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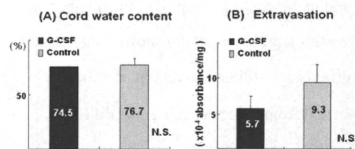FIGURE LEGENDS

(A) Cord water content (B) Extravasation

Figure 1. Assessment of blood spinal cord barrier (BSB) integrity (n=4 in each group). BSB integrity was evaluated by measuring cord water content (A) and detecting extravasation of sodium fluorescein (B). There was no significant difference in spinal cord water content or extravasation between the two groups.
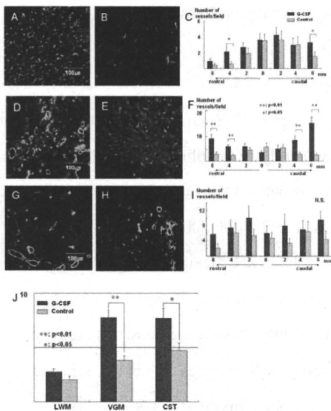

Figure 2. Angiogenesis in the spinal cord after SCI assessed by immunohistochemistry (n=5 in each group). Vessels were immunostained with anti-vWF as an endothelial cell marker. Within the LWM (A-C), a relatively small number of vessels were observed in the G-CSF-treated group (A), and in the control group (B). The number of vessels larger than 20 μm showed significant differences at four mm rostral and six mm caudal from epicenter (C). In VGM (D-F), vigorous angiogenesis were observed in the

G-CSF-treated group (D), whereas faint angiogenesis was seen in the control group (E). There was a significant difference between the two group at four and six mm rostral and caudal from the epicenter (F). In CST (G-H), there was no statistically significant difference between the G-CSF group (G), and the control group (H). The total number of vessels in the G-CSF group was larger than that in the control group (J). Bars = 100μm.
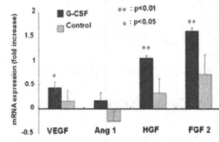

Figure 3. Expression of mRNAs for angiogenic cytokines assessed by real-time RT-PCR (n=4 in each group).

Expression of cytokine mRNAs was compared to sham group and assessed by quantitative analysis. Expression of each mRNA increased in the G-CSF group. Especially, *VEGF*, *HGF* and *FGF2* were expressed at significantly higher levels.
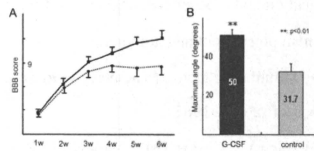

Figure 4. Hindlimb functional assessment with the Basso, Beattie, Bresnahan test. The G-CSF-treated group (square) exhibited

significantly higher scores four weeks after injury compared with the control group (circle and dotted line)(n=8 in control group, n=11 in G-CSF group)(A). The mean value of the inclined plane test was significantly higher in the G-CSF group than in the control group (n=5 in each group)(50.0 to 31.7, B).

Transplantation of murine induced pluripotent stem (iPS) cell-derived astrocytes increases sensitivity to mechanical stimulus in a rat spinal cord injury model

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Abstract

Object: Clinical use of autologous induced pluripotent stem (iPS) cells could circumvent immune rejection and bioethical issues associated with embryonic stem cells. Spinal cord injury (SCI) is a devastating trauma with long lasting disability and current therapeutic approaches are not satisfactory. In the present study, we used the neural stem sphere (NSS) method to differentiate iPS cells into astrocytes which were evaluated after their transplantation into injured rat spinal cords.
Methods: iPS cell-derived astrocytes were differentiated using the NSS method and injected three and seven days after spinal cord contusion injury. Control rats were injected with DMEM in the same manner. Locomotor recovery was assessed for eight weeks and sensory and locomotion tests were evaluated at eight weeks. Then, immunohistological parameters were assessed.
Results: Transplant recipients lived for eight weeks without tumor formation. Transplanted cells stretched their processes along the longitudinal axis but they did not merge with the processes of host GFAP-positive astrocytes. Locomotion was assessed in three ways, but none of the tests detected statistically significant improvements compared to DMEM controls after eight weeks. Rather, iPS cell transplantation caused even greater sensitivity to mechanical stimulus compared to the DMEM control.
Conclusion: Astrocytes can be generated by serum treatment of neural stem sphere-generated cells

derived from iPS cells. However, transplantation of such cells is poorly suited for repairing SCI.

Introduction

Recently, induced pluripotent stem (iPS) cells were established by introducing three or four genes (such as *Oct3/4*, *SOX2*, *KLF4*, c-*MYC*) into mouse or human fibroblasts.[35, 36] iPS cells can be established from individual somatic cells, and they possess potent differentiative capacities. If autologous iPS cells could be established from patients, it would be possible to avoid bioethical concerns and immune rejection.

