Research article

Transplantation of human urine-derived neural progenitor cells after spinal cord injury in rats

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ABSTRACT

Spinal cord injury (SCI) is a worldwide problem and transplantation of neural progenitor cells (NPCs) represents a promising treatment strategy. Urine derived induced pluripotent stem cells (UiPSCs) which enable the generation of patient-specific NPCs, provide an invaluable source of autologous cells for future therapeutic applications after SCI. However, the fate and potential contribution of transplanted human UiPSCs-derived NPCs (UiPSC-NPCs) into injured spinal cords remain largely unknown. In this study, using a rat contusive SCI model, we evaluated the survival, migration and differentiation of UiPSC-NPCs after transplantation at subacute phase. Transplanted cells survived and migrated from the site of grafting towards the lesion epicenter. More than 25% cells survived over 4 weeks post transplantation, with a few of them differentiated into neurons and astrocytes. Cytokine and chemokine levels within the injured spinal cord tissues were measured using multiplex immunoassays to evaluate the immune response. Pro-inflammatory factors and chemokines were significantly decreased at 3 days after UiPSC-NPCs transplantation. At 7 days post transplantation, a lower level of pro-inflammatory factor IFN-γ and a higher level of pro-inflammatory IL-2 were found in UiPSC-NPCs group than in the control. Transplantation of UiPSC-NPCs showed little effect on microglia activation at the lesion epicenter. However, the number of microglia cells at 4 mm rostral to the injury site was significantly decreased. The size of lesion cavity was reduced after transplantation of UiPSC-NPCs. In conclusions, the UiPSC-NPCs transplanted at the subacute phase of SCI showed a beneficial effect on tissue repairing.

1. Introduction

Spinal cord injury (SCI) accompanied by the disruption of neural networks often causes permanent motor and sensory dysfunctions and its treatment is still a worldwide problem [1]. Neural stem cell / neural progenitor cell (NPC) transplantation is considered as one promising strategy to restore the neural circuitry after SCI. Transplantation of NPCs has been widely investigated in animal models of SCI with proven therapeutic potential [2]. However, many factors such as cell source, ethics, immune rejection, safety, and efficacy need collectively to be considered for clinical applications. Induced pluripotent stem cells (iPSCs) generated from adult somatic cells through reprogramming have great promise for regenerative medicine as they have the potential to differentiate into any cell types in the body including neural progenitor cells (NPCs). Induced pluripotent stem cell-derived neural progenitor cells (iPSC-NPCs) therefore provide an attractive source of autologous cells for transplantation after SCI [3]. The cells could be obtained from SCI patients themselves to avoid ethical concerns and reduce the risk of immune rejection.

Human iPSC-NPCs generated from different tissue sources including adult human skin fibroblasts [3], human intervertebral disc cells [4], human fetal lung fibroblasts [5], adult human urine stem cells [6], have been studied for transplantation in animal models of SCI. The grafted cells survive, migrate and fill the lesion cavity, differentiate into neurons and glia cells. Some studies yielded functional recovery after iPSC-NPCs transplantation [5,7], whereas the others did not behave functional benefits [8,9]. The survival rate/period and the differentiation potential varied among studies. These could attribute partly to the variability of parent cells used for generating iPSC, and the different methods of iPSC reprogramming and NPC differentiation [2,10].

A variety of somatic cells collected from various human tissues have been used for generating iPSCs [11–13]. Optimally, collection of...
samples from patients should be easily accessible and less invasive. Urine offers an ideal unlimited somatic cell source for reprogramming as they can be easily obtained in a noninvasive way with a low cost. Human urine-derived epithelial cells could be reprogrammed into iPSCs with high efficiency, and the urinary iPSCs (UiPSCs) maintained the differential potential [14]. Previous studies showed that NPCs generated from human UiPSCs (UiPSC-NPCs) could differentiate into neurons and astrocytes in culture [15]. However, the fate and potential contributions of these cells in vivo after transplantation into injured spinal cord remain unknown. In this study, we transplanted the UiPSC-NPCs at the subacute phase of SCI in rats and investigated their survival, differentiation and revealed the potential effect on neuroinflammation.

