the effective concentration for the second step.

Four groups of animals were prepared for the second step: (1) control group, (2) I/R group, (3) I/R + 0.1 mg/kg/d rGDF11 group (rGDF11 group), (4) I/R + 0.1 mg/kg/d rGDF11 + SB431542 group (rGDF11 + SB group). We explored whether downstream signals, ALK and Smad2/3, mediated GDF11-induced angiogenesis in the peri-infarct cerebral cortex.

2.2. Right middle cerebral artery occlusion/reperfusion (MCAO/R) model

Right MCAO was induced with an intraluminal suture occlusion as previously described (Longa et al., 1989; Belayev et al., 1996). In brief, anesthesia was induced with 3.5% chloral hydrate (1 mL/100 g). After the right common carotid artery was exposed, the external carotid artery was separated and ligated. A 40 mm-length nylon filament suture, which was blunted at the tip (diameter = 0.26–0.28 mm) by a flame and coated with melted paraffin wax (Zuo et al., 2012), was inserted into the internal carotid artery for a distance of about 12 mm. The rats were then placed in a stereotaxic frame, and a laser Doppler flowmeter probe (PeriFlux System 4001; PeriMed, Stockholm, Sweden) monitored changes in regional cerebral blood flow (rCBF) in the right middle cerebral artery (MCA) region (Sun et al., 2012) to confirm successful MCAO. Then, the nylon filament was inserted more deeply to about 20 mm to occlude blood flow at the origin of the MCA. After 2 h, the animals were anesthetized again and the nylon suture was removed to allow reperfusion. The standard I/R model was defined as a decrease in cortical rCBF to 70–80% of baseline during the first 30 min and > 70% flow recovery within the initial 10 min of reperfusion. Rats that did not meet these requirements, died during surgery or showed no neurological deficits were excluded from the study. In the sham I/R group, the embolus was inserted at a distance of 12 mm and removed immediately. Throughout the duration of the experiment, animals were anesthetized and body temperature was maintained at 37 ± 0.5 °C with a heating pad.

2.3. GDF11 and inhibitor administration

Rats in designated groups were treated with a daily tail vein injection of rGDF11 (PeproTech, Rocky Hill, NJ, USA). The first injection was administered 2 h after reperfusion when the rat was awake from anesthesia. The last injection was given 2 h before euthanasia. Investigators were blinded to treatment. Animals were weighed every day before dosing.

SB431542 (MedChem Express, Monmouth Junction, NJ, USA) was dissolved in 10% ethanol at a concentration of 0.5 mg/mL and administered *via* intraperitoneal injection daily for 7 or 14 consecutive days at a concentration of 4.2 mg/kg according to previous studies (Caraci et al., 2015, Ma et al., 2013) in the rGDF11 + SB group.

2.4. Neurobehavioral evaluation

A battery of neurobehavioral tests, which included the modified neurological severity scores (mNSS) test and the adhesive removal somatosensory test (Ning et al., 2014), was performed 7 d after reperfusion (the first step) as well as before MCAO (baseline) and then 7 d, and 14 d after reperfusion (the second step) by an investigator who was blinded to the experimental groups.

2.4.1. mNSS test

The mNSS test (Chen et al., 2001; Shen et al., 2007) is a composite of motor, sensory, balance, and reflex tests. Neurological function was graded on a scale of 0–18 (normal score: 0, maximal deficit score: 18). In the severity scores of injury, 1 point was awarded for a specific abnormal behavior or for lack of a tested reflex. Thus, a higher score reflected more severe injury. Before induction of ischemia, rats were pretrained in the beam-walking test for 3 d until each rat could readily undergo the beam-traversing task without slipping (Qu et al., 2015). Their performances were video recorded, and 3 trials were recorded for analysis.

2.4.2. Adhesive removal somatosensory test

The adhesive removal somatosensory test (Shen et al., 2007; Shehadah et al., 2014) is a sensitive method to assess sensorimotor deficits (Bouet et al., 2009). All rats were familiarized with the testing