Nakayama and Inoue reported that astrocyte-derived factors modulate the differentiation of ES cells into neurons, a process they termed the "neural stem sphere" (NSS) method.[27,28,29] They also reported that ES-derived neural stem cells (NSC) could be differentiated almost exclusively into astrocytes by withdrawing fibroblast growth factor-2 (FGF-2) from the medium.[30] Recently, dopaminergic neurons derived from monkey ES cells (using the NSS method) were transplanted into a monkey model of Parkinson's disease and the technique promoted locomotion.[24] We hypothesized that the NSS method could also be applied to iPS cells to achieve targeted differentiation into neuronal cells.

Spinal cord injury (SCI) is a devastating type of trauma for patients due to the resulting long-lasting disability and the limited responsiveness to acute drug administration and rehabilitation. In assessing the pathology of SCI, it was noted that reactive astrocytes proliferate, form a glial scar and secrete inhibitory agents such as chondroitin sulfate proteoglycan (CSPG).[20] Because glial scar formation can become an obstacle to axonal regeneration,[6,7,10,20] astrocytes' actions have been regarded as harmful after SCI. More recently, there have been reports that astrocytes may be beneficial following SCI. For example, widespread infiltration of inflammatory cells results in severe motor deficits after SCI.[33] Reactive astrocytes can surround inflammatory tissue, and prevent inflammatory spread.[25] Knockdown studies of genetically modified GFAP-positive cells have reinforced the constructive role of astrocytes.[8] Ablation of reactive astrocytes has been found to reduce locomotor recovery[9] and exacerbate the inflammation and pathologies associated with autoimmune diseases of the CNS.[38] Disappearance of immature astrocytes in the region of spinal cord transection influences the regrowth of neurofilament-positive axons.[15] Transplantation of a purified population of a specific sub-type of astrocytes (derived from BMP-treated embryonic glial cell-restricted

precursors) promotes axon regeneration and functional recovery after acute transection injuries of the adult rat spinal cord.[4] Thus, transplantation of astrocytes following SCI could have beneficial effects on recovery.

In the present study, modified NSS methods were used to generate NSC from mouse iPS cells. Using that approach, we also differentiated neurons, astrocytes, and oligodendrocytes from iPS cell-derived NSCs. Conducting the transplant three days after spinal cord injury differed from previous studies. We chose acute transplantation in the present investigation in order to save damaged tissue at the lesion epicenter by replacing astrocytes soon after SCI. Although it has been well documented that the vast majority of neuronal death following SCI occurs in the first 24 hours, it is impossible to inject cells soon after SCI in the clinical setting. For that reason, we transplanted astrocytes derived from iPS cells three and seven days after SCI. We assessed locomotor recovery for eight weeks after SCI and assessed thermal and nociceptive thresholds eight weeks after SCI. This is the first report in which the NSS method has been used for iPS cell differentiation and transplantation of derived cells into injured spinal cords.

Methods

Cultivation of iPS cells and their differentiation into neural stem cells

Mouse iPS cells (iPS-MEF-Ng-20D-17; No. APS0001) were purchased from the Riken Cell Bank (Ibaraki,

Japan, http://www.brc.riken.jp/lab/cell/). These mouse iPS cells express GFP via the Nanog promoter.[35] We purchased feeder cells (SNL76/7) from DS Pharma Biomedical Co, Ltd (Osaka, Japan), and astrocyte-conditioned medium (ACM) from Sumitomo Bakelite Co, Ltd (Tokyo, Japan).

Figure 1 shows the schema for iPS colony generation and differentiation into neurons, oligodendrocytes, and astrocytes. To induce iPS colony formation, cells were seeded on a mitomycin C-treated feeder layer in ES medium (Dulbecco's modified Eagle's medium (DMEM), 15% knockout serum replacement, 1 mM non-essential amino acids, 0.1 mM •-mercaptoethanol, 1% penicillin-streptomycin) (all from GIBCO Invitrogen, Carlsbad, CA). Colonies of undifferentiated iPS cells, 300 - 500 μm in diameter, were picked up from the feeder layer using a Pipetman (Gilson, Inc. Middleton, WI) and transferred in ACM to non-treated bacteriological dishes containing an equal amount of DMEM/F2/N2 supplement (GIBCO), with 20 ng/mL recombinant FGF-2 (R&D Systems, Minneapolis, MN). The colonies were cultured for four days, giving rise to NSS, which were plated on Matrigel-coated dishes (BD Biosciences, Bedford, MA) and cultivated for seven days in NSC medium (Neurobasal medium supplemented with 2% B-27 (both from GIBCO), and 20 ng/mL FGF-2). At this stage, NSS gave rise to spherical clusters of NSCs in the outer layer which migrated from the surface of the NSS to the surrounding areas. The migrating NSCs were collected with a

Pipetman after NSS were detached using 0.25% trypsin treatment. The collected cells were expanded in NSC medium, and proliferating cells were frozen at –80 $^{\circ}$C in DMEM, 10% fetal bovine serum (FBS: BIOWEST, Nuaillé, France) plus 10% DMSO. When required, the cryopreserved cells were thawed and expanded in NSC medium.

