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TRANSPLANTATION OF NSC-DERIVED CHOLINERGIC NEURON-LIKE CELLS IMPROVES COGNITIVE FUNCTION IN APP/PS1 TRANSGENIC MICE

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Abstract—The ability to selectively control the differentiation of neural stem cells (NSCs) into cholinergic neurons *in vivo* would be an important step toward cell replacement therapy. First, green fluorescent protein (GFP)-NSCs were induced to differentiate into cholinergic neuron-like cells (CNLs) with retinoic acid (RA) pre-induction followed by nerve growth factor (NGF) induction. Then, these CNLs were transplanted into bilateral hippocampus of APP/PS1 transgenic mice. Behavioral parameters showed by Morris water maze (MWM) tests and the percentages of GFP-labeled cholinergic neurons of CNL transplanted mice were compared with those of controls. Brain levels of choline acetyltransferase (ChAT) mRNA and proteins were analyzed by quantitative real-time PCR (RT-PCR) and Western blotting, ChAT activity and acetylcholine (ACh) concentration were also evaluated by ChAT activity and ACh concentration assay kits. Immunofluorescence analysis showed that 80.3 ± 1.5% NSCs differentiated into CNLs after RA pre-induction followed by NGF induction *in vitro*. Three months after transplantation, 82.4 ± 6.3% CNLs differentiated into cholinergic neurons *in vivo*. APP/PS1 mice transplanted with CNLs showed a significant improvement in learning and memory ability compared with control groups at different time points. Furthermore, CNLs transplantation dramatically increased in the expressions of ChAT mRNA and protein, as well ChAT activity and ACh concentration in APP/PS1 mice. Our findings support the prospect of using NSC-derived CNLs in developing therapies for Alzheimer's disease (AD). © **2015 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: neural stem cells, cholinergic neuron-like cells, transplantation, Alzheimer's disease, retinoic acid, nerve growth factor.

INTRODUCTION

Alzheimer's disease (AD) is the most common neurodegenerative disorder among senile people. It is characterized by progressive and irreversible cognitive decline (Wu et al., 2010; Park et al., 2012). One of the fundamental neuromorphological changes of AD is the serious loss of cholinergic neurons and widespread degeneration of cholinergic functions in the brain (Nakao et al., 2006; Moghadam et al., 2009). Current treatments for AD mainly focus on augmenting residual cholinergic neurotransmission by using actylcholinesterase (AChE) inhibitors (Zhang et al., 2010). However, because drug therapies could only improve symptoms in mild to moderate patients rather than reverse neuronal loss and some of these drugs have serious side-effects (Iqbal and Grundke-Iqbal, 2010). Therefore, there is an urgent need for effective therapies for AD.

It is well known that intrinsic factors in the brain environment induce differentiation of stem cells. For example, Park et al. found that adipose tissue-derived mesenchymal stem cells (ADSCs) were differentiated into neurons (NF-positive) as well as cholinergic neurons (ChAT-positive) after intracerebrospinal transplantation (Park et al., 2013). Previous studies also showed transplanted neural stem cells (NSCs) differentiated into neurons and astrocytes and improved the learning and memory deficits of AD animals (Blurton-Jones et al., 2009; Park et al., 2012; Zhang et al., 2014). However, other studies indicated that transplanted NSCs may remain in an undifferentiated state and formed glioblastoma or most of them differentiated into astrocytes *in vivo* and compromise the effects (Teng et al., 2002; Lu et al., 2003; Casalbore et al., 2009; Wang et al., 2011). Therefore, additional data or experiments would be required and stem cell-derived progenitor cells may offer hope for cell replacement therapies for neurodegenerative diseases.

Retinoic acid (RA) is the most potent natural form of vitamin A and is well-known to be one of the most potent inducers for the acquisition of neuronal fate and cholinergic phenotype in many different cell types and cell lines (Hill and Robertson, 1998), including septal cell lines (Holler et al., 1996) and hES cells (Nistor et al., 2011). Nerve growth factor (NGF) is the best characterized trophic factor for cholinergic neurons in the adult brain and studies indicated that NGF can induce maturation of precursors toward cholinergic differentiation. In this study, we described the sequential use of RA and NGF to induce NSCs to differentiate *in vitro* and

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Abbreviations: ACh, acetylcholine; AChE, actylcholinesterase; AD, Alzheimer's disease; ChAT, choline acetyltransferase; CNLs, cholinergic neuron-like cells; GFP, green fluorescent protein; MWM, Morris water maze; NGF, nerve growth factor; NSCs, neural stem cells; PBS, phosphate-buffered-saline; PFA, paraformaldehyde; RA, retinoic acid; RARs, retinoic acid receptors; TQ, target quadrant.

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transplanted these cholinergic neuron-like cells (CNLs) into the bilateral hippocampus of APP/PS1 mice. The purpose of the current study was twofold: (1) to determine whether NSCs could differentiate effectively into CNLs during *in vitro* culture after RA pre-induction followed by NGF induction and (2) to evaluate whether CNL transplantation could improve cognitive ability and restore cholinergic function in APP/PS1.

EXPERIMENTAL PROCEDURES

Animals and groups

All surgical procedures were approved by Shanghai Ethics Committee and all experiments were conducted in accordance with guidelines from the Chinese Animal Welfare Agency.

