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Rapid communication

Treatment of hypoxic–ischemic encephalopathy in mouse by transplantation of embryonic stem cell-derived cells

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Abstract

A 7-day-old hypoxic–ischemic encephalopathy (HIE) mouse model was used to study the effect of transplantation of embryonic stem (ES) cell-derived cells on the HIE. After the inducement *in vitro*, the ES cell-derived cells expressed Nestin and MAP-2, rather than GFAP mRNA. After transplantation, ES cell-derived cells can survive, migrate into the injury site, and specifically differentiate into neurons, showing improvement of the learning ability and memory of the HIE mouse at 8 months post-transplantation. The non-grafted HIE mouse brain showed typical pathological changes in the hippocampus and cerebral cortex, where the number of neurons was reduced, while in the cell graft group, number of the neurons increased in the same regions. Although further study is necessary to elucidate the precise mechanisms responsible for this functional recovery, we believe that ES cells have advantages for use as a donor source in HIE.

Keywords: Hypoxic–ischemic encephalopathy; Embryonic neural stem cell; Neonatal mouse; Cells transplantation

1. Introduction

Hypoxic–ischemic encephalopathy (HIE), a common cause of neurological disability in adults and children (Davenport and Dennis, 2000; Vannucci and Perlman, 1997), causes extensive loss of the cerebral parenchyma and of the cells and connections that reside there (Johnston et al., 2001). Although promising neuroprotective strategies have been studied, current management of HI brain injury has been limited to supportive measures; no strategy for reconstitution has yet been found clinically effective (Deacon et al., 1998; Du Plessis and Johnston, 1997; Vannucci and Perlman, 1997; Del Zoppo et al., 2000).

Stem cells or progenitor cells therapies are a promising strategy for the treatment of stroke and HIE (Chen et al., 2006). Human embryonic mesenchymal stem cells (hMSCs) transplantation can increase the survival rate of rats with HIE, and improve their learning and memory ability (Xie et al., 2006; Zhu et al., 2005). After transplantation of multipotent astrocytic stem cells (MASCs) into a rat model of neonatal HIE, MASCs can survive and differentiate into neurons and astrocytes in the post-injury milieu of the neonatal brain injured by hypoxic ischemia (HI) (Zheng et al., 2006). Recently, there has been a growing interest in the therapeutic potential of ES cell-derived neural precursors for therapy in stroke and HIE. Following transplantation into the central nervous system (CNS) of the rodent, ES cell-derived neural precursors have been shown to integrate into the host tissue and yield a functional improvement (Kim et al., 2002). However, it remains to be determined whether ES cell-derived precursors can differentiate into neural cells and yield functional improvement in neonatal HIE mice.

In this study, we have determined that ES cell-derived precursors could integrate into the host brain, differentiate into neuronal cells, and yield a functional improvement.

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2. Experimental procedures

2.1. Neural stem cell culture

A mouse ES cell line, S8, was kindly provided with marker gene LacZ from Prof. Sheng Huizhen (Key Laboratory of Development Biology). Undifferentiated ES cells were plated on mitotically-inactivated primary mouse fibroblasts and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with (final concentrations) 10% fetal calf serum (FCS), 1000 U/ml of mouse recombinant leukaemia inhibitory factor (LIF), 0.1 mM 2-mercaptoethanol, 0.1 mM non-essential amino acids, 2 mM glutamine, 100 U/ml of penicillin, and 100 mg/ml streptomycin. After trypsinized (0.05% trypsin–EDTA), the ES cells were resuspended and seeded at about 5×10^5 cells in 15 ml of DMEM supplemented with 10% FCS, 500 nM all-trans retinoic acid (RA, Sigma), 100 U/mL penicillin, and 100 mg/ml streptomycin in a 100 mm Petri dish. In culture for 6 days, cells developed into round spheres that were determined to be embryonic bodies (EBs). To induce neural differentiation, the formed EBs were collected and maintained on gelatin-coated dishes supplemented with medium B (DMEM, 10% FBS, 10% Horse Serum, 1% Chick Embryo Extract Ultrafiltrate, 1% Penicillin–Streptomycin) in culture for 9 days. Cells were then enzymatically dissociated into single cells and resuspended with PBS at a density of 10^4 cells/μl^{-1}.

2.2. RT-PCR analysis

Total RNA was extracted from EBs (1 week after passage) or three mice at 2 months and 8 months after cell transplantation using Trizol reagent (Invitrogen). cDNA synthesis was carried out using the SuperScript II first-strand synthesis system (Invitrogen). The resulting complementary DNA was amplified by polymerase chain reaction (PCR) with the TaKaRa Ex Taq PCR system. Three independent PCRs were done: loop (15 s 94 °C, 30 s 56–58 °C, 45 s 72 °C). The number of cycles varied between 25 and 35, depending on the abundance of particular mRNA. The primers and the length of the amplified products were shown in Table 1. RT-PCR products were analyzed in an agarose gel containing ethidium bromide. To analyze the relative expression of different mRNAs, the amount of cDNA was normalized by amplifying glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene transcripts.