Differentiation of NSCs into neurons, astrocytes, and oligodendrocytes

To induce differentiation into neurons, NSCs were transferred to Matrigel-coated dishes and cultivated for seven days in ACM with 20 ng/mL FGF-2. This method was applicable to primary expanded or thawed NSCs. To induce differentiation into oligodendrocytes, we used a commercial oligodendrocyte differentiation kit (R&D systems, Minneapolis, MN) developed for ES cells. Briefly, to induce A2B5-positive cells from NSCs, expanded NSCs were plated on poly-L-ornithine/ fibronectin-coated plates for 12 days. The medium was changed every four days as follows: N2 plus/FGF medium for the first four days, followed by N2-plus/FGF/EGF medium for an additional four days, and N2 plus/FGF/EGF/PDGF-AA medium for the final four days. To direct differentiation into oligodendrocytes, the medium was changed to N2 plus/T3 medium, and the cells were cultivated for an additional seven days. To differentiate NSC into astrocytes, expanded NSCs (passage two to six) were transferred to Matrigel-coated dishes and cultivated for 14 days in DMEM with 10% FBS and 1%

penicillin-streptomycin without FGF-2.

Fluorescent immunocytochemistry

For *in vitro* experiments, cells cultured on chamber slides or NSS attached to chamber slides were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS). Immunocytochemistry was performed using standard protocols and antibodies as follows: rabbit polyclonal anti-nestin antibody (1:400; Chemicon, Temecula, CA, USA), mouse anti-•-tubulin III (Tuj-1, 1:800; Covance, Berkeley, CA), mouse anti-glial fibrillary acidic protein (GFAP, 1:400; Sigma-Aldrich), rabbit anti-s100 (1:400; Dako Cytomation Co. Ltd, Copenhagen, Denmark), mouse anti-A2B5 (1:100; R&D Systems, Minneapolis, MN), mouse anti-O4 (1:50; Chemicon), mouse anti-GalC (1:200; Chemicon), and rabbit anti-green fluorescent protein (GFP, 1:1600, Molecular Probes, Eugene, OR). Cell nuclei were stained with DAPI (1:1000, Molecular Probes). After reacting with primary antibodies, the sections were incubated with Alexa-Fluor 488-conjugated anti-mouse or anti-rabbit IgG (Molecular Probes), Alexa-Fluor 488-conjugated anti-mouse IgM (Molecular Probes), and Alexa-Fluor 594-conjugated anti-mouse or anti-rabbit IgG (Molecular Probes).

Real-Time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

The samples consisted of undifferentiated iPS cells, NSCs (passage number three), and astrocytes derived from

NSCs (passage number five). Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) reagent and RNAeasy Mini Kit (Qiagen, Hilden, Germany). Using the Agilent 2100 Electrophoresis Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA), we ascertained that both 18 S and 28 S rRNAs were present without genomic contamination. Also, we characterized RNA by absorption spectroscopy, using the NanoDrop ND-2000 (Thermo Fisher Scientific, Waltham, MA) to confirm that the absorption value of A260/280 fell between 1.8 and 2.1. Total RNA (2 µg) was reverse transcribed into cDNA by Super Script III (Invitrogen). Samples were analyzed by ABI PRISM 7900HT Sequence Detection System and Taq-man Gene Expression Assay Products (Applied Biosystems Inc., Warrington, UK). Gene Symbols and Assays ID were as follows: Nanog (Mm02384862_g1), *Oct3/4* (Mm03053917_g1), Nestin (Mm00450205), *GFAP* (Mm01253033_m1). *GAPDH* (Mm99999915_g1) was used for the internal control gene. Standard curves and amplification plots of each gene were prepared using one of three RNAs (Nanog, *Oct3/4*: iPS cells; nestin: NSC; *GFAP*: astrocytes). We confirmed that all values fell within the range of the standard curve. To compensate for differences in RNA quantity, all data were normalized to *GAPDH* gene expression.

SCI and cell transplantation

Forty-five female Sprague-Dawley rats (eight weeks old; SLC Inc. Hamamatsu, Japan) were subjected to SCI. Anesthesia was induced by inhalation of 5% halothane in 0.5 L/min oxygen and maintained with 1.3% halothane in 0.5 L/min oxygen. A laminectomy was performed at the T9-T10 levels. The moderate contusion injury was introduced using Infinite Horizon impactor (2 mm diameter impactor head, 200 Kdyn; Precision Systems and Instrumentation, Lexington, NY). After injury, muscles and skin were sutured layer to layer and the rats placed in a warm cage overnight. All animals were given antibiotics (500 µL/ day; Bactramin, Chugai Pharmaceutical, Tokyo, Japan) by subcutaneous administration once a day for three days. Food and water were provided *ad libitum*. Manual bladder expression was performed twice a day until recovery of the bladder reflex.

