Nerve growth factor promotes in vitro proliferation of neural stem cells from tree shrews

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Abstract

Neural stem cells promote neuronal regeneration and repair of brain tissue after injury, but have limited resources and proliferative ability in vivo. We hypothesized that nerve growth factor would promote in vitro proliferation of neural stem cells derived from the tree shrews, a primate-like mammal that has been proposed as an alternative to primates in biomedical translational research. We cultured neural stem cells from the hippocampus of tree shrews at embryonic day 38, and added nerve growth factor (100 μg/L) to the culture medium. Neural stem cells from the hippocampus of tree shrews cultured without nerve growth factor were used as controls. After 3 days, fluorescence microscopy after DAPI and nestin staining revealed that the number of neurospheres and DAPI/nestin-positive cells was markedly greater in the nerve growth factor-treated cells than in control cells. These findings demonstrate that nerve growth factor promotes the proliferation of neural stem cells derived from tree shrews.

Key Words: nerve regeneration; tree shrews; hippocampus; neural stem cells; cell proliferation; nerve growth factor; neurosphere; embryo; cell number; cell therapy; in vitro; neural regeneration
Introduction

Neural stem cells (NSCs) are multipotent, self-renewing cells in the mammalian central nervous system, and promote neuronal regeneration and nervous tissue repair (Anderson and Michelsohn, 1989; Gould and Gross, 2002; Gritti et al., 2002). In addition to being used as a vector for gene therapy against nervous system diseases, NSCs can be induced to differentiate into neurons and promote the repair of brain tissue (Aleksandrova et al., 2005; Eslamboli et al., 2005).

NSCs derived from rats can facilitate the recovery of neuronal injury; however, when implanted in vivo they show limited ability to proliferate and differentiate (Tran et al., 2010; Chen et al., 2015). Changes in the culture environment influence the growth and metabolism of NSCs, and three-dimensional cell vectors can facilitate growth of NSCs in vitro (Song et al., 2014). Therefore, it is necessary to isolate NSCs and make them proliferate well in vitro first. The environment may affect the biological features of NSCs (Laywell et al., 2000; Lin et al., 2003; Zhang et al., 2003). Suitable conditions and drugs that can promote the proliferation of NSCs in the brain will pave a new way for rehabilitative therapy of nervous system diseases.

Nerve growth factor (NGF) plays a crucial role in the proliferation and development of stem cells (Xu et al., 2012), regulating normal cell development and promoting neuronal survival and regeneration (Benoit et al., 2001). However, it remains unknown whether NGF can promote the proliferation of NSCs derived from primates. The tree shrew is a small, arboreal mammal from the family Tupaiidae, which lives in the tropics and sub-tropics. Genomically, metabolically and anatomically, tree shrews are relatively closer to humans than rodents; coupled with their small size and ease of breeding (Janecka et al., 2007; Fan et al., 2013; Xu et al., 2013), they are a promising convenient and low-cost substitute for rodents and primates in animal studies. They have already been widely used in studies of physiology, anatomy, virology, neuropsychiatry, neural development, and nervous system diseases (Wang et al., 2011, 2012; Ruan et al., 2013; Xu et al., 2014). NSCs derived from the tree shrew could provide a suitable cell model for studying human diseases that may require stem cell therapy.

Therefore, in the present study, we explored the conditions for the culture of tree shrew-derived NSCs, and studied the effect of NGF on their growth.

Materials and Methods

Animals

Six clean, pregnant female tree shrews, at 38 days of gestation and weighing 170 g, were provided by the Experimental Animal Center of Kunming Medical University, China (Animal license No. SYXK (Dian) K2015-0002). Tree shrews were housed in individual cages under a 12 hour light/dark cycle and in a dry and ventilated room at 23–25°C, with free access to food and water. All surgery was performed under anesthesia, and all efforts were made to minimize pain and distress in the experimental animals. All procedures were carried out in accordance with the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals (Publication No. 85-23, revised 1986). Animal care and all experimental protocols were approved by the guidelines of the Institutional Medical Experimental Animal Care Committee of Sichuan University, West China Hospital, China.

Sample harvest

The animals were sacrificed after intraperitoneal anesthesia with 3.6% chloral hydrate (1 mL/100 g) (SG1019; TongYao Biological Technology Co., Ltd., Shanghai, China). On a superclean bench, the fetuses were extracted under sterile conditions, and placed into culture dishes containing D-Hank’s solution. The skulls were carefully dissected, and the brains removed and kept in pre-cooled phosphate buffered saline (PBS). Under an anatomical microscope, the meninges, olfactory bulb, cerebellum and brain stem were removed to expose the hippocampus. These were harvested and washed twice with pre-cooled PBS.