2.3. HI brain injury mouse model

The right common carotid artery of anesthetized 1-week-old C57BL/6 mice was ligated with a surgical silk suture through a ventral midline neck incision. The incision was closed, and the animals were kept warm until they awoke. The mice were then returned to their dams for 2 h. Environmental temperature during surgery and recovery was kept at 28 °C. Mice were then placed for 90 min in a tightly sealed hypoxia chamber, with constant flow (350 ml/min) of a hypoxic atmosphere of 8% O_2 and 92% N_2. The hypoxia chamber was kept in a temperature-controlled water bath to maintain the ambient temperature inside the chamber at a normal range (37–38 °C). Following hypoxic exposure, pups were returned back to their mothers for recovery. C57bL/6 mouse are small enough to observe the physiological markers, we performed the operation under microscope, and the operation time is only ten minute, there is no big change of physiological markers during the short time. We found, during 20 min after the hypoxia process, most of the mouse had spasm onset and retard in action. Survival ratio of HIE mice in this experimental paradigm is 83.3%.

2.4. Cell transplantation

One-week-old C57BL/6 mice from the animal center of the China Science Academy were randomly divided into three groups as follows: the control group, the control operated group (HIE/PBS) and the stem cell graft group (HIE/Esc). Each group contained nine mice. At 2–3 days after HI brain injury, each anaesthetized mouse received 3 μl of PBS or 3 μl of ES cells transplants (1×10^4 cells/μl) in HIE/PBS group or HIE/Esc group, respectively, using a Kopf stereotaxic frame (Kopf Instruments, Tujunga, CA), grafted into the right side of the lateral ventricle of the brain (AP, 0.02 mm; ML, 0.75 mm; DV, 2 mm) over a period of 3 min and retracted after an additional 2 min. The procedures were overseen by the Animal Care and Use Committees of Xinhua Hospital.

2.5. X-gal histochemistry and immunohistochemistry

Three mice at 2 months or 8 months after cell transplantation were anaesthetized with 10% SU-MIAN-XIN injected into the abdominal cavity and perfused transcardially with 30 ml of fixative composed of 4% PFA in sodium phosphate buffer (PB; 0.1 M, pH 7.4). The brains fixed in 4% PFA in PBS for 60 min at 4 °C were rinsed three times with PBS for 30 min, and dehydrated in 30% sucrose overnight at 4 °C. Serial coronal sections were cut on a cryostat microtome at 10 μm thickness. After washing three times with buffer (0.1% NaH_2PO_4/Na_2HPO_4, pH7.4, 2 mM $MgCl_2$, 0.1% Triton X-100), the sections were incubated in X-gal staining solution (1 mg/ml X-gal, 5 mM ferricyanide, and 5 mM ferrocyanide at pH 7.4 in wash buffer) for 16–24 h at 37 °C and then subjected to immunofluorescence labeling with the following primary antibodies: rabbit antibody against NF (1:100; Sigma) or GFAP (1:200; DAKO). All subsequent procedures were performed using the TSA Trade Mark-Plus Fluorescence Palette System kit (NEN Life Science). For a simultaneous detection of X-gal with cell type-specific marker, the sections were exposed to visible light and fluorescent light within the same field.

2.6. Hematoxylin and eosin staining

The sections were washed three times with buffer (0.1% NaH_2PO_4/Na_2HPO_4, pH 7.4, 2 mM $MgCl_2$, 0.1% Triton X-100) and then were incubated with hematoxylin and eosin. Sections were dehydrated through graded alcohols, cleared in xylene, and coverslipped in Canadian balsam solution. Cell loss in brain sections was determined using a light microscope (Olympus PM-20).

2.7. Cell counting

Ten slices each mouse taken from the same part of the hippocampus (when it came to CA1 region, we pick one every 3 slice, 10 slices in total) were performed the hippocampal cell counting under microscope (400×) especially for CA1 region with double blind method. Two continuous fields from each slice were counted; we kept the hippocampal cell at the diameter of the field for equal condition. The mean number of pyramidal neuron in the 10 slices is used as the hippocampal cell number of each mouse.

Table 1
The following forward (F) and reverse (R) primers nucleotide sequences were used for RT-PCR and PCR

Gene	Primer	Length (bp)
For RT-PCR		
Nestin	F: 5′-CAGAGAGGCGCTGGAACAGAGATT-3′; R: 5′-AGACATAGGTGGGATGGGAGTGCT-3′	469
MAP-2	F: 5′-AGCCGCAACGCCAATGGATT-3′, R: 5′-TTTGTTCCGAGGCTGGCGAT-3′;	313
GFAP	F: 5′ -GCGCTCAATGCTGGCTTCAA-3′, R: 5′-ACGCAGCCAGGTTGTTCTCT-3′;	346
GAPDH	F: 5′-CTCGTCTCATAGACAAGATGGTGAAG-3′; R: 5′-AGACTCCACGACATACTCAGCACC-3′	305
For PCR		
LacZ	F: 5′-CCAGTTCAACATCAGCCGCTACAG-3′; R: 5′-CACCAGACCAACTGGTAATGGTAG-3′	200
GAPDH	F: 5′ ATTCCTTGCCAAGAGGTGAC-3′; R: 5′-CAGCAAGGCAAGGTAGTGTG-3′	300