iPS-derived astrocytes were detached from dishes using 0.25% trypsin, washed one time with DMEM, and resuspended in DMEM. In that time, we verified that iPS-derived astrocytes were not GFP-positive. Derived astrocytes were labeled for transplantation studies with a PKH26 Red Fluorescent Cell Linker Kit per the manufacturer's instruction (Sigma-Aldrich, St Louis, MO). In preliminary trials, we confirmed that PKH26-treated astrocytes could survive more than two months on PLL-coated dishes in DMEM with 10% FBS and 1% penicillin-streptomycin without FGF-2. Three or seven days after injury, the injured site was re-exposed and transplantation was performed. Astrocytes derived from iPS cells (100,000/ 5 µL; three day astrocyte group, n = 20,: seven day astrocyte group n = 9,) or DMEM (5 µL; three day DMEM group, n = 10;

seven day DMEM group n = 7) were injected into injured sites using glass micropipettes attached to a 10 μL Hamilton syringe (Hamilton Company, Reno, NV). The injection was made at a one millimeter depth over a period of one minute. The needle was left in the spinal cord for an additional two minutes following injection in order to minimize reflux. The sites of injection were at the lesion epicenter. All animals were immunosuppressed subcutaneously with cyclosporine A (10 mg/kg, Novartis, Basel, Switzerland) for 14 days immediately after cell transplantation or DMEM injection. Thereafter, cyclosporine (Neoral, Novartis) was mixed in drinking water at 50 μg/mL concentration until sacrifice. None of the animals showed abnormal behavior. All the experimental procedures were performed in compliance with the guidelines established by the Animal Care and Use Committee of Chiba University.

Assessment of sensory motor functions

BBB open field locomotor test

Hind limb function was assessed in an open field (100 × 60 cm plastic pool) using the BBB open field locomotor test.[2] Measurements were performed one week after contusion injury and weekly thereafter for eight weeks. Tests were videotaped for five minutes and scored by a trained observer who was unaware of the treatment group.

Inclined plane test

Eight weeks after SCI, animals were placed in a head up position on an inclined plane and the angle of the slope was gradually increased. The angle at which the animal fell down from the slope was recorded, with three trials per animal. The median values were compared between the astrocyte group and the DMEM group. We also performed the test with normal rats (n = 5).

SCANET test

We performed movement analysis using the SCANET MV-40 (Melquest, Toyama, Japan). Rats were allowed to move freely in the attached wide plastic box (460 mm x 460 mm x 303 mm (H)). The SCANET system consists of a cage equipped with two crossing infrared sensor frames arranged at different heights, by which small (M1) and large (M2) horizontal movements can be monitored. The authors assessed locomotor function by determining the M1 scores for five and 30 minutes. The quantity of two dimensional motions was accumulated automatically.[23]

Sensory tests

Thermal nociceptive thresholds in rat hind limbs were evaluated using a Hargreaves device (Ugo Basile, Varese, Italy). The rats were placed in individual transparent acrylic boxes with the floor maintained at 28°C. A heat stimulus (150 mcal/ s/ cm^2) was delivered using a 0.5 cm diameter radiant heat source positioned under the plantar surface of the hind limb. The heat source was placed alternately under each hind limb to avoid anticipation by the animal. A cutoff time of 22 seconds was used, as it had been ascertained that no tissue

damage would result within this time period. The withdrawal threshold was calculated as the average of six consecutive tests.

Mechanical withdrawal thresholds in rat hind limbs were tested using a dynamic plantar aesthesiometer (Ugo Basile), in which a mechanical stimulus was applied via an actuator filament (0.5 mm diameter), which under computer control applies a linear ramp 5.0 g/s to the plantar surface of the hind limb. The withdrawal threshold was calculated as the average of six consecutive tests. Both tests were performed eight weeks after contusion. For comparison with baseline, we also performed both tests with normal rats (n = 5).

Tissue preparation

For histological evaluation, eight weeks after SCI, all animals were perfused transcardially with 4% paraformaldehyde in PBS (pH 7.4) under pentobarbital anesthesia (50 mg/kg, Abbott Laboratories, IL). A tissue block of the spinal cord including the lesion epicenter (Th 9-11) was removed and treated overnight in the same fixative, stored in 20% sucrose in PBS at 4°C, embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan) and frozen at -80°C. Spinal cords were freeze-mounted on a holder, cut into 25 µm sagittal or transverse sections, and placed on poly-L-lysine-coated glass slides. For midsagittal sections (three day astrocyte group, n = 10; seven day astrocyte group, n = 3), spinal cords (1.2 cm length centered upon the lesion) were sliced and mounted on six sequential slides. Each slide had 6 - 8 slices

spaced at 150 µm intervals for each sequential section, which correspond to one millimeter spinal cord width. For cross sections of the spinal cords (three day astrocyte group, n = 10; three day DMEM group, n = 5; seven day astrocyte group, n = 6; seven day DMEM group, n = 6), we sliced 8.0 mm long spinal cord pieces centered on the lesion. Every third slice was mounted on six sequential slides. Each slide had 16 – 18 sequential sections spaced at 450 µm intervals, which corresponded to a seven to eight millimeter length of spinal cord.