Preparation of cell suspension

Hippocampal tissue was kept in pre-cooled Dulbecco’s modified Eagle’s medium (DMEM)/F12 (1:1; Gibco, New York, NY, USA). The tissue was cut into 1 mm³ blocks using microscissors, and transferred into centrifuge tubes. Subsequently, all samples were digested with 0.25% trypsin (1:250; Millipore, Temecula, CA, USA) for 20 minutes, treated with DMEM/F12 to stop the digestion, and then centrifuged at 800 × g for 5 minutes. Supernatant was discarded, 100 mL DMEM/F12 (1:1; Gibco) was added to the cell suspension, with 2 mL B27 (Gibco), 2 mM glutamine, 2 μg basic fibroblast growth factor (PeproTech, Rocky Hill, NJ, USA) 1% N2 (Gibco), 10,000 U/L penicillin and 10 mg/mL streptomycin. The cell suspension was then harvested.

Cell inoculation

The density of the cell suspension was adjusted to 5 × 10⁶/mL. The cells were placed in an incubator (Thermo, Marietta, OH, USA) containing 5% CO₂ at 37°C for 48 hours and half of the culture medium was replaced every other day for 7 days. After 2 days in culture, the cultures were centrifuged at 800 × g for 5 minutes, the supernatant was discarded; this process was repeated two more times. All samples were then digested with 1 mL 0.25% trypsin at room temperature for 15 minutes and centrifuged again at 800 × g for 5 minutes. Stem cell culture medium was added and the culture plates were inoculated. Cells were cultured in an incubator (Thermo) at 5% CO₂ and 37°C.

NSC subculturing

NSCs were subcultured once every seven days when the neurospheres reached 100 μm in diameter. The NSC suspension was collected into 15 mL of sterile centrifuge tubes and centrifuged at 800 × g for 5 minutes. The supernatant was discarded. The cells were resuspended in the DMEM/F12 medium containing 20 ng/mL fibroblast growth factor and 20 ng/mL epidermal growth factor. Finally, the cell suspension was mixed gently.
and incubated at 37°C in 25 mL culture bottles at a density of 1.5–2.5 × 10⁵/mL.

**NSC identification**

The third passage of NSCs was digested with 0.25% trypsin, and the digestion was terminated with culture medium containing the serum. The cell suspension was dropped onto the cover slips for 3 days of incubation. Nestin immunocytochemistry (1:200; Chemicon, Lansing, NC, USA) was used to identify the cultured cells as NSCs. Red-staining nestin-immunoreactivity showed that the red color mainly existed in the cytoplasm. 4′,6-Diamidino-2-phenylindole (DAPI) was used to label nuclei, which appeared blue. Together, this confirmed that the cultured cells were NSCs.

**NGF administration and morphology**

Cultured NSCs were divided into two groups: NGF-induced (NGF-NSC), and control. NGF (100 μg/L; Gibco) was added to DMEM/F12 culture medium in the NGF-NSC group but not the control group. After 3 days of incubation, in vitro samples were prepared in 6-well plates. To determine the number of neurospheres and the NSC area, cells were collected from five fields of each well, and photographed with a fluorescent microscope (Leica, Wetzlar, Germany) at 200× magnification. Neurosphere number and NSC area were calculated with Image-Pro Plus 6.0 software (MediaCybernetics, Silver Spring, MD, USA). The neurosphere count was processed by three experimenters blinded to the type of treatment. The mean of their counts was the final result. For NSC area, we used the average pixels / sum area. The thresholds for NSC staining intensity were determined after all images were acquired. The total pixel count per image field (300 × 300) was measured. Average pixel count was calculated as the ratio of total pixels per image field relative to the total area (average pixels = sum pixels / sum area).

**Statistical analysis**

Data are expressed as the mean ± SD, and were analyzed using SPSS 18.0 software (IBM Corporation, Armonk, NY, USA). Student’s t-test with two-tailed distribution was used to compare means. P < 0.05 was considered statistically significant.

**Results**

**Growth of NSCs derived from tree shrews**

At 12 hours of primary culture, small, transparent, round or oval single cells were mainly observed in the suspension. Most cells had no processes and good refraction (Figure 1A). After 2 days in culture, tens of cells aggregated to form neurospheres that gradually enlarged over time (Figure 1B). Suspended growth of surviving neurospheres was observed until 7 days in culture (Figure 1C). The passaged neurospheres grew further in number and volume, and became spheres that consisted of tens to hundreds of cells. After subculturing, single cells and small cell clumps could be seen. Some cells died, and others proceeded to the cleavage phase.

**Identification of NSCs**

Nestin immunoreactivity was present in the neurospheres, mainly in the cytoplasm (Figure 2A). DAPI labelling was mainly distributed in the nuclei (Figure 2B). Nestin- and DAPI-positive staining co-existed in the cultured NSCs (Figure 2C), indicating that we had successfully isolated NSCs from tree shrews. This provided a good foundation for the subsequent experiments.

