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Neurotrophy and immunomodulation of induced neural stem cell grafts in a mouse model of closed head injury

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ABSTRACT

Closed head injury (CHI) usually results in severe and permanent neurological impairments, which are caused by several intertwined phenomena, such as cerebral edema, blood-brain barrier (BBB) disruption, neuronal loss, astroglial scarring and inflammation. We previously reported that induced neural stem cells (iNSCs), similar to neural stem cells (NSCs), can accelerate neurological recovery in vivo and produce neurotrophic factors in vitro. However, the effects of iNSC neurotrophy following CHI were not determined. Moreover, whether iNSCs have immunomodulatory properties is unknown. Mouse models of CHI were established using a standardized weight-drop device and assessed by neurological severity score (NSS). Although these models fail to mimic the complete spectrum of human CHI, they reproduce impairment in neurological function observed in clinical patients. Syngeneic iNSCs or NSCs were separately transplanted into the brains of CHI mice at 12 h after CHI. Neurological impairment post-CHI was evaluated by several tests. Animals were sacrificed for morphological and molecular biological analyses. We discovered that iNSC administration promoted neurological functional recovery in CHI mice and reduced cerebral edema, BBB disruption, cell death and astroglial scarring following trauma. Implanted iNSCs could up-regulate brain-derived neurotrophic factor (BDNF) and glial-derived neurotrophic factor (GDNF) levels to support the survival of existing neurons after CHI. In addition, engrafted iNSCs decreased immune cell recruitment and pro-inflammatory cytokine expression in the brain post-injury. Moreover, we found significant nuclear factor-kappaB (NF-κB) inhibition in the presence of iNSC grafts. In short, iNSCs exert neurotrophic and immunomodulatory effects that mitigate CHI-induced neurological impairment.

1. Introduction

Closed head injury (CHI) is the result of an immediate mechanical insult to the central nervous system (CNS). Patients with CHI suffer from neurological impairments and require long-term care (Bramlett and Dietrich, 2015). CHI-induced neurological deficits are caused by several intertwined phenomena, such as cerebral edema, blood-brain barrier (BBB) disruption, neuronal loss, astroglial scarring and inflammation (Abdul-Muneer et al., 2015; Sofroniew, 2015; Das et al., 2012). Accumulating evidence indicates that inflammation following trauma, including immune cell recruitment and cytokine production, plays a crucial role in neural injury and functional reconstruction (Das et al., 2012; Corps et al., 2015; Gage and Temple, 2013). Therefore, inflammation modulation, which may promote recovery of damaged brain tissue, may represent a promising therapeutic intervention for CHI patients.

In recent decades, scholars have noted that in addition to facilitating cell replacement, transplanted neural stem cells (NSCs) can support resident CNS cells by secreting neurotrophic factors and can improve neurological outcomes by immunoregulating various disorders (Ager, 2015; Lemmens and Steinberg, 2013; Dooley et al., 2014). Currently, with advances in reprogramming technology, induced neural stem cells (iNSCs) generated directly from autologous somatic cells are more suitable for clinical use than NSCs because their capacities for self-renewal and multi-lineage differentiation are similar to those of NSCs, and their use is not fraught with the ethical concerns or resource limitations associated with NSC use (Yao et al., 2015; Gao et al., 2016).

We previously reported that iNSCs directly reprogrammed from mouse embryonic fibroblasts can expand and give rise to neurons, astrocytes and oligodendrocytes (Yao et al., 2015). Moreover, we noted that there were no significant differences in the levels of several neurotrophic factors between iNSC and NSC culture supernatants. In particular, we found that iNSC grafts accelerated neurological recovery in middle cerebral artery occlusion (MCAO) animals and a portion of iNSCs differentiated into glial fibrillary acidic protein (GFAP)-positive

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astrocytes and beta III tubulin (Tuj1)-positive neural cells in MCAO-damaged brains. These findings suggested that iNSCs, similar to NSCs, can play roles in cell replacement and neurotrophy in CNS regeneration.

INSCs have many NSC-like characteristics, but they are not identical to NSCs because transdifferentiation technology may cause genetic or epigenetic variations (Zhao et al., 2011). For instance, induced pluripotent stem cells (iPSCs), which had previously been considered identical to embryonic stem cells (ESCs), can express different sets of genes and proteins that may evoke unwanted immune rejection after grafting (Zhao et al., 2011). Hence, it is essential to carefully determine iNSC potential in pre-clinical research. Moreover, whether iNSCs have immunomodulatory properties remains uncertain, and few studies have evaluated the effects of iNSC grafts in syngeneic mouse models of CHI.

In this study, we aimed to determine the role of engrafted iNSCs in CNS recovery following CHI-induced neurological impairment, as well as the mechanisms underlying this role. Mouse models of CHI were established using a standardized weight-drop device and assessed by neurological severity score (NSS) (Flierl et al., 2009; Xiong et al., 2013). Although these models fail to mimic the complete spectrum of human CHI, they represent a clinically encountered injury mechanism and reproduce impairment in neurological function observed in CHI patients (Flierl et al., 2009). Syngeneic iNSCs or NSCs were separately transplanted into the brains of CHI mice at 12 h after CHI. Neurological impairment post-CHI was evaluated by several tests. Animals were sacrificed for morphological and molecular biological analyses.

2. Material and methods

2.1. Closed head injury models

Healthy adult male C57BL/6 (B6) mice weighing 24–30 g (Vital River Laboratories, Beijing, China) were housed in a temperature- and humidity-controlled room with food and water ad libitum. All experimental procedures were in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH) and approved by the Committee on the Ethics of Animal Experiments of the P.L.A Army General Hospital (Permit number: 2014–044).