Histological analyses

Transplanted cell counts

Sagittal sections of the spinal cords were used to estimate counts of viable transplanted cells eight weeks after SCI. The PKH28 red fluorescence was observed by fluorescence microscopy (DP71, Olympus, Tokyo, Japan). Three slices per animal were observed with a 10x objective lens. One slice was divided into four pictures. We counted twelve pictures for each animal and averaged the cell densities. When we counted surviving cells in the spinal cord, we assumed that the spinal cord was a 4.0 mm cylinder whose diameter averaged 0.92 mm. One cross section had an area of $\cdot (0.46)^2$ mm^2. We hypothesized that the height of transplanted cells under these conditions was approximately 40 µm. If a spinal cord is assumed to be cylindrical, a 4.0 mm long spinal cord segment would be composed of 100 cross sections. If the surviving cell density is X cells/ mm^2, then the surviving cell number in the 4.0 mm long

spinal cord is given by $100 \cdot (0.46)^2$ X cell number.

We analyzed transplanted cells for the presence of GFAP-positive astrocytes, Tuj-1-positive neurons, or GST-• -positive oligodendrocytes. We used mouse anti-GFAP antibody (1:400, Sigma-Aldrich), mouse anti-• -tubulin III antibody (1:800, Covance), and mouse anti- GST-• (1:1000, BD Pharmingen, Franklin Lakes, NJ) for primary antibodies, followed by Alexa-Fluor 488-conjugated anti-mouse IgG (1:800, Molecular Probes) for secondary antibody. We used LSM5 Pascal confocal laser scanning microscopy to observe double positive cells (Carl Zeiss, Oberkochen, Germany).

Luxol Fast Blue (LFB) staining

We performed LFB staining for the three day astrocyte group and the three day DMEM group to measure the area of spared white matter eight weeks after SCI. One slide per animal was stained with LFB for myelin staining. Slices were photographed using an optical microscope (DP71, Olympus) with a 4x objective lens. The percentage of spared white matter was calculated from the number of pixels representing the area of spared white matter divided by the number of pixels for total cross sectional area using Scion Image computer analysis software (Scion Corporation, Frederick, MA, USA). We calculated the data from three regions (the lesion epicenter, 2.5 mm rostral, and 2.5 mm caudal from the lesion epicenter).

Quantitative analysis of CGRP-positive area

For the three day astrocyte andthe DMEM group, one cross section per animal was incubated with rabbit anti-calcitonin gene related peptide (CGRP) antibody for overnight at $4^{\circ}C$ (1:1000, ImmunoStar, Inc. Hudson, WI) and reacted with Alexa-Fluor 488 goat anti-rabbit IgG secondary antibody for one hours at room temperature. Three slices which were three to four millimeter rostral or caudal from the lesion epicenter were picked and photographed with a 10x objective lens using a fluorescence microscope (DP71, Olympus). Images were inverted in black and white and assessed for the number of CGRP-positive immunoreactive pixels with Scion Image. We divided the superficial posterior horn into two layers and deeper regions into three layers for separate measurements.

Quantitative analysis of the GFAP-positive area

We performed GFAP staining to measure the GFAP-positive area eight weeks after SCI. One slide per animal was incubated with mouse anti-GFAP antibody for overnight at $4^{\circ}C$ and reacted with Alexa-Fluor 488 goat anti-mouse IgG for one hour at room temperature. Slices were photographed by fluorescent microscopy (DP71, Olympus) using a 4x objective lens. The percentage of the GFAP-positive area was calculated from the number of pixels in the GFAP-positive area divided by the total pixels of the cross sectional area using Scion Image. We calculated the data from three points (the lesion epicenter, 2.5 mm rostral, and 2.5 mm caudal from the lesion

epicenter).

Statistical analysis

The fractional BBB locomotor score, the inclined plane test, the extent of motion (SCANET MV40), and the histological studies were subjected to Student's *t*-test. The time course of recovery of BBB scores was subjected to repeated measures ANOVA followed by *post-hoc* testing using Student's *t*-test. Hypersensitivity of mechanical stimulus and thermal hyperalgesia were subjected to one-way ANOVA followed by Scheffe's *post-hoc* test. Results are presented as mean values ± S.E.M. Values of $p < 0.05$ were considered statistically significant.

Results

Formation of neural stem spheres in ACM

iPS cells grew rapidly on the SNL feeder layer, and remained in an undifferentiated state (Fig. 2A, B). iPS colonies picked up from SNL feeder layers gave rise to floating spheres after four days of cultivation in ACM + FGF-2 (Fig. 2C-F). GFP green fluorescence declined gradually at the surface (Fig. 2G-J). The spheres were composed of three regions. The core region was still undifferentiated, and generated GFP green fluorescence (Fig. 2K). The surface region consisted of neural stem cells (NSCs) which expressed nestin (Fig. 2K white arrows, Fig. 2L), a marker of neural stem cells. The intermediate layer was a transitional stage of differentiation from iPS cells to NSC (Fig. 2K). Subsequently, NSS were cultured in neurobasal

medium + FGF-2 and a large number of NSCs migrated from the NSS to the surrounding area (Fig. 2M). After removing NSS from the dish, NSCs were retrieved by trypsin (0.25%) treatment. Retrieved NSCs could be expanded for more than four passages, remaining undifferentiated. We could retrieve 120,000 NSCs from twelve NSS. Thus, one NSS gave rise on average to 10,000 NSCs. Moreover, NSCs could be cryopreserved by a standard protocol.

