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Improvement by Human Oligodendrocyte Progenitor Cells of Neurobehavioral Disorders in an Experimental Model of Neonatal Periventricular Leukomalacia

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Abstract

The effects of human oligodendrocyte progenitor (F3.olig2) cells on improving neurobehavioral deficits were investigated in an experimental model of periventricular leukomalacia (PVL). Seven-day-old male rats were subjected to hypoxia-ischemia-lipopolysaccharide injection (HIL), and intracerebroventricularly transplanted with F3.olig2 (4×10^5 cells/rat) once at postnatal day (PND) 10 or repeatedly at PND10, 17, 27, and 37. Neurobehavioral disorders were evaluated at PND14, 20, 30, and 40 via cylinder test, locomotor activity, and rotarod performance, and cognitive function was evaluated at PND41–45 through passive avoidance and Morris water-maze performances. F3.olig2 cells recovered the rate of use of the forelimb contralateral to the injured brain, improved locomotor activity, and restored rotarod performance of PVL animals; in addition, marked improvement of learning and memory function was seen. It was confirmed that transplanted F3·olig2 cells migrated to injured areas, matured to oligodendrocytes expressing myelin basic protein (MBP), and markedly attenuated the loss of host MBP in the corpus callosum. The results indicate that the transplanted F3.olig2 cells restored neurobehavioral functions by preventing axonal demyelination, and that human oligodendrocyte progenitor cells could be a candidate for cell therapy of perinatal hypoxic-ischemic and infectious brain injuries including PVL and cerebral palsy.

Keywords

periventricular leukomalacia, neurobehavioral deficit, oligodendrocyte progenitor cell (OPC), myelination

Introduction

Cerebral palsy (CP), one of the most devastating neural diseases, exhibits diverse neurobehavioral symptoms following periventricular leukomalacia (PVL), and results from perinatal asphyxia as well as intrauterine infection[1,2]. In particular, it is well known that respiratory dysfunction is a predominant factor in pre-term infants in which there is a very high incidence of CP[3,4]. Motor, perceptual, visual, behavioral, and/or cognitive disorders occur in the majority of cases with PVL[5,6].

The process of oligodendrocyte development begins with the oligodendroglial progenitor cells (OPCs, preoligodendrocytes) differentiating into immature oligodendrocytes that sheathe axons, and finally into myelin-producing oligodendrocytes[7,8]. In the human central nervous system (CNS) during the active myelination period [gestational weeks (GW) 24–40], late preoligodendrocytes and immature oligodendrocytes are particularly susceptible to hypoxia-ischemia (HI) insults, lipopolysaccharide (LPS), and pre-immunocytokines[7,9–13]. Therefore, loss of myelin and delayed myelination by oligodendrocytes are key features of PVL in premature infants.

Current therapeutic strategies are very limited and restricted to supportive intensive care. For example, clinical hypothermia[14–16] and experimental administration with erythropoietin, minocycline or *N*-acetyl-L-cysteine[17–19] were

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found to delay the progress of bodily dysfunction. Recent research has reported that various stem cells such as mesenchymal stem cells (MSCs), neural stem cells (NSCs), and multipotent progenitor cells have beneficial effects on HI brain injury[20–23]. It has also been suggested that MSCs can differentiate into neurons and oligodendrocytes, and thereby contribute to the repair of the injured brain[24,25].

Human NSCs have emerged as a highly effective source of cells for treatment of CNS diseases, where genetically modified NSCs survive, integrate into host tissues, and differentiate into neurons and glial cells after transplantation[26]. F3.olig2 OPCs were established by transducing the Olig2 transcription factor gene to a human NSC line (F3)[27]. In previous studies, it was suggested that transplantation of the F3.olig2 cells may be an effective strategy to improve functional outcomes following spinal cord injury[28] as well as in demyelinating experimental allergic encephalopathy (EAE)[29].

Based on these results and the underlying mechanisms, the present study was carried out to determine whether transplantation of F3.olig2 OPCs to neonatal rats with hypoxia-ischemia-LPS (HIL) brain injury improves neurobehavioral and cognitive functions, in addition to neuroprotection.

Materials and Methods

Establishment of F3.olig2 OPCs

In order to establish F3.olig2 OPCs we used HB1.F3 (F3), which is an immortalized human NSC line previously established from primary cultures of a 15-week gestational human fetal brain by infecting with a retroviral vector encoding the v-*myc* oncogene[30,31]. Permission to use the fetal tissues was granted by the Clinical Research Screening Committee involving Human Subjects of the University of British Columbia, Canada, and the fetal tissues were obtained from the Anatomical Pathology Department of Vancouver General Hospital.

To generate Olig2 overexpressing OPCs, Olig2 cDNA was ligated into multiple cloning sites of the retroviral vector pLPCX. The PA317 amphotropic packaging cell line was infected with the recombinant retroviral vector, and the supernatants from the packaging cells were added to the F3 cells. Stably transfected colonies were selected by puromycin resistance, and the immortalized F3.olig2 OPC line exclusively differentiated into O4- and CNPase-positive oligodendrocytes, overexpressing Olig2 *in vitro*[27–29].

