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Transplantation of neural stem/progenitor cells at different locations in mice with spinal cord injury

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Abstract

Transplantation of neural stem/progenitor cells (NS/PCs) promotes functional recovery after spinal cord injury (SCI); however, few studies have examined the optimal site of NS/PC transplantation in the spinal cord. The purpose of this study was to determine the optimal transplantation site of NS/PCs for the treatment of SCI. Wild-type mice were generated with contusive SCI at the T10 level and NS/PCs were derived from fetal transgenic mice. These NS/PCs ubiquitously expressed *ffLuc-cp156* protein (Venus and luciferase fusion protein) and so could be detected by *in vivo* bioluminescence imaging 9 days post-injury. NS/PCs (Low: 250,000 cells per mouse; High: 1 million cells per mouse) were grafted into the spinal cord at the lesion epicenter (E) or at rostral and caudal (RC) sites. Phosphate buffered saline was injected into E as a control. Motor functional recovery was better in each of the transplantation groups (Low-E, High-E, Low-RC and High-RC) than in the control group. The photon counts of the grafted NS/PCs were similar in each of the four transplantation groups, suggesting that the survival of NS/PCs was fairly uniform when more than a certain threshold number of cells were transplanted. Quantitative RT-PCR analyses demonstrated that brain-derived neurotrophic factor expression was higher in the RC segment than in the E segment, and this may underlie why NS/PCs more readily differentiated into neurons than into astrocytes in the RC group. The location of the transplantation site did not affect the area of spared fibers, angiogenesis, or the expression of any other mediators. These findings indicated that the microenvironments of the E and RC sites are able to support NS/PCs transplanted during the sub-acute phase of SCI similarly. Optimally, a certain threshold number of NS/PCs should be grafted into the E segment to avoid damaging sites adjacent to the lesion during the injection procedure.

Key words

Spinal cord injury, Neural stem/progenitor cells, Cell transplantation, Transplantation site, Sub-acute phase.

INTRODUCTION

The central nervous system including the spinal cord was thought to be difficult to regenerate after injury. However, many reports show that transplantation of neural stem/progenitor cells (NS/PCs) promotes functional recovery in animal models of spinal cord injury (SCI) (1,2,7,10,19,22,23,28,31-34,48,49). These NS/PCs were derived from various origins such as hair follicle stem cells, fetal ganglionic eminence, embryonic stem cells, and induced pluripotent stem cells of rodent and human; moreover, various host animals were used such as rodents and non-human primates. These studies contributed to the clinical application of NS/PCs, and transplantation of these cells into injured spinal cords is being clinically developed. Stem Cells Incorporation is currently performing clinical trials in which human fetal NS/PCs are transplanted into the injured spinal cord during the chronic phase, according to the pre-clinical studies using rodent early chronic models (43).

We previously reported that intralesional transplantation of NS/PCs is more effective than intrathecal and intravenous injections to treat SCI in mice (46). Only a small amount of grafted cells survive at the injury site following intrathecal injection. Grafted cells are mainly trapped in the lungs and kidneys following intravenous injection and few cells are engrafted at the injury site. Therefore, we concluded that intralesional application is the most effective method for the transplantation of NS/PCs and that intrathecal and intravenous injections do not deliver cells to the lesion site reliably.

NS/PCs were transplanted into various sites of the injured spinal cord in previous studies including the lesion epicenter (26,28,33,47), one segment rostral or caudal to the lesion (8,25), rostral and caudal sites (9,14), and two bilateral rostral and caudal regions (16,20). For clinical trials of NS/PCs, it is critical to determine the optimal transplantation site of these cells in the injured spinal cord; however, few studies have addressed this issue. The purpose of this study is to investigate the optimal transplantation site of NS/PCs to treat SCI in adult mice.