Differentiation into neurons, astrocytes, and oligodendrocytes

When NSS were transferred to Matrigel-coated plates and cultivated in ACM + FGF-2, Tuj-1-positive neurons migrated from the NSS (Fig. 3A, B). NSS were plated on poly-L-ornithine/fibronectin-coated plates for four days in N2 plus/FGF medium, then N2-plus/FGF/EGF medium for an additional four days, and finally N2 plus/FGF/EGF/PDGF-AA medium for the final four days. Most NSCs then expressed A2B5 (a marker of glial precursor cells) (Fig. 3C). About 30% of A2B5-positive cells differentiated into O4-positive oligodendrocytes after seven days in N2 plus T3 medium (Fig. 3D). High magnification views show that induced oligodendrocytes were both O4 and GalC-positive, stellate-shaped cells (Fig.3E, F). NSCs derived from NSS differentiated exclusively into astrocytes when FGF-2 was removed from neurobasal medium. To differentiate cells into GFAP-positive astrocytes more efficiently, we cultivated NSCs

in DMEM with 10% FBS for 14 days (Fig. 3G). Induced astrocytes had both GFAP and abundant s100 expression in the cytoplasm (Fig. 3H, I).

Gene expression analysis of iPS cells, NSCs, and astrocytes

To investigate gene expression profiles during iPS cellular differentiation into astrocytes, Nanog, *Oct3/4*, nestin, and *GFAP* expression were analyzed quantitatively by RT-PCR. The samples included undifferentiated iPS cells, NSCs (passage number three), and astrocytes differentiated from NSCs (passage number five). Nanog and *Oct3/4* were mainly expressed in iPS cells rather than in NSCs or astrocytes derived from iPS cells (Fig. 4). Nestin expression was upregulated almost two-fold in NSCs compared to astrocytes derived from iPS cells. Conversely, *GFAP* expression was upregulated nearly two-fold in astrocytes derived from iPS cells compared to NSCs.

Behavioral assessments

We transplanted astrocytes derived from iPS cells three and seven days after SCI and measured the BBB locomotor score for eight weeks. The control group received a DMEM injection under the same conditions. There was no statistically significant difference between the astrocyte group and the DMEM group in the BBB locomotor score (repeated-measures ANOVA: three days, p = 0.99, Fig. 5A; seven days, p = 0.115, Fig. 5B).

In the inclined plane test, the three day astrocyte transplant group scored 42.5 ± 1.2 degrees and the three day DMEM group scored 42.5 ± 3.1 degrees. Normal animals averaged 70 ± 0 degrees (n = 5). There was no statistically significant difference between the three day astrocyte transplantation group and the three day DMEM group (p = 1). The extent of locomotion measured by SCANET MV40 for five minutes and 30 minutes also did not show a statistically significant difference (p = 0.84 for five min and p = 0.55 for 30 min, Fig. S1 A and B). The average quantity of locomotion in all groups was 1107.4 ± 138.6 for five min and 2012.5 ± 220.4 for 30 min. Locomotion rapidly slowed in the SCANET box within a few minutes, making it difficult to measure without some stimulus to induce locomotion. These experiments also did not show a statistically significant difference between seven day astrocyte and seven day DMEM groups (data not shown).

Sensory tests

Mechanical thresholds using a dynamic plantar aesthesiometer showed a mean of 41.3 ± 1.7 g in normal rats, decreasing to 21.7 ± 1.6 g in the three day astrocyte transplant group versus 29.5 ± 1.5 g in the three day DMEM control group eight weeks after contusion (Fig. 6A). All contused rats showed more sensitivity to mechanical stimulus than did normal rats (p < 0.001, normal vs. the three day astrocyte group; p = 0.0051, normal versus the three day DMEM group). The three day astrocyte group showed a lower threshold toward mechanical stimulus compared to the

three day DMEM control group (p = 0.013). In the seven day transplantation group, mechanical thresholds averaged 25.5 ± 2.7 g versus 28.4 ± 3.3 g in the seven day DMEM group (Fig. 6B). Although all contused rats showed more sensitivity to mechanical stimulus than did normal rats (p < 0.0066, normal vs. the seven day astrocyte group; p = 0.0324, normal versus seven day DMEM group), there was no statistically significant difference between the seven day astrocyte group and the seven day DMEM group (p = 0.76).

Analysis of normal rats with a Hargreaves device revealed a mean thermal latency of 22.3 ± 0 seconds (Fig. 6C) compared to mean values of 17.2 ± 0.9 s in the three day astrocyte group, and 17.8 ± 1.1 s in the three day DMEM group eight weeks after contusion. The three day astrocyte transplant group showed significant thermal hyperalgesia compared to normal rats (p = 0.018). In the seven day transplantation group, the mean thermal latency was 16.5 ± 1.6 S and 18.8 ± 1.7 S in the DMEM group. There was no statistical significance (p = 0.52). The seven day astrocyte group showed more thermal hyperalgesia compared to normal rats, but the difference did not reach statistical significance (p = 0.056).