Animal Model of CP

Pregnant Sprague-Dawley rats were purchased from Daehan-Biolink (Eumseong, Korea). The animals ($n = 7$/group) were maintained at a constant temperature (23 ± 2°C), relative humidity of $55 \pm 10\%$, and 12-hour light/dark cycle, and fed with standard rodent chow and purified water *ad libitum*. Neonates were obtained from natural delivery, and male pups of post-natal day (PND) 7 underwent HIL; i.e. their left common carotid artery was occluded and placed in an 8% oxygen/92% nitrogen incubator (36°C) for 2 hours[18,32]. Finally, the pups were intraperitoneally injected with LPS after 1-hour recovery to induce inflammation, and returned to their dam. Control animals underwent only sham operation (exposure of the left common carotid artery without occlusion) and vehicle treatment. All the animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Chungbuk National University (CBNU), Korea, and conducted according to the Standard Operation Procedures of the Laboratory Animal Research Center, CBNU. In order to avoid possible biased interpretation, the behavioral tests were conducted in a blind manner.

Transplantation of F3.Olig2 Cells

HIL rats were intracerebroventricularly transplanted with F3.olig2 cells (4×10^5 cells/rat) in 2 μl saline at the following coordinates: anterior/posterior +1 mm, right lateral 2 mm, and ventral 3 mm from bregma[33,34]. The rats received the cells once at only PDN10 in the single-dose group, or repeatedly at PND10, 17, 27, and 37 in repeated-dose groups.

Cylinder Test

For the evaluation of forelimb use asymmetry by ipsilateral brain damage, the ratio of contralateral forelimb use was analyzed at PND14, 20, 30, and 40. Each animal was individually placed in a glass cylinder (21 cm in diameter, 34 cm in height) for 3 min. The weight-bearing forepaw to contact the cylinder wall during a full rear was recorded as left (normal), right (impaired), or both[18,35]. Paw preference was calculated as [(normal forepaw–impaired forepaw)/(normal forepaw + impaired forepaw + both) × 100%].

Rotarod Performance

Motor balance and coordination were evaluated using a rotarod test system (Panlab Technology, Barcelona, Spain) at PND14, 20, 30, and 40. Rats were placed on a rotating rod at a constant speed of 12 rpm, and the time for the rats to fall off the rod was recorded[18,35,36]. The average latency was calculated from three consecutive measurements.

Locomotor Activity

Spontaneous activities and exploratory behaviors were evaluated using a video tracking system (Smart v2.5; Panlab Technology), connected to a CCTV (Samsung, Changwon, Korea) at PND14, 20, 30, and 40. Rat pups were placed in a quiet chamber with a dim (200 Lux) light. The types of movement, i.e. resting (<250 cm/s), slow-moving (250–800 cm/s), and fast-moving (≥800 cm/s) times, were recorded for 5 min, and the ratio was analyzed[18,35,36].

Passive Avoidance Performance

For the evaluation of memory acquisition, the rats were subjected to Shuttle box (ENV-010MD; Med associates Inc., Vermont, USA) once a day for 5 consecutive days from PND41. The Shuttle box apparatus consists of two light and dark compartments; a light chamber equipped with a lamp, and a dark chamber with a steel-grid floor for electric shock. On the trials, electric shock (1 mA for 5 s) was delivered when rats entered the dark compartment from the light room through a guillotine door. The latency time in the light room was recorded during 5-day trials[33–36]. The end-point was set at 300 s, denoting full acquisition of memory.

Morris Water-Maze Performance

For the evaluation of spatial memory, the rats were subjected to Morris water-maze system (Smart v2.5; Panlab Technology). Water-maze trials were performed in a circle water pool (180 cm in diameter) filled with water (27 cm in depth) maintained at 22 $\pm$ 2°C. The pool was divided into four quadrants, and a hidden escape platform (10 cm in diameter, 25 cm in height) was submerged in the center of one quadrant, 2 cm below the surface of the water. The rats were subjected to five trials, once a day from PND41, to find the platform hidden by white Styrofoam granules (5 cm in diameter) on the surface of water, based on several cues external to the maze. The position of the cues remained unchanged throughout the experiments. Escape latency time—time taken to escape onto the platform during trials—was recorded[33–36].

Double Immunostaining

To detect the distribution and survival of transplanted F3.olig2 cells, the rat brain was perfusion-fixed with 10% paraformaldehyde solution and post-fixed in the same solution for 48 h, followed by cryoprotection in 30% sucrose for 72 h. Coronal cryosections of 30 μm thickness were prepared and processed for double immunostaining for human mitochondria (hMito) and Olig2 or myelin basic protein (MBP) as markers of human-derived cells and oligodendrocytes, respectively[18,29,35]. Brain sections were incubated with primary antibodies specific for hMito (1:100, mouse monoclonal, Chemicon, Temecula, USA) and/or Olig2 (1:200 rabbit polyclonal, Chemicon) or MBP (1:200 rabbit polyclonal, Chemicon) overnight at 4°C, and with secondary antibodies conjugated with Alexa Fluor-488 or -594 (1:200, Molecular Probes, Eugene, USA) for 2 h at room temperature.

