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Significant therapeutic effects of adult human multipotent neural cells on spinal cord injury

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ABSTRACT

Neural stem cells are emerging as a regenerative therapy for spinal cord injury (SCI), since they differentiate into functional neural cells and secrete beneficial paracrine factors into the damaged microenvironment. Previously, we successfully isolated and cultured adult human multipotent neural cells (ahMNCs) from the temporal lobes of epileptic patients. In this study, we investigated the therapeutic efficacy and treatment mechanism of ahMNCs for SCI using rodent models. When 1×10^6 ahMNCs were transplanted into injured spinal cords at 7 days after contusion, the injection group showed significantly better functional recovery than the control group (media injection after contusion), which was determined by the Basso, Beattie and Bresnahan (BBB) score. Although transplanted ahMNCs disappeared continuously, remained cells expressed differentiated neural cell markers (Tuj1) or astrocyte marker (GFAP) in the injured spinal cords. Moreover, the number of CD31-positive microvessels significantly increased in the injection group than that of the control group. The paracrine pro-angiogenic activities of ahMNCs were confirmed by *in vitro* tube formation assay and *in vivo* Matrigel plug assay. Together, these results indicate that ahMNCs have significant therapeutic efficacy in SCI *via* replacement of damaged neural cells and pro-angiogenic effects on the microenvironment of SCI.

1. Introduction

Spinal cord injury (SCI) provokes physical discontinuance of ascending and descending neural pathways at damaged regions, which is deteriorated more by secondary reactions such as demyelination, inflammation, and gliosis (Rowland et al., 2008). Although there are medical interventions to prevent secondary damages, functional recovery of the damaged spinal cord has not been achieved in patients with SCI. For the regeneration of damaged neural cells in SCI, various types of stem cells including Schwann cells, olfactory ensheathing cells, activated macrophages, embryonic stem cells (ESCs), mesenchymal stem cells (MSCs), and induced pluripotent stem cells (iPSCs) has been preclinically examined (Barnabe-Heider and Frisen, 2008; Fujimoto et al., 2012; Lindvall and Kokaia, 2010; Mothe and Tator, 2012; Sahni and Kessler, 2010), and some transplantation studies reported significant functional recovery in SCI animal models (Alastrue-Agudo et al., 2014; McDonald et al., 1999; Qu and Zhang, 2017; Rodriguez-Jimnez et al., 2012; Suzuki et al., 2017; Teng et al., 2002). However, the experimental transplantation efforts have made few significant progresses in clinical applications, indicating their limitations in manufacture, efficacy, and/or safety (Nam et al., 2015; Ronaghi et al., 2010).

Neural stem cells (NSCs) have merits in clinical application compared with other stem cell types; regeneration of functional neural cells

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via their differentiation capacity (Gincberg et al., 2012; Lindvall and Kokaia, 2010; Lunn et al., 2011) and long-term safety due to their low tumorigenic potential (Lee et al., 2016; Lu et al., 2012; Yan et al., 2007). These strengths have made clinical trials of NSCs for SCI carried out by various national and private groups. Presently, there are four active phase I and/or II clinical trials registered with clinicaltrial.gov (NCT02326662, NCT02688049, NCT01772810, and NCT02302157) for SCI. However, they are using NSCs derived from MSCs (NCT02326662), MSCs or NSCs with scaffold (NCT02688049), spinal cords of aborted fetuses (NCT01772810), and oligodendrocyte progenitor cells derived from iPSCs (NCT02302157), which might not overcome limited differentiation capacity, ethical considerations, and possible *in vivo* tumorigenic potential, respectively. Therefore, ethically and functionally safe NSC sources with regeneration potential still need to be developed further for SCI treatment.

Previously, we reported that adult human multipotent neural cells (ahMNCs) could be cultivated from the neurogenic regions of the temporal lobes of epileptic patients that were voluntarily donated (Joo et al., 2013). Functionally, ahMNCs have differentiation potential into both neurons and astrocytes, and they showed significant treatment effects on stroke preclinically (Joo et al., 2013). Moreover, ahMNCs showed little *in vivo* tumorigenic potential even when they were immortalized by artificial delivery of the human telomerase reverse transcriptase (hTERT) gene (Lee et al., 2016). Based on our observations about the advantages of ahMNCs, we determined their treatment efficacy on SCI and elucidated their therapeutic mechanisms in this study.

2. Materials and methods

2.1. Study approval

Informed written consents were obtained from each patient according to the guidelines approved by the Institutional Review Board (IRB File No. SMC 2009-07-071-004) to acquire surgical samples. Animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the Samsung Biomedical Research Institute (SBRI, Seoul, South Korea) under approval numbers of 20140916001 and 20160719001.