2. Materials and methods

2.1. Neural progenitor cell (NPC) preparation

NPCs were generated from a human UiPSC clone (reprogrammed from human urine epithelial cells, provided by Dr. Pei’s lab, Chinese Academy of Sciences) as previously described [14], by dual inhibition of BMP and TGF-β pathways using Dorsomorphin (Selleck, Texas, USA) and SB4315242 (Selleck). In brief, UiPSCs were cultured on matrigel-coated 6-well plates with mTeSR™1 medium (STEMCELL Technologies, Vancouver, Canada). Cells were detached with EDTA and replated onto 12-well plates upon reaching 95% confluency. After cells became full confluency, cell culture medium was switched to the induction medium (N2B27 supplemented with 5 μM Dorsomorphin and 5 μM SB4315242) to induce neural differentiation. After induction for 8 days, cell sheets were mechanically divided into fragments and transferred to matrigel-coated 6-well plates. Cells were growing in proliferation medium I (N2B27, Thermo Fisher Scientific, MA, USA) for around one week. Cell clones were then mechanically scraped into floating fragments and transferred to uncoated T25 flasks for floating culture with proliferation medium II (N2B27 + 20 ng/mL bFGF + 20 ng/mL EGF, Thermo Fisher Scientific). Neurons were usually form on the second day and the proliferation of NPCs in the neurospheres was maintained by the bFGF and EGF contained in the proliferation medium II. Neurons were dissociated into single cells for transplantation using Accutase (Thermo Fisher Scientific).

UiPSCs were characterized by anti-SSEA4 (1: 1000, MA1-021, Invitrogen, CA, USA) and anti-OCT4 (1: 1000, ab181557, Abcam, Cambridge, UK) immunofluorescent staining. Neurospheres were stained with anti-PAX6 (1: 500, ab5790, Abcam) and anti-Nestin (1:1000, ab6320, Abcam) antibodies to confirm their identities. To induce the differentiation of NPCs in culture, neurospheres were dissociated into single cells suspension using Accutase (Thermo Fisher Scientific) and were then plated on matrigel-coated dishes with neuron differentiation medium (N2B27 + 1 μM dibutyryl-cAMP + 20 ng/mL BDNF, Thermo Fisher Scientific). After differentiation for 9 days, cells were fixed and stained with anti-TUJ1 (1:1000; ab18207, Abcam) and anti-GFAP (1:1000; 13-0300, Thermofisher) antibodies.

2.2. Rat SCI model and cell transplantation

Animal experiment was followed the guideline of the Experimental
rats received gentamicin (5 mg/kg, Guangdong Bangmin, China) in one group, the same volume of cell culture medium was applied instead. The glass micropipette was inserted from the dorsal midline surface into the spinal cord to a depth of 1.6 mm and then withdrawn to 1.2 mm depth prior to injection. During injection, the delivery was stopped for 10 s every 0.5 μL and the needle (Glass electrode, WPI, Florida, USA) was remained in position for 1 min after injection. All animals were received daily injection of cyclosporine A (15 mg / kg; Novartis, Prague, Czech Republic) [18,19] from two days before transplantation until to the end of experiments in order to prevent immune rejection. All animals were kept in environment-controlled rooms (22–24 °C, light / dark cycle of 12 h).

2.3. Cytokine analysis

At 3 and 7 days after transplantation, the 1-cm long spinal cord segment centered on the injury site was collected from each animal for cytokine analysis. Spinal cord tissues were lysed by RIPA solution containing phenyl methane sulfonyl fluoride (PMSF, 1:100, Solarbio Bioscience and Technology, Beijing, China) and analyzed by the bioplex system (BioRad, CA, USA) using a 23-plex cytokine kit (Cat No.: 12005641, BioRad). Three animals were used in each group at each time point.