For determination of the integrity of host myelins, immunostaining for MBP was performed using anti-MBP (1:200 rabbit polyclonal, Chemicon) as primary antibody. Secondary antibody conjugated with Alexa Fluor-594 (1:200, Molecular Probes, Eugene, USA) was used for the procedures same as Olig2 staining[18,35]. All samples were evaluated immediately after staining and photographed with a laser-scanning confocal microscope (LSM710; Zeiss, New York, USA). In order to quantify the immunoreactivity of MBP, the photographs were analyzed with a Digital Image Analyzer (Image Inside; Focus, Seoul, Korea) for the red intensity and expressed as a percentage of the sham control group.

Statistical Analysis

Data are presented as mean $\pm$ SEM. Statistical analyses were performed using the SAS program (version 6.12; SAS Institute Inc., Cary, USA; http://www.sas.com). We analyzed the difference in HIL alone group against sham control, followed by HIL plus stem cell treatment groups against HIL alone. Continuous variables, including passive avoidance and Morris water-maze performances, were compared by one-way analysis of variance (ANOVA), followed by Scheffe's multiple comparison test when the results were significant. Other behavioral data, such as ratios in the cylinder test and locomotor activity and the duration of rotarod performance, were compared using Kruskal–Wallis nonparametric ANOVA, followed by a Mann–Whitney *U*-test when appropriate. The intensity of MBP immunoreactivity was compared using Fisher's exact probability test. P values <0.05 were considered statistically significant.

Results

Effects of F3.olig2 Cells on Neurobehavioral Deficits

In the cylinder test, sham control animals used their left and right forelimbs in similar ratios (50:50) at PND14, 20, 30, and 40 (Fig. 1). However, rats subjected to HIL at PND7 showed significantly decreased (<40%) use of the forelimb contralateral to the brain injury at PND20, 30, and 40. Such reduced use of the contralateral forelimb was recovered to near-normal levels at PND30 and 40 by a single transplantation of F3.olig2 cells at PND10. A similar restoration of physical dysfunction of the contralateral forelimb induced by HIL was achieved with repeated transplantations of F3.olig2 cells at PND10, 17, 27, and 37.

The latency time of sham control animals on a 12-rpm rotarod gradually increased according to their growth from mean 12.5 s at PND14 to 270–290 s at PND30–40 (Fig. 2). However, HIL caused impairment of motor function and coordination, leading to a drastic reduction of the latency time by 35% at PND14 and 65–70% at PND20–40. Interestingly, such decreased rotarod performances were near-fully restored at all time points tested by a single transplantation of F3.olig2 cells at PND10. Also, the functional impairments of HIL rats were significantly recovered following repeated transplantations of F3.olig2 cells.

In sham control animals, moving time was longer than resting time at PND14–40 in the global activity analysis (Fig. 3). However, the resting time greatly increased in HIL rats, leading to significant decreases in slow-moving and fast-moving times from PND14 to PND40. In comparison, single transplantation with F3.olig2 cells near-fully recovered the HIL-induced decrease in locomotor activity from PND20. Such