2.2. Cell culture

Primary culture and *in vitro* differentiation of adult human multipotent neural cells (ahMNCs) were performed according to the previous report (Joo et al., 2013). Fetal human neural stem cells (fhNSCs) were purchased from Millipore (Billerica, MA, USA) and maintained in Re-Ncell maintenance medium (Millipore) supplemented with 20 ng/ml human epidermal growth factor (EGF) (R&D Systems, Minneapolis, MN, USA), 20 ng/ml human basic fibroblast growth factor (bFGF) (R&D Systems), and 100 U/ml penicillin/streptomycin. Human umbilical vein endothelial cells (HUVECs) (Gibco, Carlsbad, CA, USA) were cultured in EGM-2 medium (Lonza, Walkersville, MD, USA). U87MG, glioblastoma cell line, was cultured in DMEM (Gibco) and 10% FBS (Gibco).

2.3. Immunocytochemistry

Cells were fixed with ice-cold 4% paraformaldehyde (4% PFA) (Biosesang, Gyeonggi, South Korea) for 10 min at room temperature (RT). Primary antibodies were treated overnight at 4 °C; anti-human specific cytoplasmic protein (STEM121) (Stem Cells, Vancouver, Canada), CD133 (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), Nestin (Novus, Saint Charles, MO, USA), SOX2 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), NeuN (Millipore), GFAP (Abcam, Cambridge, UK), or O4 (Novus).

2.4. Contusion SCI animal model

Under isoflurane (Ifran™, Hana Pharma, Hwasung-si, South Korea) anesthesia, the T8–10 level of the spinal cord of an adult female Sprague-Dawley rat (12 weeks old, body weight 250–300 g) (Orient Bio.) was exposed by laminectomy. Using a Multicenter Animal Spinal Cord Injury Study (MASCIS) impactor (Rutgers University, NJ), a 10-g rod was dropped from a vertical distance of 25 mm onto the T9 level. After contusion, the muscle, subcutaneous layer, and skin were sutured in their respective anatomical layers. All animals had an intramuscular injection of 10 mg/kg ketoprofen (Uni Biotech, Seoul, South Korea) every day to reduce pain after the surgery. Bladder compression was performed once a day until the volume of extracted urine was < 0.5 ml. The negative control group underwent laminectomy without neither contusion injury nor stem cell transplantation (Normal, $n = 5$).

2.5. Transplantation of ahMNCs

Seven days after SCI, the rats were randomly classified into the control (Control, $n = 34$) or injection (Injection, $n = 32$) group. Under isoflurane anesthesia, the contusion site was re-exposed and placed in a stereotaxic frame (Model 900, Small Animal Stereotaxic Instrument, DAVID KOPF INSTRUMENTS, Tujunga, CA). ahMNCs (1×10^6) at *in vitro* passage 9 in 20 µl HBSS (Gibco) for the Injection group and 20 µl HBSS alone for the Control group were administered into the center of the lesion (0.5 mm from the midline and 2 mm down from the dura at 30 degrees from the cranial to caudal) at a rate of 2 µl/min using a 26-G Hamilton syringe. The needle was kept in place for 5 min after the injection to prevent leakage. Muscle, subcutaneous, and skin layers were closed in layers. For immunosuppression, cyclosporine A (CSA, 10 mg/kg) (Chong Kun Dang Pharmaceutical Corp., Seoul, South Korea) was intraperitoneally administered daily from 24 h before transplantation. The concentration of CSA was diminished by half every week.

2.6. Functional analysis

The Basso, Beattie, and Bresnahan (BBB) scale was used to evaluate the locomotor function of the paralyzed hind limb (Basso et al., 1995). The rats' behavior ($n = 17$ for the Control group, $n = 7$ for the Injection group) was observed by two independent observers who were blinded to the treatment. The final score was calculated as the average of the scores obtained by the two observers.

2.7. Immunofluorescence

Rats were sacrificed for immunofluorescence at 3 days, 6 days, 12 days, and 6 weeks after the contusion injury ($n = 6$, 5, 6, and 17 in the Control group; $n = 8$, 9, 8, and 7 in the Injection group, respectively) or 3 days after laminectomy ($n = 5$ in the Normal group). After euthanasia, rats were perfused by injection of ice-cold PBS (20–40 ml/min for 10 min) and then 4% PFA (20–40 ml/min for 30–50 min until tail stiffness) into the left ventricle of the heart using a peristaltic pump (MINIPULS, GILSON, Rochester, NY). After perfusion, 2 cm spinal cords centered on the injury site were removed, fixed for 48 h in 4% PFA at 4 °C, and then embedded in paraffin as 1 cm thick blocks containing transverse planes of the graft site. The paraffin blocks were sectioned at 4 µm thickness. After deparaffinization, antigens were retrieved by heating in antigen retrieval solution (Dako, Glostrup, Denmark) at boiling temperature for 30 min. Slides were treated with primary antibodies overnight at 4 °C; anti-human specific cytoplasmic protein (Stem Cells), Nestin (Novus), Tuj1 (Abcam), GFAP (Abcam), myelin oligodendrocyte glycoprotein (MOG) (Novus), CD31 (Santa Cruz), GFP (Abcam), or alpha smooth muscle actin (αSMA) (Dako) antibody. Nuclei were stained by DAPI (1:1000) (Invitrogen, ThermoFischer Scientific, Carlsbad, CA, USA) for 5 min at RT.