2.4. Immunofluorescence staining

At different time points after UiPSC-NPC transplantation, rats were perfused with 4% paraformaldehyde. The 1-cm long spinal cord tissue centered on the injury site was collected for preparation of serial transverse or horizontal cryostat sections with a thickness of 20 μm. Ten sets of transverse serial sections or 5 sets of horizontal serial sections were collected for each spinal block. For immunofluorescence staining, serial sections were incubated with primary antibodies overnight at 4 °C followed by incubation with Alexa Fluor 488, 546 secondary antibodies (1:1000; A21202/A10040/A31571, Thermor Fisher) for 1 h at room temperature. The following primary antibodies were used: mouse anti-human nuclear antigen (HUNA; 1:1000; mab1281, Millipore, Massachusetts, USA), rat anti-GFAP (1:1000; 13-0300, Thermor Fisher), rabbit anti-βIII Tubulin (TUJ1; 1:1000; ab18207, Abcam), rabbit anti-βII Tubulin (TUJ1; 1:1000; ab18207, Abcam), rabbit anti-Olig2 (1:1000; ab6910, Abcam), rabbit anti-Iba1 (1:1000; 019-19741, Wako, Japan). Images were captured by a fluorescence microscope (Imager z2, Zeiss, Germany) or by a confocal microscope (LSM700, Zeiss) and were analyzed using ImageJ. One set of serial sections each were used for the quantification of cell survival, differentiation, lesion cavity measurement and microglia counting.

2.5. UiPSC-NPCs survival and differentiation after transplantation

The total HUNA-positive cells within one set of transverse sections of each animal with a total of 50 spinal cord sections spaced 200 μm apart were counted and the cell number was corrected by the Abercrombie formula [20]. The ratio of the number of HUNA-positive cells to the total number of transplanted cells was calculated as the survival rate. Cell differentiation was evaluated after double labeling of HUNA with TUJ1, GFAP or Olig2. The ratio of the number of double-labeled cells to the total number of HUNA-positive cells was calculated as the differentiation rate. Six animals were used in each group for each time point.

Animal Ethics Committee of Jinan University, and human stem cell applications were approved by the Medical Ethics Committee (No. 2016041303, 2016) of Jinan University, which is in line with the Helsinki Convention. Adult female Sprague-Dawley rats (aged 10 weeks, 250 ± 25 g, Guangdong Laboratory Animals Center, China, license No. SCXK (Yue) 2018-0002, n = 60) were used to establish contusive SCI models as described previously [16]. In brief, rats underwent a laminectomy at the T10 level after anesthetized with 1.0–2.0 % isoflurane (RWD, Shenzhen, China). After clamping the transverse processes of T10 vertebrae to stabilize the spine, the exposed dorsal surface of T13 spinal segment was subjected to a drop injury using a LISA impactor (Louisville Injury System Apparatus, Kentucky, USA) with 1.0 mm displacement and contact duration of 0.5 s. After surgery, rats received gentamicin (5 mg/kg, Guangdong Bangmin, China) intramuscularly for 3 consecutive days and their bladders were emptied manually twice daily until the rats were able to urinate freely. Locomotor behavior of hindlimbs were evaluated the second day after SCI, animals with Basso-Beattie-Bresnahan (BBB) score over 0 were excluded from the study. At 9 days post injury, rats were randomly assigned to 2 groups: UiPSC-NPCs group (n = 30) and control group (n = 30).