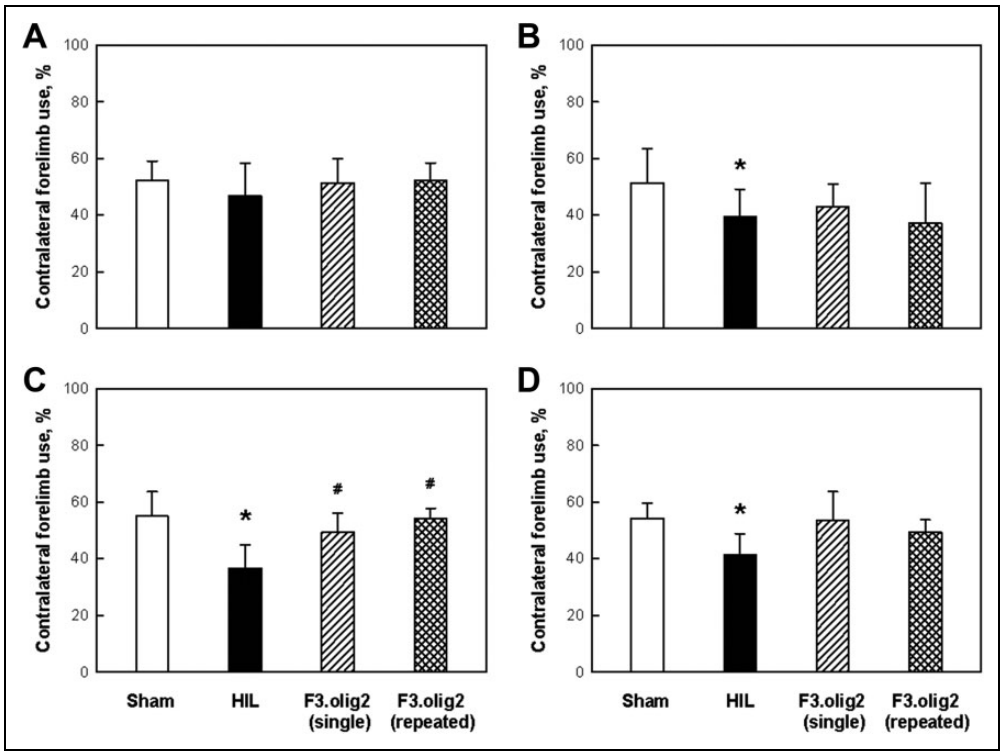

Figure 1. The using ratio (%) of forelimb contralateral to hypoxia-ischemia-lipopolysaccharide injection (HIL) in the cylinder test at PND14 (A), 20 (B), 30 (C), and 40 (D). Rats ($n = 7$/group) were subjected to HIL, and intracerebroventricularly transplanted with F3.oilg2 (4×10^5) cells once at PND10 or four times at PND10, 17, 27 and 37. *Significantly different from sham control ($P < 0.05$). #Significantly different from HIL ($P < 0.05$).

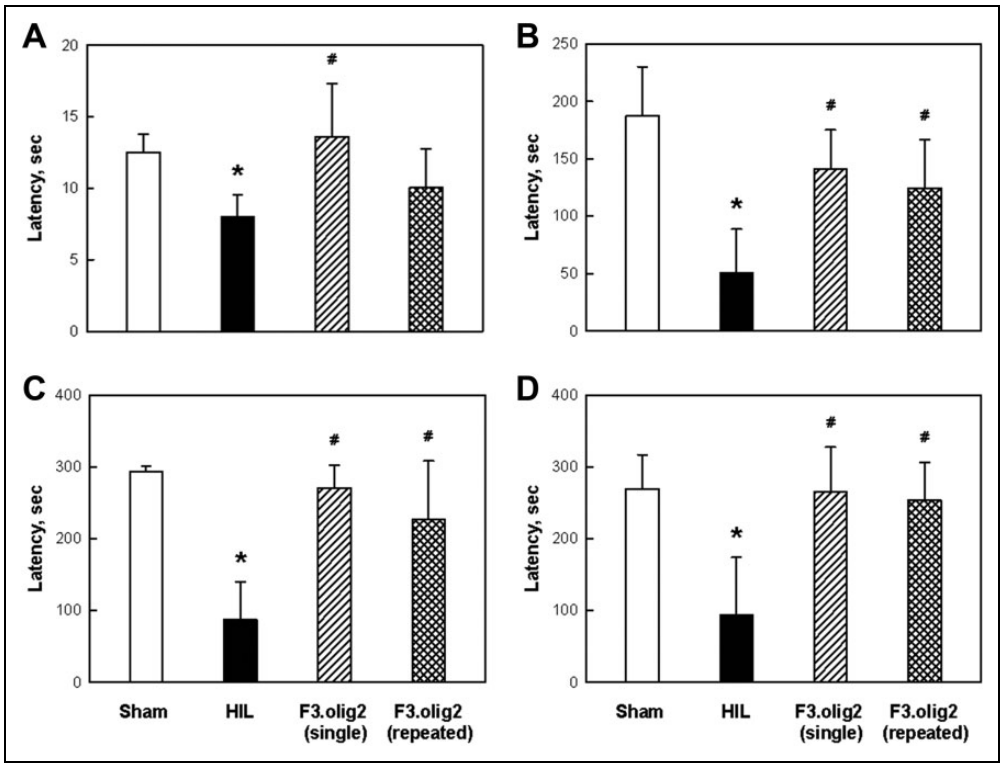

Figure 2. Latency time (sec) in constant-speed (12 rpm) rotarod performances at PND14 (A), 20 (B), 30 (C), and 40 (D). Rats ($n = 7$/group) were subjected to hypoxia-ischemia-lipopolysaccharide injection (HIL), and intracerebroventricularly transplanted with F3.oilg2 (4×10^5) cells once at PND10 or four times at PND10, 17, 27, and 37. *Significantly different from sham control ($P < 0.05$). #Significantly different from HIL ($P < 0.05$).

