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Research article

Basic fibroblast growth factor enhances cell proliferation in the dentate gyrus of neonatal rats following hypoxic–ischemic brain damage

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ABSTRACT

Background: Perinatal hypoxic–ischemic insult is considered a major contributor to child mortality and morbidity and leads to neurological deficits in newborn infants. There has been a lack of promising neurotherapeutic interventions for hypoxic–ischemic brain damage (HIBD) for clinical application in infants. The present study aimed to investigate the correlation between neurogenesis and basic fibroblast growth factor (bFGF) in the hippocampal dentate gyrus (DG) region in neonatal rats following HIBD.

Material and methods: Cell proliferation was examined by detecting BrdU signals, and the role of bFGF in cell proliferation in the DG region following neonatal HIBD was investigated.

Results: Cell proliferation was induced by HIBD in the hippocampal DG of neonatal rats. Furthermore, bFGF gene expression was upregulated in the hippocampus in neonatal rats, particularly between 7 and 14 days after HIBD. Moreover, intraperitoneal injection of exogenous bFGF enhanced cell proliferation in the hippocampal DG following neonatal HIBD.

Conclusions: Taken together, these data indicate that cell proliferation in the DG could be induced by neonatal HIBD, and bFGF promotes proliferation following neonatal HIBD.

1. Introduction

Perinatal hypoxic–ischemic injury leads to neurological deficits in newborn infants and is considered a major contributor to child mortality and morbidity [20]. Hypoxic–ischemic brain damage (HIBD) encompasses complex pathological and cellular injuries to the brain caused by hypoxia, ischemia, cytotoxicity, or a combination of these conditions [21]. Despite significant progress in elucidating the mechanism(s) underlying hypoxia–ischemia (H-I), there is currently a lack of promising neurotherapeutic interventions following HIBD for clinical application in infants.

It is well-known that the brain has some capacity for neurogenesis in response to various types of damage that result in the loss of neurons in adult mammalian brains following H-I, particularly in two regions, the dentate gyrus (DG) subgranular zone (SGZ) and subventricular zone in the lateral ventricles [11]. Endogenous neurogenesis after ischemic injury produces new cells that can integrate into neural networks and play active roles in the recovery from neurological deficits [6]. However, there is contradictory evidence as to whether neonatal H-I increases or decreases the number of hippocampal precursor cells [3,7,25].

Endogenous neurogenic capacity depends on not only the intrinsic properties of neural precursor cells (NPCs) but also the regional differences in extrinsic regulatory signals and receptor expression. Therefore, harnessing the abilities of such extrinsic factors to amplify neurogenesis represents a neurotherapy for neonatal H-I. The basic fibroblast growth factor (bFGF, also called FGF2) is a member of the FGF superfamily proteins. It binds to the tyrosine kinase receptors FGFR1–4 to activate downstream signaling [24]. bFGF plays an important role in neurogenesis and also promotes neural stem cell (NSC) proliferation and differentiation into neurons during development as well as in the adult mouse brain *via* pharmacological addition or blockade of bFGF [4,12,22]. However, bFGF-deficient mice exhibit no obvious deficit in NSC proliferation in the adult DG but exhibit impairments in the differentiation of NPCs [23]. bFGF efficiently promotes the regeneration of neurons after injury [2,14] and has also been shown to modulate axonal branching and synaptic plasticity *in vitro* and *in vivo* [1,18]. As a neurotrophic factor, bFGF is highly significant for the development of therapies as it has the potential to restore neurological deficits in neurodegenerative diseases and acute stress [8,9,15]. However, the role

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of bFGF in neurogenesis following neonatal H-I remains unexplored.

The present study aimed to investigate the correlation between neurogenesis and bFGF in the hippocampal DG region of neonatal rats following H-I. Our data indicated that cell proliferation in the DG is induced by neonatal H-I and that cell proliferation is promoted by bFGF following neonatal H-I. bFGF may have neuroprotective and neurorestorative properties for neurological deficits following neonatal H-I.

2. Material and methods

2.1. Materials

Postnatal day 7 Sprague–Dawley (SD) rats (weighing 12.70 ± 1.14 g; male and female) were purchased from the Animal Laboratory at the Medical College of Southeast University (Nanjing, China). BrdU *In-Situ* Detection Kits were purchased from BD Bioscience. Oligonucleotide primers for bFGF and GADPH used for RT-PCR were synthesized by SANGON Co., Ltd. TriPure isolation reagent was purchased from Roche Diagnostics Corp. RNA PCR Kits (AMV) version 3.0 were purchased from TaKaRa Biotechnology Co., Ltd (Dalian).

2.2. Animals

Procedures for the use of laboratory animals were approved by the institutional animal use and care committee, and the methods were conducted in accordance with the approved guidelines. For immunohistochemistry (IHC), SD rats were randomly divided into six groups (eight rats per group): sham, hypoxia, ischemia, H-I, normal saline (NS), and bFGF groups. Exogenous bFGF (10 μg/kg) was intraperitoneally injected every day for 7 days following H-I in the bFGF group, and saline was used as a control in the NS group. The rats were intraperitoneally injected with 100 mg/kg BrdU twice every week 48 h after different injuries. For RT-PCR, SD rats were randomly divided into four groups: sham, hypoxia, ischemia, and H-I groups. Animals were not injected with BrdU for RT-PCR analysis.