After opening of spinal dura matter, UiPSC-NPCs were transplanted stereotaxically using a glass micropipette attached to a microinjector (Hamilton, Bonaduz, Switzerland) into two sites (2.5 μL / site, 1 × 105 cells / μL) [17] rostral and caudal to the injury site. In the control group, the same volume of cell culture medium was applied instead. The glass micropipette was inserted from the dorsal midline surface into the spinal cord to a depth of 1.6 mm and then withdrawn to 1.2 mm depth prior to injection. During injection, the delivery was stopped for 10 s every 0.5 μL and the needle (Glass electrode, WPI, Florida, USA) was remained in position for 1 min after injection. All animals were received daily injection of cyclosporine A (15 mg / kg; Novartis, Prague, Czech Republic) [18,19] from two days before transplantation until to the end of experiments in order to prevent immune rejection. All animals were kept in environment-controlled rooms (22–24 °C, light / dark cycle of 12 h).
2.6. Microglia cell counting

Iba1-positive cells were counted from the sections of 0 mm (lesion epicenter), +4 mm (rostral to the epicenter) using ImageJ software. Three random frames at a size of 0.25 mm x 0.25 mm each around the lesion cavity (0 mm), or at a size of 0.5 mm x 0.5 mm each in dorsal part of the spinal cord at 4 mm rostral (+4 mm) for each section were chosen and the cell density was extrapolated. Six animals perfused at 56 days after UiPSC-NPCs transplantation were used in each group.

2.7. Measurement of lesion cavity

One set of transverse sections of each spinal block stained with GFAP were used to measure the size of lesion cavity. Three adjacent slices at distances of +4 mm, +2 mm, 0 mm, -2 mm, -4 mm to the epicenter were chosen. “+” referred to “rostral” and “-” referred to “caudal”. The area of lesion cavity and the total area of the spinal cord for each section were measured by Image J. The percentage of lesion cavity to the corresponding spinal cord area was calculated.

2.8. Statistical analysis

Results were presented as mean ± SEM. Data of cells survival rate was analyzed using one-way ANOVA, others were using the two-way ANOVA with Bonferroni’s post-hoc correction or (Prism®, 7.0c, GraphPad, San Diego, CA, USA). The significant difference level was set to 0.05.

3. Results

3.1. Survival and migration of transplanted UiPSC-NPCs in vivo

Before transplantation into animals, characterization of the cultured UiPSCs and UiPSC-NPCs were performed. The results showed that UiPSCs were positive for SSEA4 and OCT4, makers of pluripotent stem cells (Fig. 1A), and the neurospheres induced from UiPSCs expressed neural progenitor markers PAX6, SOX2 and Nestin (Fig. 1B). UiPSC-NPCs could be differentiated into neurons and astrocytes in vitro as confirmed by anti-TUJ1 and anti-GFAP immunofluorescent staining (Fig. 1C)

UiPSC-NPCs were transplanted into the two adjacent spinal segments rostral and caudal to the injury site at 9 days after SCI. An anti-HUNA antibody was used to trace the UiPSC-NPCs at different times after transplantation. From the horizontal spinal sections, a large number of HUNA-positive cells were found in the lesion epicenter at 28 days after transplantation. Interestingly, more cells appeared in the lesion epicenter than in the original grafting site (Fig. 2A), indicating that the transplanted UiPSC-NPCs were attracted towards the lesion site. The survival rate of transplanted UiPSC-NPCs was 49.47±14.75 at day 7, 41.47±10.93 at day 14, and 27.95±5.81 at day 28 (Fig. 2B–E), showing no statistical differences among different time points.

3.2. Differentiation of transplanted UiPSC-NPCs

Differentiation of transplanted UiPSC-NPCs were evaluated from spinal cord samples collected at 28 days after transplantation. Some HUNA labeled transplanted cells were found positive for neuronal marker TUJ1 (Fig. 3A, A’), and a few of them expressed GFAP (Fig. 3B, B’), an astrocyte marker. No olig2 expression was seen in the HUNA-positive cells (Fig. 3C, C’). The differentiating rate was relatively low; about 7.9% of the HUNA-positive cells were positive for TUJ1 and 1.79% of them were positive for GFAP as seen at 28 days after transplantation.