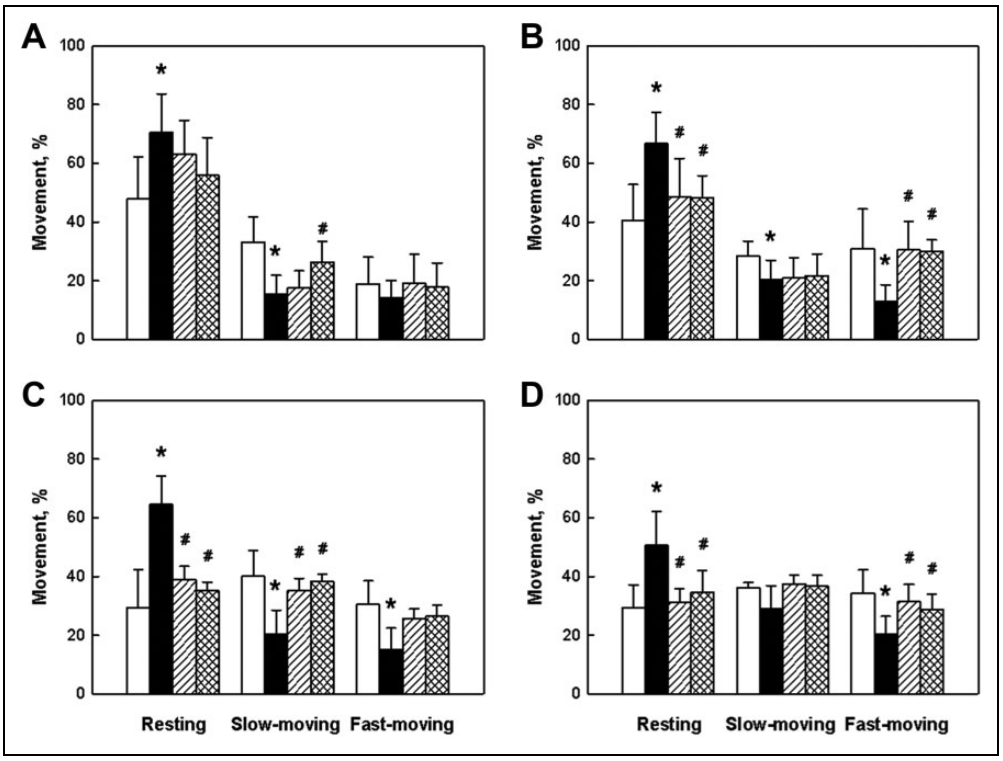

Figure 3. Locomotor (global) activity analyzed based on spontaneous movements at PND14 (A), 20 (B), 30 (C), and 40 (D) ($n = 7$/group). White, sham control; black, hypoxia-ischemia-lipopolysaccharide injection (HIL); shaded, single dose of F3.olig2 (4×10^5) cells at post-natal day (PND) 10; checked, repeated doses of F3.olig2 cells at PND10, 17, 27, and 37. *Significantly different from sham control ($P < 0.05$). #Significantly different from HIL ($P < 0.05$).

effects on the restored activity were also obtained by repeated transplantations of F3.oligs cells at PND10, 17, 27, and 37.

Effects of F3.Olig2 cells on Cognitive Dysfunction

HIL at PND7 induced severe impairment of learning and memory functions as assessed by both passive avoidance and Morris water-maze performances at PND41–45. That is, HIL rats displayed much delayed increase in retention time and long latency time during repeated trials in passive avoidance and Morris water-maze performances, respectively, whereas full memory acquisition was completed on the 4th trial in sham control animals (Fig. 4). By comparison, transplantation of F3.olig2 cells once at PND10 or four times at PND10, 17, 27, and 37 significantly recovered the impaired learning and memory function in passive avoidance trials. In addition, transplantation of F3.olig2 cells improved the cognitive dysfunction in Morris water-maze performance test, in which the effect of repeated transplantations was superior to that of single treatment.

Distribution and Maturation of Transplanted F3.olig2 cells

Immunostaining for human mitochondria revealed that hMito-positive F3.olig2 cells, intracerebroventricularly transplanted at PND10, were found in the injured white matters (Fig. 5), suggestive of a lesion-tropic property as shown in F3.olig2 OPCs and their parental F3 NSCs in different CNS disease models[29,33,34]. The numbers of transplanted cells in the white matter at PND45 were 89.60 and 256.15 cells/mm^2 following single and repeated transplantation, respectively (Fig. 5A). Furthermore, the hMito-positive cells strongly expressed Olig2 (Fig. 5B), confirming through double immunostaining for hMito and Olig2 that the transplanted cells were oligodendrocyte lineage cells. In addition, the transplanted cells were found to be matured to myelin-producing oligodendrocytes, as confirmed by immunostaining for hMito and MBP (Fig. 5C).

Effects of F3.Olig2 Cells on Demyelination

In sham control animals, intensive immunoreactivity for MBP of oligodendrocytic myelins was observed in white matter such as corpus callosum (Fig. 6). By comparison, the MBP immunoreactivity markedly reduced in HIL animals as observed at PND45, indicative of severe demyelination. Notably, however, the HIL-induced loss of MBP was remarkably attenuated by a single transplantation of F3.olig2 cells at PND10. More marked protective effects on MBP depletion were achieved by repeated treatments with F3.olig2 cells at PND10, 17, 27, and 37.