Histology

Survival of transplanted cells

Transplanted cells labeled with PKH26 red survived eight weeks after transplantation (Fig.7A). These cells fluoresce bright red and stretched their processes in the direction of the longitudinal axis (Fig. 7B,

GFAP: green). High magnification images showed that transplanted cells (PKH26 red positive) stretched their process along astrocytes' processes (GFAP: green). Immunofluorescent labeling of GFAP did not co-localize with the PKH26 red fluorescent marker of the transplanted cells (Fig. 7C). Transplanted cells labeled with PKH26 red also did not co-localize with cells marked with the neuronal markers •-tubulin III or GST-•. We counted 69.2 ± 26.2 PKH26-positive transplanted cells / mm^2 in a spinal cord area four mm in length centered on the lesion epicenter. Thus, •(0.46)^2 × 69.2 × 100 = 4598 cells survived eight weeks after transplantation, or about 4.6% of the original number transplanted. In the seven day astrocyte group, only one rat within three rats processed for sagittal sections had PKH26 red positive transplanted cells in the spinal cord eight weeks after cell transplantation.

White matter sparing

We evaluated the ratio of the retained myelinated area to the whole transverse area of the spinal cord at three points (rostral 2.5 mm from lesion epicenter, lesion epicenter, caudal 2.5 mm from lesion epicenter). There were no statistically significant differences between the controls and animals transplanted at three days at any of the points (p = 0.91 for rostral, p = 0.96 for lesion epicenter, p = 0.44 for caudal, Fig. S2 A-C). We also did not see any statistically significant differences between controls and the animals transplanted after seven days at any points along the spinal cord

(data not shown).

Quantitative analysis of the CGRP-positive area

Supplemental Figure 3 shows CGRP-immunoreactivity 3 - 4 mm rostral from the lesion epicenter (A) and 3 - 4 mm caudal from the lesion epicenter (B) for animals transplanted after three days. We divided the posterior horn into five layers and measured superficial layers (I-II) and deep layers (III-V) in animals receiving astrocytes or DMEM. In the superficial layers, the astrocyte transplant group had greater immunoreactivity for CGRP, but there was no statistical significance to the difference (p = 0.64 for rostral, p = 0.27 for caudal). In deep layers, the CGRP-immunoreactive area was quite limited, and there was no statistically significant difference between the astrocyte group and the DMEM group (p = 0.61 for rostral, p = 0.40 for caudal). We also did not see any statistically significant difference for animals transplanted after seven days at any points along the spinal cord (data not shown).

Quantitative analysis of the GFAP-positive area

Supplemental Figure 4 shows the proportions of the whole cross sectional areas which were GFAP-positive 2.5 mm rostral from the lesion epicenter, at the lesion epicenter, and 2.5 mm caudal from the lesion epicenter. Although the GFAP-positive areas were abundant in the astrocyte groups at all three sites, there were no statistically significant differences (p = 0.09 for rostral, p = 0.79 for

lesion epicenter, and p = 0.27 for caudal).

Discussion

The present study consisted of two parts. In the first portion, we documented that the NSS method can successfully differentiate mouse iPS cells into astrocytes. In the second portion of the study, we described the results obtained by transplanting iPS-derived cells into SCI lesions.

NSS method for iPS cell differentiation

Several methods have been developed to prepare neural cells from ES cells. These include the use of embryoid bodies,[32] induction via retinoic acid,[1] and the use of stromal cell-derived inducing activity (SDIA).[18] Nakayama et al. reported that astrocyte-derived factors can mediate differentiation of ES cells into neurons.[27] They also described a NSS method in which FGF-2 was withdrawn from medium leading NSCs to differentiate into astrocytes.[29,30] Differentiation of neurons from ES cells using the NSS method is well established. Using the NSS method, dopaminergic neurons have been derived from monkey ES cells and have been transplanted to monkeys with Parkinson's disease, resulting in improved motor function.[24] Human neurons, generated from ES cells by the NSS method, survive in murine brains following xenotransplantation.[34] Nakayama reported that the NSS method was very efficient because each ES cell generated 1000 NSCs over 11 days.[28] Moreover, xeno-free induction is possible because chemically defined N2

medium can generate NSS without having to resort to rodent ACM.[34] We hypothesized that the NSS method could be applied to iPS cell differentiation. In the present study, we transferred iPS cell colonies to floating cultures, and differentiated NSCs on the surface of the NSS. NSCs rapidly migrated away after the shift to adhesion culture. We could propagate NSCs in this fashion, followed by cryopreservation. NSCs were successfully differentiated into astrocytes, neurons, and oligodendrocytes. The results of quantitative RT-PCR study were reasonable because GFAP transcripts were more abundant in the astrocytes derived from iPS cells than in the NSCs. We also showed that astrocytes derived from iPS cells have abundant S-100 and GFAP proteins in the cytoplasm.