3.3. Effect of UiPSC-NPCs transplantation on neuroinflammation after SCI

Inflammatory cytokines are important modulators of the secondary injury after SCI. To assess whether cytokine levels were altered after UiPSC-NPC transplantation, we collected spinal cord samples at 3 days and 7 days after transplantation and measured the levels of cytokines using multiple immunoassay (Fig. 4A). A total of 9 cytokines were altered after UiPSC-NPC transplantation compared to control animals. Three pro-inflammatory factors including IFN-γ, IL-1β and IL-6, and four chemokines including MCP-1, RANTES, MIP-3α and GRO/KC, were significantly decreased at 3 days after UiPSC-NPC transplantation.
Notably, the level of IFN-γ was reduced both at 3 days and 7 days in the UiPSC-NPC group compared to the control group. A lower level of anti-inflammatory factor IL-4 was detected at 3 days but not at 7 days in UiPSC-NPC transplanted animals than that in control animals. On the other hand, pro-inflammatory factor IL-2 was found unchanged at 3 days but was increased at 7 days after UiPSC-NPC transplantation.

Activation of microglial cells represents an important feature of neuroinflammation after SCI [21]. We therefore performed anti-Iba1 immunostaining on transverse spinal sections of 56 days after UiPSC-NPC transplantation. At the lesion epicenter, microglia displayed an activated state characterized by a round shape with enlarged cell body and shortened processes (Fig. 4C). While in the spinal cord segment 4 mm rostral to injury sites were stained with anti-Iba1. Quantitative result showed that fewer Iba1+ cells in UiPSC-NPCs group than those in the control group at +4 mm from the epicenter, and no difference in cell density was found at the epicenter (0 mm) (E). Values represent the means ± SEM, Two-way ANOVA with Bonferroni’s post-hoc correction; *, p < 0.05; **, p < 0.01; ***, p < 0.001; n= 6 in each group. Scale bars as indicated in each row of panels.

3.4. Transplantation of UiPSC-NPCs reduces the size of lesion cavity

The formation of a cystic cavity surrounded by the glia scar constitutes one major obstacle of axonal regeneration after SCI [1]. To evaluate whether UiPSC-NPC transplantation have any effect on the lesion cavity formation, we stained serial transverse sections collected 56 days after UiPSC-NPC transplantation with GFAP (Fig. 5A). The overall GFAP intensity within the glia scar surrounding the lesion cavity showed no obvious differences between the UiPSC-NPCs and control groups (Fig. 5B). The lesion cavity extending up to 4 mm rostral and caudal to the epicenter in control animals, was significantly restrained after UiPSC-NPC transplantation. The size of lesion cavity, presented by the percentage of corresponding spinal cord area, was much smaller either at the lesion epicenter (0 mm) or at the positions rostrally/caudally in the UiPSC-NPCs group compared to the control group (UiPSC-NPCs versus Control: p < 0.05 at +2 mm; p < 0.001 at 0 mm; p < 0.01 at -2 mm). The results indicate that UiPSC-NPC transplantation reduces tissue loss after spinal cord injury.
4. Discussion