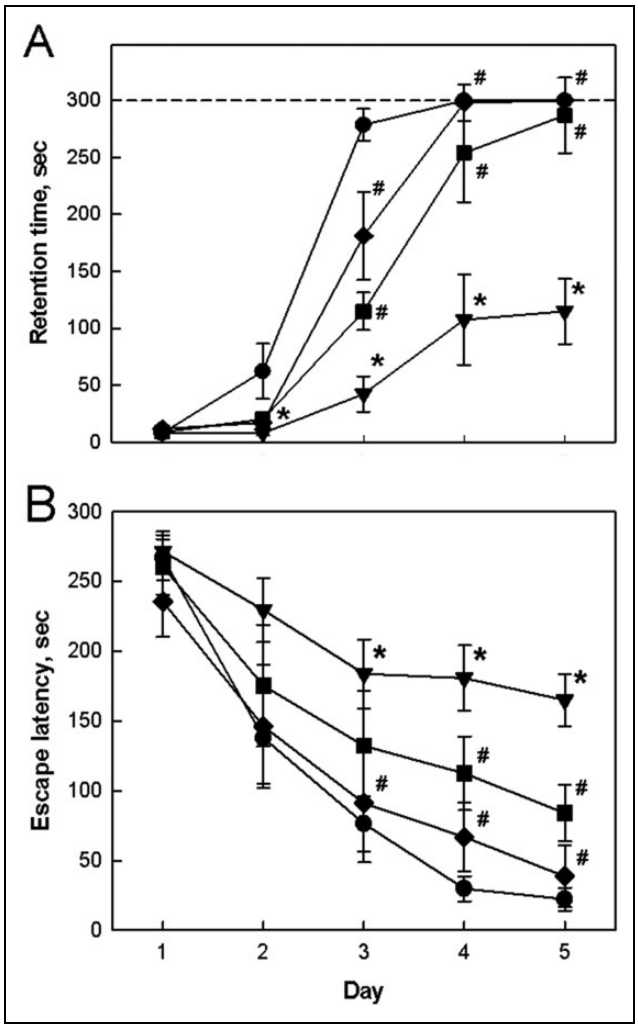

Figure 4. Learning and memory functions in passive avoidance (A) and Morris water-maze (B) performances at post-natal day (PND) 41–45 ($n = 7$/group). Circle, sham control; inverted triangle, hypoxia-ischemia-lipopolysaccharide injection (HIL); square, single dose of F3.olig2 (4×10^5) cells at PND10; lozenge, repeated doses of F3.olig2 cells at PND10, 17, 27, and 37. *Significantly different from sham control ($P < 0.05$). #Significantly different from HIL ($P < 0.05$).

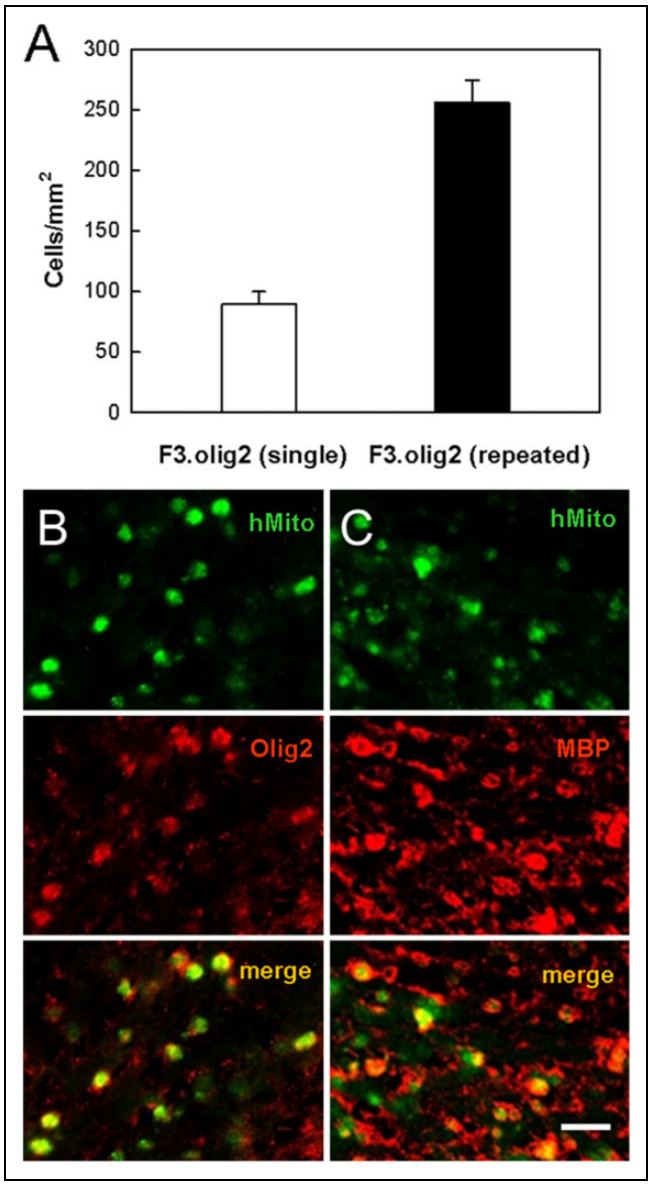

Figure 5. Distribution and maturation of F3.olig2 cells. Transplanted F3.oilg2 cells were found in injured area after single or repeated transplantation (A, $n = 7$/group) and confirmed to be oligodendroglia (expressing Olig2, B) producing myelin basic proteins (MBP, C) by double immunoreactivities to markers specific for human mitochondria (hMito) and Olig2 or MBP 5 weeks post-transplantation. Scale bar = 20 μm.