Recently, two papers demonstrated that human pluripotent stem cells could differentiate into oligodendrocytes[14] or spinal motor neurons.[13] They showed that neural cells could be differentiated by way of neuroepithelial cells over more than two months. Okuno et al. demonstrated that human ES cells could differentiate into neural cells within one month by the NSS method.[34] We emphasize that the NSS method could be quite rapid if applied clinically. Additional advantages of the NSS method include obtaining NSCs with high purity and the option of cryopreservation. The generation of NSCs from NSS is efficient and a useful option for regenerative medicine. Neural progenitors were produced from murine ES cells by a combination of non-adherent conditions and serum starvation.[21] They showed that stem cell markers Oct4 and Nanog decreased and nestin increased after differentiation of ES cells to neural progenitors in floating spheres as in the present study. While the sphere-like structures resembled those observed in the present study, the proliferation efficacy of neural progenitor cells remains unclear. The generation of NSCs from NSS is still efficient and is a useful option for regenerative medicine.

Astrocyte transplantation causes greater sensitivity to mechanical stimulus

Astrocytes derived from iPS cells were injected into rat spinal cord lesions three and seven days after SCI. Although the astrocyte group did not show locomotor recovery that was significantly better than the DMEM group, transplanted cells survived in the spinal cords eight weeks after transplantation. Transplanted astrocytes changed their character to GFAP-negative cells in the injured spinal cord eight weeks after transplantation. Astrocyte transplantation increased the recipients' sensitivity to mechanical stimulus and thermal hyperalgesia compared to normal rats. Also, three day astrocyte transplantation caused greater sensitivity to mechanical stimulus compared to DMEM controls. Greater sensitivity to mechanical stimulus is mainly caused by reactive astrocytes, perhaps through the secretion of diffusible chemical transmitters which may augment primary afferent neuronal signaling or sensitize second order neurons in the spinal cord.[11] Transplanted astrocytes

derived from glial precursors induced allodynia after SCI.[5] Transduction of neural stem cells with neurogenin-2 before transplantation suppressed astrocytic differentiation of engrafted cells and prevented graft-induced sprouting and allodynia.[12] These reports support our finding that astrocytes derived from iPS cells caused greater sensitivity to mechanical stimulus. Neural stem cell transplantation also causes allodynia of the forelimbs after thoracic SCI without functional recovery.[22] The onset of allodynia might be a general occurrence because it is difficult to envision a causal association between forelimb allodynia and thoracic SCI.

Early astrocyte transplantation

　　In the present study, early astrocyte transplantation alone did not promote motor recovery as described above. We expected cell survival factors, like neurotrophins, would be up-regulated by astrocyte transplantation, leading to improved salvage of neuronal cells. We were unable to demonstrate a statistically significant difference in white matter sparing using Luxol fast blue staining. Thus, early transplantation of astrocytes to save damaged tissue at the lesion epicenter did not show the expected results. Many studies have reported that the transplantation of NSCs promotes motor recovery.[16,31] Possible mechanisms of recovery include remyelination by oligodendrocytes differentiated from transplanted NSCs,[3,19] and donor-derived immature neurons forming both efferent and afferent synapses with host neurons.[3,37] In the

present study, iPS-derived astrocytes changed their character to GFAP-negative cells after transplantation and contacted GFAP-positive host astrocytes. Trans-differentiation of transplanted cells in the injured spinal cord could explain their change in function. In the present study, approximately 5% of the 100,000 cells injected into the injured spinal cord survived eight weeks. Immediately after injury, the levels of many neurotoxic inflammatory cytokines (such as interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-alpha) increase and then decline sharply within 24 hr.[26] When neurosphere-derived neural progenitor cells were transplanted 24 hr after the injury, almost none of the grafted cells survived.[31] Transplanted cells could be damaged by inflammatory cytokines after cell transplantation. Thus, astrocyte transplantation did not proceed as anticipated in the injured spinal cord. These results suggest that iPS-derived astrocyte transplantation three and seven days after SCI is insufficient to achieve locomotor recovery. In the setting of traumatic SCI, neurons, oligodendrocytes, and astrocytes are damaged simultaneously. Karimi et al. reported that adult neural stem/ progenitor cell transplantation with Chondroitinase and growth factor improved functional recovery.[17] We conclude that transplanting a single type cell for spinal cord injury is insufficient to improve functional recovery. On the other hand, a critical aspect of regenerative medicine is guaranteeing the safety of the patient. In that regard, we suggest that the NSS method, which is quite simple and efficient, could be utilized

with confidence.

Conclusions

Astrocytes derived from iPS cells were injected into rat spinal cord lesions three and seven days after SCI. Although the astrocyte group did not show significantly better locomotor recovery than the DMEM group, transplanted cells survived in the spinal cords eight weeks after transplantation. Astrocyte transplantation increased the recipients' sensitivity to mechanical stimulus compared to DMEM controls. We conclude that transplanting astrocytes differentiated by serum treatment of neural stem cells (generated from iPS cell-derived, neural stem spheres), is poorly suited for repairing SCI.

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Disclosure

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

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