iPSCs are an attractive source for generating NPCs enabling autologous cell transplantation after SCI [2]. Multiple tissue-derived iPSC-NPCs have been shown effectiveness in animal SCI models [4,5]. However, the survival and differentiation of iPSC-NPCs vary according to their sources. Human urine derived iPSC-NPCs have some significant advantages considering future clinical applications. However, their biological characteristics in vivo as well as the safety and efficacy in the treatment of SCI still require further investigation using animal models. In this study, we evaluated the survival, migration, and differentiation of UiPSC-NPCs after transplantation into the injured spinal cord at the subacute phase. Ideally, transplanted NPCs are expected to differentiate into neurons and glia cells replacing the lost neurons and myelin, and therefore achieve neural circuitry remodeling and functional recovery. After a contusive injury to the spinal cord, activated microglia cells, hematogenous macrophages and other penetrating inflammatory cells are recruited to the lesion site which constitutes an inflammatory environment hostile to NPC survival [1]. Therefore, we chose to transplant at the subacute phase and injected cells into the adjacent relatively normal spinal cord area. The results could be attributed at least partly to the fact that the majority of transplanted UiPSC-NPCs migrated into and accumulated in the lesion core filled with cell debris and penetrating inflammatory cells. The cystic lesion cavity presents a nonpermissive microenvironment for NPCs to survival and differentiation, although it provides cues for stem cell homing. Improving microenvironment is critical to enhance the efficacy of UiPSC-NPCs transplantation. Considering the future clinic appliance, we did not use additional neurotropic factors or inducible factors in this study, which may also account for the low survival and differentiation rates after transplantation. In a previous report, Lu et al. [9] embedded human skin derived iPSC-NSCs into a fibrin matrix containing growth factor cocktails and transplanted them into SCI animals, robust cell survival, differentiation and integration were achieved. Scaffolds to bridge the cavity and additional growth factors to build a permissive microenvironment should be considered in the combination with UiPSC-NPC transplantation in the future.

Considering the fact that transplanted UiPSC-NPCs migrated into and accumulated at the lesion core surrounded by a gliotic scar, it made us speculate that they might act on the inflammatory cells in the lesion and alter the neuroinflammation to some extent. Indeed, through multiplex cytokine profiling, we found pro-inflammatory cytokines such as IL-1β and INF-γ were decreased after UiPSC-NPCs transplantation. This implicates that the transplanted UiPSC-NPCs could impact on the host cells within the injured spinal cord to downregulate their expression of these pro-inflammatory cytokines. However, the anti-inflammatory effect was found more prominent at 3 days after transplantation. This might be attributed to the ongoing death of the UiPSC-NPCs as around 50 % of the transplanted cells died by 7 days after transplantation. Transplantation of UiPSC-NPCs also resulted in a reduction in the extent of microglia activation, which further support a potential role of UiPSC-NPCs in suppressing neuroinflammation.

In addition to cell replacement, transplantation of NPCs have also been implicated to promote tissue repairing after SCI through neuroprotective and immune modulatory actions mediated by undifferentiated NPCs [23–25], which might also be potential...
mechanisms of UiPSC-NPCs to reduce tissue loss after SCI. Subacute transplantation of mouse NPCs after SCI has previously been shown to instruct phagocytes and reduce secondary tissue damage. Consistent with our results, they found most survived NPCs remained undifferentiated after transplantation and showed a significant impact on modulating the expression of inflammatory gene transcripts and reducing the infiltration of inflammatory cells including M1-like macrophages [24]. Transplanted NPCs could induce the expression of growth factors that prevent neural apoptosis [25]. In our study, in the UiPSC-NPC group, we found an increased expression trend of VEGF (see the heatmap), which favored neuronal and vascular regeneration and prevented tissue atrophy and demyelination in rats underwent SCI [26].

In summary, this study showed that UiPSC-NPCs could survival, migrate and differentiation after transplanted into the injured spinal cord. However, optimal strategies to improve the survival and differentiation rate remain to be explored. We also revealed a role of UiPSC-NPCs in modulating neuroinflammation which might contribute to the reduction of lesion cavity.

Author contributions

Aimei Liu: Methodology, Literature search, Experimental studies, Data acquisition, Formal analysis, Roles/Writing - original draft. Sai Kang: Experimental studies, Data acquisition, Formal analysis. Lingling Shi: Experimental studies, Data curation, Literature search, Writing - review & editing, Supervision. Panpan Yu: Project administration, Data curation, Writing - review & editing, Funding acquisition. Libing Zhou: Conceptualization, Validation, Methodology, Literature search, Writing - review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare no competing interests.

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