Discussion

Compared with OPCs during the gestational period, OPCs of neonatal rats are also susceptible to HI insults, because PND5 in rats corresponds to GW24–30 in humans regarding white matter maturation[9,37], and MBP synthesis by oligodendrocytes begins from around PND7[9,38]. In previous studies, it was confirmed that ischemia (carotid artery occlusion) followed by hypoxia (5–8% O_2 for 0.5–3.5 h) caused delayed myelination and neurobehavioral disorders[9,21,32]. Thereafter, HIL, a combinational model of carotid artery ligation–exposure to a hypoxic environment–LPS injection, was demonstrated for the induction of CP, displaying profound neurobehavioral and cognitive dysfunctions following PVL[35,39].

In the present and previous studies, it was confirmed that HIL in PND7 rats produced severe loss of myelin and pathological changes (rarefaction) in periventricular white matter including corpus callosum[18]. Such brain lesions led to physical and cognitive abnormalities as observed in the cylinder test, rotarod performance, locomotor (global) activity, and passive avoidance and Morris water-maze performances[18,32,35]. Therefore, these diverse pathological and neurobehavioral alterations may support that HIL at PND7 could be a good CP model for the evaluation of preventive

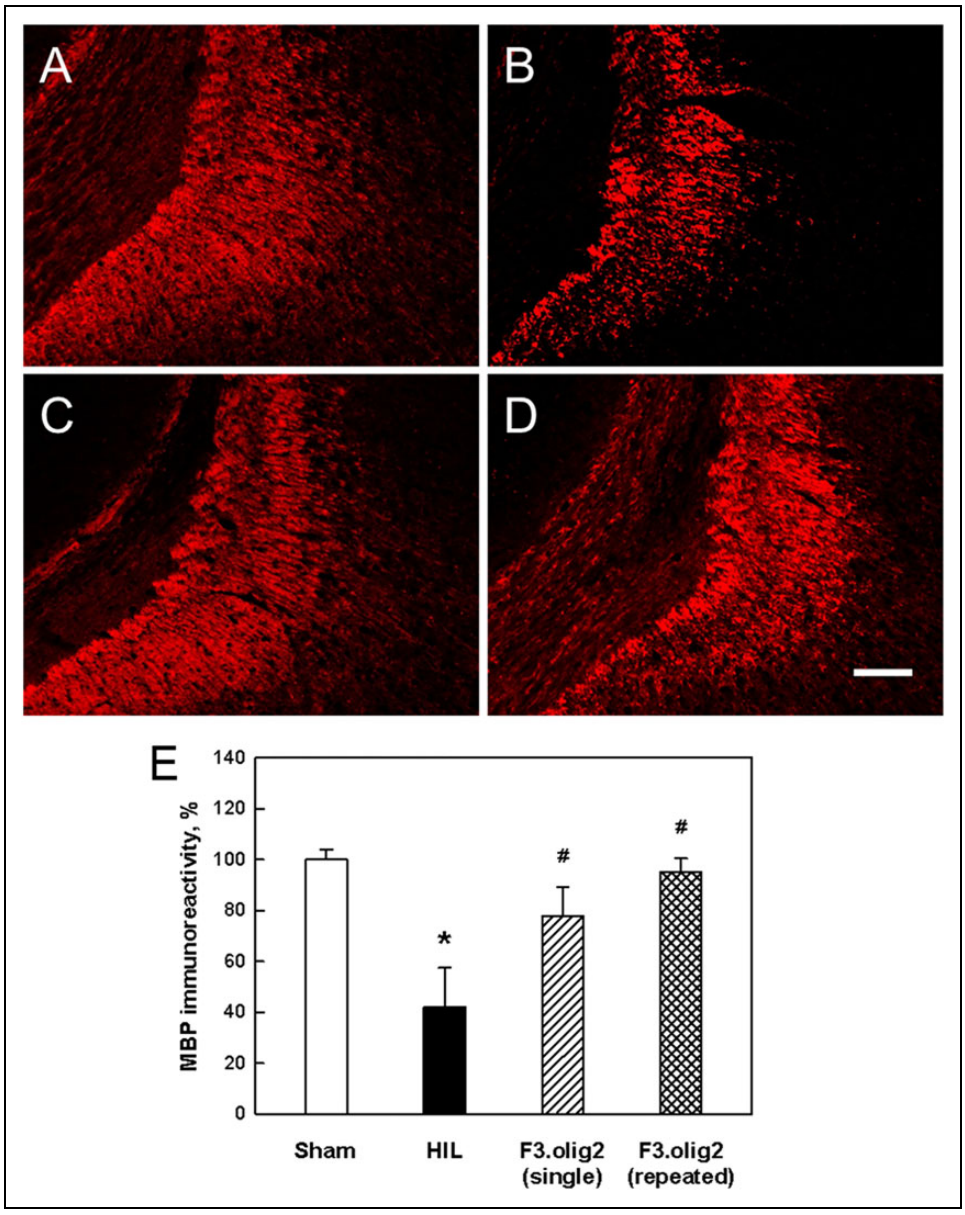

Figure 6. Representative photomicrographs of myelin basic protein (MBP) immunostaining in the corpus callosum of post-natal day (PND)45 rat brains. Scale bar = 100 μm. A, sham control; B, hypoxia-ischemia-lipopolysaccharide injection (HIL); C, single dose of F3.olig2 (4×10^5) cells at PND10; D, repeated doses of F3.olig2 cells at PND10, 17, 27, and 37; D, immunoreactivity of MBP ($n = 7$/group). *Significantly different from sham control ($P < 0.05$). #Significantly different from HIL ($P < 0.05$).

and/or therapeutic candidates. The characteristics of the HIL model are similar to the neurobehavioral development of human PVL[15,40]. This is a reason why we adopted the HIL model to expand functional parameters including physical and cognitive impairments, rather than the HI model which produces mild and limited disorders[9,21,32]. Myelin abnormalities and deficiencies are also known to play important roles in cognitive dysfunction in other neurologic diseases such as schizophrenia and multiple sclerosis (MS)[41,42]. It is well demonstrated that oligodendrocyte lineage degeneration and ultimately reduced myelin production cause problems in CP, schizophrenia, and MS, as a result of the saltatory conduction of nervous impulses along axons[43].

In the present study, F3.olig2 cells, intracerebroventricularly transplanted 3 days after HIL, were distributed in damaged areas such as corpus callosum and survived longer than 5 weeks *in vivo*, as detected with double immunostaining for hMito and Olig2 markers. Such lesion-tropism of F3.olig2 cells was also demonstrated in EAE mice, an animal model of human MS, another demyelinating disease[33]. The lesion-tropic migration of F3.olig2 cells might be triggered by chemoattractants such as hepatocyte growth factor, stromal