Seventy-five 9-month-old male APP/PS1 double transgenic mice (B6C3-Tg (APPswe, PSEN1dE9)85Dbo/J, the Jackson Laboratory, USA) and 25 control wild-type littermates (Wt group) were used. Mice were raised in separate cages in a 12-h light/dark cycle at constant temperature (22 °C ± 1) with free access to food and water. APP/PS1 mice were divided into two groups randomly: APP/PS1 group (received no treatment, $n = 25$), PBS group (received PBS injection, $n = 25$) and CNL group (received CNLs injection, $n = 25$).

NSCs culture and cholinergic-like neuronal differentiation

NSCs were isolated from embryonic day-14 GFP-transgenic C57BL/6 mice fetal forebrain (Cyagen Biosciences Inc., China). This cell line constitutively expresses green fluorescent protein (GFP), thus allowing us to distinguish between transplanted and host cells. Cells were cultured as free-floating "neurospheres" in ultra-low binding 25 cm^2 or 75 cm^2 flasks (Corning, USA). The expanding medium consists of DMEM/F12 serum-free culture (Invitrogen, Carlsbad, CA, USA), supplemented with 2% B27 supplement (Invitrogen, USA), 20 ng/ml recombinant human basic fibroblast growth factor (bFGF) (Invitrogen, USA), 20 ng/ml recombinant human epidermal growth factor (EGF) (Invitrogen, USA) and 1% penicillin and streptomycin (Beyotime, China). The basic neuronal differentiation medium consists of neurobasal medium (Invitrogen, USA), 2% N2 supplement (Invitrogen, USA), 2 mM glutamine (Invitrogen, USA), 1 μM cAMP (Sigma, USA).

For cholinergic differentiation, NSCs were dissociated by accutase (Gibco, USA) and plated on polyornithine/laminin-coated T-75 flasks. The differentiation protocol consisted of a pre-induction stage using 10 μM RA (Sigma, USA) per day for 5 days followed by an induction stage using 100 ng/ml NGF (R&D, USA) per day for 9 days.

Immunocytochemistry and cellular quantification

For immunocytochemistry assay, NSCs were dissociated by treatment with accutase and plated on polyornithine/laminin-coated coverslips at equal density (5×10^5 cells/ml). After preinduction by RA (10 μM) for 5 days, NGF (100 ng/ml) was added into the differentiation every other day for 9 days. Then, cells were fixed with 4% paraformaldehyde (PFA) (Sigma, USA) for 30 min, permeabilized with 0.25% Triton X-100 for 30 min, then blocked with 10% donkey serum albumin (Abcam, USA) and incubated with primary antibodies in PBS at 4 °C overnight. The primary antibodies were mouse anti-nestin (1:300, Abcam, USA), mouse anti-β-tubulin-III (TuJ1) (1:200, Sigma), mouse anti-Map2 (1:200, Abcam, USA), rabbit anti-GFAP (1:200, Abcam, USA), rabbit anti-Galc (1:200, Abcam, USA), rabbit anti-ChAT (1:200, Abcam, USA), rabbit anti-p75 (1:200, Abcam, USA). Then cells were washed with PBS and incubated with fluorescent secondary antibodies (Alexafluor-555 or Alexafluor-647 conjugated, Abcam, USA) for 2 h at 37 °C. Meanwhile, nuclei were counterstained with Hoechst33342 (Beyotime, China). Images were visualized using a Nikon ECLIPSE Ti-S fluorescence microscope (Nikon Instruments, Japan) or a confocal microscope (Karl Zeiss 710, Germany). The quantification analysis was performed as described (Marjanska et al., 2005).

Cell transplantation

Fourteen days later, CNLs were dissociated with accutase (Invitrogen, USA) and centrifuged at 250*g* for 5 min. The pellet was resuspended in PBS and two more centrifugations and resuspensions were performed to wash the cells and remove non-viable cells. Mice were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and placed on a stereotaxic apparatus. Rectal temperature was regulated at 37–38 °C by means of a thermostatically controlled heating blanket. For the CNL group, about 1×10^5 CNLs in 2 μl PBS were injected into each side of the hippocampus in 5 min at the following stereotaxic coordinates: −2.00 mm anterior, ±1.85 mm lateral, and −2.50 mm ventral relative to bregma (Kiyota et al., 2009, 2011). The cannula was left *in situ* for 5 min to allow diffusion into the surrounding tissue before being slowly withdrawn. Sham-operated mice received the same volume of PBS.

Behavioral tests

To examine spatial learning and memory abilities, Morris water maze (MWM) tests were performed as described (Liu et al., 2013). In this study, MWM were performed 2 weeks before transplantation, and 1 month, 2 months and 3 months after cell transplantation. The MWM system consists of a black pool (120 cm in diameter) filled with water (22 ± 2 °C). A circular transparent platform (12 cm in diameter) was placed in the middle of the target quadrant (TQ) and submerged 2 cm below the water surface. The behavioral analysis was performed on a laptop computer using ANY-maze software (Stoelting Company, USA).

The MWM tests consisted of two procedures: the place navigation test and the spatial probe test. During the place navigation test mice were trained with a protocol of four trials per day, with an interval of 20 min, for four consecutive days, and the latency to find the

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