Animals were anesthetized with inhalative isoflurane (induction: 3% isoflurane; maintenance: 1.25% isoflurane) and received fentanyl (0.05 mg/kg body weight per day, intraperitoneal injection) as the analgesic agent. CHI models were established using a standardized weight-drop device as previously reported (Flierl et al., 2009; Xiong et al., 2013). In brief, the parietal bone was exposed by a midline scalp incision after shaving and cleaning the skin. A free-falling rod with a blunt tip of 3.0 mm diameter dropped onto mouse skull (2.0 mm anterior to the lambda suture and 2.0 mm lateral to the middle line) and the falling height was 3.0 cm. Then the scalp wound was sutured and treated with povidone-iodine solution. After surgery, mice were allowed to recover on a heating pad until fully awake and they were evaluated at 1 h post-CHI using NSS by two blinded, trained investigators (Supplementary Table 1) (Flierl et al., 2009). Briefly, one point was given for a tested reflex's absence or for failing to perform an individual task. Mice having an NSS of 4–8 were randomly assigned to three groups: the iNSC group (mice receiving iNSC transplantation), the NSC group (mice receiving NSC transplantation) and the phosphate-buffered saline (PBS) group (mice receiving PBS treatment). NSS was further assessed at several time intervals up to 7 days.

2.2. Cell transplantation

B6 mouse iNSCs labeled with green fluorescent protein (GFP) were generated as described previously (Yao et al., 2015). In brief, GFP-expressing mouse embryonic fibroblasts (MEF) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) (Invitrogen) and 0.1 mM non-essential amino acids (NEAA) (Invitrogen), infected with combinations of lentiviruses (TetO-FUW-*Oct4*, *Sox2*, *Klf4* and *c-Myc*), and subsequently treated with doxycycline (DOX, 2–8 μg/ml) (Sigma-Aldrich, St. Louis, MO, USA) for 6 days. In the final stage of reprogramming, the medium was changed to iNSC/NSC culture medium (iNSC/NSCcm, Neurobasal: DMEM/F12 (1:1) containing 2% B27 supplements, 20 ng/ml basic fibroblast growth factor (bFGF), 20 ng/ml epidermal growth factor (EGF), 0.05% bovine serum albumin (BSA) and 2 mM L-glutamine) (all from Invitrogen). INSC clones appeared in 13–26 days after induction and were expanded. Furthermore, B6 mouse NSCs were generated and cultured in iNSC/NSCcm as described above. For transplantation, cultured cells were digested with accutase (Invitrogen) and washed three times with PBS. After the density of single-cell suspension was adjusted, the cells were maintained on ice. At 12 h after CHI, mice were anesthetized again and mounted in a stereotaxic apparatus (Stoelting, Wood Dale, IL, USA). Cell suspension or PBS was separately injected into the brains (5.0 mm anterior to the lambda suture, 1.0 mm lateral to the middle line and 2.0 mm under the dura) via different sterile 25-μl 22 s Hamilton syringes. Each site received 5 μl of cell suspension containing 1×10^6 cells or PBS at a speed of 0.5 μl/min. Approximately 5 min after injection, the syringe was slowly withdrawn.

2.3. Evaluation of neurological function, cerebral edema and BBB permeability

We assessed fine-motor coordination deficits using a beam-walk task at 1, 3 and 7 days post-CHI (Yan et al., 2011). Briefly, mice were trained to cross a standardized narrow wooden beam and the number of foot faults for the right hind limb was recorded over 50 steps. A basal level of competence at this task was established before surgery with an acceptance level of <10 faults per 50 steps.

For morphological and molecular biological analyses, mice were sacrificed after anesthesia and their fresh or perfused-fixed brain tissues were respectively collected. Cerebral edema and BBB permeability were measured by two blinded, trained investigators as previously described (Flierl et al., 2009). The percentage of brain water was calculated as [(wet weight-dry weight) / wet weight] × 100%. The concentration of Evans blue was quantified as micrograms of the dye per gram of the tissue.

2.4. Morphological analysis

Brain tissues were postfixed in 4% Paraformaldehyde (PFA) in 0.1 M PBS (PH 7.4) at 4 °C overnight, and then sectioned (10 μm) on a cryostat (Leica CM 1950, Leica Biosystems, Nussloch, Germany) and mounted on adhesion microscope slides. The pathologic features of brain tissues transverse sections from the width of the grafting/lesion site were assessed by hematoxylin and eosin (HE) staining. Furthermore, slides of brain tissues were blocked for 1 h using 10% BSA/0.3% TritonX-100 and then incubated overnight at 4 °C with primary antibodies (Supplementary Table 2). After being washed in PBS, they were incubated for 1–2 h at room temperature (RT) with secondary antibodies (Supplementary Table 2). After several washes with PBS, the nuclei were stained with Dapi Fluoromount-G (SouthernBiotech, Birmingham, AL, USA) and staining was detected via fluorescent microscopy (DM3000, Leica) or confocal laser scanning microscopy (CLSM, TCS SP5 II, Leica). The number of positive cells was manually counted on the microscopy at 20× magnification and adjusted using image analysis software (Image-Pro plus 5.0) by two blinded, trained investigators. The ratio of positive cells was calculated as (number of positive cells / total number of cells) × 100%.

2.5. Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) staining

TUNEL staining was performed using the In Situ Cell Death Detection Kit, TMR red (Roche, Mannheim, Germany) as described in the