cell-derived factor-1, vascular endothelial growth factor, and stem cell factor, as well as inflammatory mediators that affect differentiation and survival of transplanted stem/progenitor cells[29,37,44–46]. Also, over-expression of Olig2 transcription factor is sufficient to induce the expression of Nkx2.2 and differentiation of neural stem/progenitor cells into oligodendrocytes[47,48].

Notably, transplantation of F3.olig2 cells into HIL rat brains markedly preserved host MBP. Such neuroprotective effects of F3.olig2 cells may come from various growth and neurotrophic factors including brain-derived neurotrophic factor (BDNF), nerve growth factor, and insulin-like growth factor-1 (IGF-1) released from the cells, as shown in previous research reporting that F3 parent NSCs also prevented demyelination and promoted endogenous remyelination process[31,49]. Several studies have demonstrated neuroprotection and improvement of cognitive function in striatum of HI model rats by intracerebroventricular pretreatment of BDNF[50,51]. In addition, IGF-1 was found to protect oligodendrocytes in a bilateral ischemia model[52]. Therefore, it is believed that F3.olig2 cells protected myelin against HI and inflammatory (LPS) insults by releasing growth and neurotrophic factors that influence neuroprotective and neuroregenerative mechanisms, together with enhanced myelination.

Most importantly, transplanted F3.olig2 cells substantially ameliorated neurobehavioral and cognitive deficits of HIL rats. Improved rotarod performances were achieved as early as 4 days after cell transplantation, and lasted longer than 5 weeks. From the full improvement of contralateral forelimb dysfunction, in addition to rotarod performances, it is assumed that physical function of the HIL animals was recovered by F3.olig2 cells, implying that the integrity of neuromuscular transmission was preserved. Moreover, analysis of the locomotor activity of HIL animals revealed that their overall physical functions were protected and restored following F3.olig2 cell transplantation, leading to enhanced global activity similar to sham control rats.

In conclusion, the present study clearly showed the protective activities of F3.olig2 cells against MBP loss in the white matter and ensuing physical and cognitive abnormalities induced by HIL at PND7. Therefore, it is suggested that transplantation of F3.olig2 OPCs restores neurobehavioral disorders as well as cognitive deficits of PVL animals by preventing neurons from demyelination in periventricular white matter, and that F3.olig2 cells could be a promising candidate for the treatment of patients with CP.

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Authors Contribution

Tae-Kyun Kim, Dongsun Park These authors provided equal contribution to this work.

Ethical Approval

Animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Chungbuk National University, Korea.

Statement of Human and Animal Rights.

This article contain studies with animal subjects, but not humans. Animal experiments were conducted according to the Standard (ethical) Operation Procedures of the Laboratory Animal Research Center of Chungbuk National University, Korea.

Statement of Informed Consent

Statement of Informed Consent is not applicable to this article.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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