H-I was induced according to previously described methods [13]. Seven-day-old SD rats were anesthetized by inhaling diethyl ether. The left common carotid artery was isolated and ligated using 4-0 type silk. The rats were placed in a hypoxic chamber (8% oxygen, 92% nitrogen) for 2 h, then in enclosed, vented chambers that were partially submerged in water (37.0 °C). Animals in the sham group were subjected to isolation but no ligation and no subsequent hypoxia, animals in the hypoxia group were only exposed to hypoxia and not ligation, and animals in the ischemia group were ligated but not exposed to hypoxia.

2.3. Immunohistochemistry and hematoxylin and eosin (HE) staining

The rats were sacrificed at 3, 7, 14, 21 days after injury for IHC and at 0 h, 6 h, 24 h, 48 h, 5 days, 7 days, and 90 days (n = 8) after injury for HE staining. Brains were removed, postfixed in 4% paraformaldehyde, and then cut into sections. Paraffin-embedded tissue was cut to 4-μm-thick sections. For HE staining, sections were deparaffinized using xylene, rehydrated, stained using HE, dehydrated with alcohol, and cleared in xylene. Paraffin was removed from the sections using xylene according to the instructions on the BrdU *In-Situ* Detection Kit. Endogenous peroxidase activity was blocked in the sections. Antigen retrieval was performed using a microwave method. Biotinylated anti-BrdU antibodies were applied to the sections on the slide and incubated for 1 h at 37 °C. Ready-to-use streptavidin–HRP was then applied to each slide and incubated for 30 min at room temperature. DAB substrates were stained in hematoxylin. Sections were dehydrated, cleared, and mounted. Results were considered positive when the cell nuclei were stained dark brown. Phosphate-buffered saline was used as a negative control in the procedure. The small intestines of BALB/c mice were stained as a positive control.

For cell number quantitation, sections at 80 μm-intervals were chosen for analysis. BrdU-positive cells were counted from the DG region in four 400 × magnification images in each section of the brain at the different time points.

2.4. RT-PCR

Isolation of total mRNA from the hippocampus was performed using TriPure reagent, following the one-step guanidinium isothiocyanate and acidified phenol method according to the manufacturer's instructions. RNA concentration was determined using optical density measurements at 260 nm, and RNA purity and integrity were identified using an ultraviolet spectrometer and agarose gel electrophoresis.

First-strand cDNA was synthesized from 1 μg of total RNA in a 20-μL reaction volume by reverse transcription insolution (4 μL $MgCl_2$, 2 μL dNTP mixture, 1 μL OligodT-Adaptor primer, 0.5 μL RNase inhibitor, and 1 μL reverse transcriptase) at 42 °C for 30 min, 99 °C for 5 min, and 5 °C for 5 min. Ten microliters of product of the reverse transcription reaction were used as a template. PCR was run at 94 °C for 2 min, followed by 30 cycles at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 60 s, and finally at 72 °C for 8 min. Forward (5′-CTT TGG GTG GAA GGC TGG TCG-3′) and reverse (5′-TGC GGG AAG CGA AGT GAT GC-3′) primers were used to amplify a 448 bp rat bFGF fragment, and 308 bp GADPH served as a control with the following primers: sense 5′- TCA CTC AAG ATT GTC AGC AA-3′ and antisense 5′- AGA TCC ACG ACG GAC ACA TT-3′. Equal amounts of corresponding bFGF and GADPH products were loaded onto 1.5% agarose gels, and the optical densities of ethidium bromide-stained DNA bands were used to quantify the expression of the bFGF gene.

2.5. Statistical analysis

Data are presented as mean ± standard deviation. Statistical comparisons were made using analysis of variance. The Student–Newman–Keuls *q*-test was used for comparisons between two groups. $P < .05$ was considered statistically significant.

3. Results

3.1. Pathological changes and apoptosis of brain cells following H-I

The H-I model was produced according to the traditional Rice model [13]. Carotid artery ligation caused the ipsilateral eye to remain unopened after 90 days following H-I (Fig. 1a). Brains were collected, and atrophy of the left brain was observed (Fig. 1b). Brain damage of experimental animals in each group was compared at different time points, and hippocampal histopathological changes in each group were detected by the HE staining method. No evident damage was found in the control and sham operated groups (Fig. 1c). However, HE staining revealed edema and apoptosis of brain cells in the hippocampus, and the number of neurons was decreased at 24 h following H-I (Fig. 1d–f). The volume of the hippocampus was significantly reduced, and the pyramidal cells were randomly arranged. Necrosis was also observed after 5 days in the H-I model (Fig. 1g and h). The above data indicate neuronal damage in the neonatal brain following H-I.

3.2. Time course of cell proliferation in the hippocampus of neonatal brains following H-I

Endogenous neurogenesis was induced after ischemic injury in the adult mice brain. In order to investigate whether neurogenesis was also stimulated in neonatal brains following H-I, animals were intraperitoneally injected with BrdU and then sacrificed at specific time points after H-I. The number of newly generated cells was estimated in the entire DG region of the hippocampus by detecting the BrdU signal. The majority of BrdU-positive cells in the DG showed a dark brown color in the nuclei (Fig. 2a–d). There was no pronounced change in the

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