**Role of NGF in the morphology of NSCs**

At 3 days of culture post NGF administration, single cells and small cell clumps were observed in the cultured NSCs. Notably, some neurospheres aggregated with stronger refraction over time, and the number of neurospheres was higher in the NGF-NSC group than in the control group (Figure 3A, B).

**NGF effects on the proliferation of neurospheres**

At 3 days of culture, the number of neurospheres was significantly greater in the NGF-NSC group than in the control group (P < 0.01; Figure 4A). However, the average area of NSCs in the first passage of cells was lower in the NGF-NSC group than in the control group (P < 0.01; Figure 4B). This suggested that NGF plays a crucial role in NSC proliferation and promotes the proliferation of neurospheres. New NSCs had a relatively small area, however.

**Discussion**

In this study, we found that primary cultured NSCs derived from tree shrews proliferated successfully, from single cells into tens, and aggregated to form neurospheres under our culture conditions. Moreover, following NGF administration, the number of neurospheres in the cultured NSCs was significantly greater, but the average cell area was markedly smaller, than in control cells. These findings show that NGF effectively promoted the growth of tree shrew NSCs in vitro, and provide new evidence to demonstrate the biological characteristics of NSCs in these primate-like mammals.

**Characteristics of NSCs from embryonic tree shrew in vitro**

Nestin is used as a marker of NSCs, because it is associated with the survival, renewal and mitogen-stimulated proliferation of neural progenitor cells (Yuan et al., 2015). In the present study, red nestin fluorescence confirmed that NSCs from the tree shrew had been successfully isolated and cultured. In addition, the number of primary cultured NSCs from tree shrews increased. Tens of cells aggregated to form neurospheres, which enlarged over time. These results suggest that the tree shrew-derived NSCs we cultured had the ability to self-renew and proliferate.

**NSC proliferation induced by NGF**

Following NGF induction, the number of neurospheres was significantly greater than without NGF, and most new neurospheres consisted of small cells. This suggests that NGF
induced proliferation in cultured tree shrew NSCs. However, the proliferative ability of NSCs is limited and the molecular mechanism of NSC proliferation and differentiation is complex. It is widely accepted that NSC proliferation and differentiation are related to the internal cellular environment (Kalyani et al., 1997; Liu et al., 1999; Zhang et al., 2002; Gao and Zheng, 2013). In rodent NSCs, various factors influence the quality of the cultured cells, including sample harvesting time, cell density, subculturing time, cytokines, and serum concentration (Li et al., 2010; Zhang et al., 2011; Marei et al., 2013; Xu et al., 2013). During NSC culture, apart from essential basic culture medium, some growth factors need to be added. Without sufficient growth factor concentration,
cell numbers in culture will not increase (Marei et al., 2013). NGF, an important neurotrophic factor, plays a crucial role in the division and proliferation of nerve cells (Doering and Snydeer, 2000; Wachs et al., 2003; Oliveira et al., 2015), and in axonal growth and cellular network formation (Guo et al., 2013). Therefore, when the nervous system is injured, NGF is involved in the growth of most neurons, especially in anti-apoptosis and promoting recovery of neural function (Carito et al., 2015; Turney et al., 2015). However, these previous studies did not address the role of NGF in the proliferation of tree shrew-derived NSCs. To our knowledge, we are the first to address this issue.

Future application in translational medicine
NSC transplantation may be a strategy to promote recovery from central nervous system diseases in translational research (Pilitti et al., 2013; Nizzardo et al., 2014). Establishing non-human primate models of human diseases is an efficient way to narrow the large gap between basic studies and clinical medicine (Wang et al., 2013). Tree shrews are advantageous as they are relatively closer to humans and primates than rodents (Fan et al., 2013; Xu et al., 2013). It is therefore important to study the effects of NGF in NSCs isolated from the tree shrew in vitro. The limited ability of NSCs implanted in vivo to proliferate and differentiate into neurons (Tran et al., 2010) means it is important to find an agent that will increase the number of NSCs in culture. From our study, we conclude that NGF promotes the proliferation of tree shrew-derived NSCs, and has the potential to increase the number of cultured NSCs for later transplantation.

The mechanisms by which NGF promotes NSC proliferation are complex (Van Kanegan and Strack, 2009; Yuan et al., 2013) and will be addressed in our future experiments.

In summary, we successfully established an in vitro cultured tree shrew NSC model. We observed the morphological changes in NSC cultures in vitro, and explored the effect of NGF. We showed that our culture conditions for tree shrew-derived NSCs were successful, and also that NGF is effective for their growth. Our study provides valuable insight into the role of NGF in NSCs derived from the tree shrew, a promising alternative to primates in translational research.

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