MATERIALS AND METHODS

Culture of NS/PCs and differentiation assay

NS/PCs were cultured and expanded as described previously (41). We previously developed CAG-*ffLuc*-cp156 mice, which are transgenic mice that ubiquitously express a fusion protein of firefly luciferase and circularly permuted Venus (13). In brief, the ganglionic eminences of these transgenic mice were dissociated using a fire-polished glass pipette (Corning, Corning, NY, USA) on embryonic day 14.5. The dissociated cells were collected by centrifugation and resuspended in culture medium, which consisted of Dulbecco's modified Eagle medium/F12 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with previously described hormone mixture (41) including human recombinant fibroblast growth factor-2 (FGF-2) (Peprotech, Rocky Hill, NJ, USA) and epithelial growth factor (EGF) (Peprotech). Neurospheres were allowed to form. The neurospheres were cultured without serum and growth factors for 5 days to allow differentiation. Then, the cells were fixed in 0.1 M phosphate buffered saline (PBS) (Nacalai tesque Inc., Kyoto, Japan) containing 4% paraformaldehyde (PFA) (Nacalai tesque Inc.) for immunocytochemistry with the following primary antibodies: anti- β -III tubulin (Tuj-1) (mouse IgG, 1:1000, Sigma-Aldrich), anti-glial fibrillary acid protein (GFAP) (rat IgG, 1:1000, Invitrogen, Carlsbad, CA, USA), and anti-2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) (mouse IgG1, 1:1000, Sigma-Aldrich). Nuclei were stained with Hoechst 33258 (10 μ g/ml, Sigma-Aldrich). In vitro images were obtained using a fluorescence microscope (Axiophot 2; Carl Zeiss, Munich, Germany).

SCI model

Adult female C57BL/6 mice (8–9-weeks old, 18–22 g, n=26; Clea Japan Inc., Tokyo, Japan) were anesthetized by intraperitoneal (i.p.) injection of ketamine (100 mg/kg body weight; Sankyo Lifetech Co., Ltd., Tokyo, Japan) and xylazine (10 mg/kg body weight; Bayer, Leverkusen, Germany). The dorsal surface of the dura mater was exposed after laminectomy at the tenth thoracic spinal vertebra (T10). SCI was induced as described previously (44), using a commercially available SCI device (IH

Impactor, Precision Systems and Instrumentation, Lexington, USA). This device creates a reliable contusion injury by rapidly applying a force-defined impact (60 kdyn) with a stainless steel-tipped impactor. All experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of Keio University School of Medicine (No 16-098-37, No 09091-(9)) and the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD, USA).

Transplantation of NS/PCs

NS/PCs were transplanted into the lesion epicenter (E group, n=8) or regions 1 mm rostral and caudal of the rim of the lesion (RC group, n=8) at 9 days post-injury (dpi). Each of these two transplantation groups was subdivided into two groups depending on the number of cells grafted; the 'Low' group (approximately 2.5×10^5 cells) and the 'High' group (approximately 1.0×10^6 cells). Thus, there were four groups in total: E-High, E-Low, RC-High and RC-Low (n=4 per group). NS/PCs were diluted with 3 μ l of PBS in both the Low and High groups and were grafted with a glass micropipette at a rate of 1 μ l/min using a Hamilton syringe (25 μ l) and a stereotaxic microinjector (KDS 310; Muromachikikai Co., Ltd., Tokyo, Japan). PBS (3 μ l) was injected into the lesion epicenter of the vehicle control mice (PBS group, n=8).

Bioluminescence imaging

A Xenogen-IVIS spectrum CCD optical macroscopic imaging system (Summit Pharmaceuticals International Corporation, Tokyo, Japan) was used for *in vitro* and *in vivo* bioluminescence imaging (BLI) (42) as previously reported (22,35,46,49). In brief, the signal intensities of various numbers of NS/PCs were assessed *in vitro* (approximate range, 1×10^2 – 1×10^6 cells). The integration time was fixed at 1 min for each image.

In vivo imaging was performed 5 min after i.p. injection of D-luciferin (0.3 mg/g body weight; Promega, Madison, WI, USA) with the field-of-view set at 13.2 cm height, as the photon count was most stable during this period. The intensity peaked 10–30 min after i.p. injection of D-luciferin. The integration time was fixed at 1 min for each image. All images were analyzed with Living Image software (Caliper Life-Sciences, Hopkinton, MA, USA) and the optical signal intensity was expressed as the photon count, which has the units photons/s/cm²/steradian. Each result was displayed as a pseudo-colored photon count image superimposed on a gray-scale anatomic image. To quantify the photon count, a region of interest (ROI) was defined in the area in which cells were transplanted, and all values at the same ROI were examined.

Behavioral analysis

The motor function of each mouse was evaluated weekly using the Basso mouse scale (BMS) up to 49 dpi (3). This assessment was performed by two investigators who were blinded to the treatment of the experimental mice.

Histological analyses

Injured animals were deeply anesthetized and transcardially perfused with PBS containing 4% PFA at 49 days post-transplantation (dpi). The spinal cords were removed, post-fixed overnight in PBS containing 4% PFA, soaked overnight in 10% sucrose (Nacalai tesque Inc.), soaked in 30% sucrose, embedded in optimal cutting temperature compound (Sakura Seiki Co., Ltd., Tokyo, Japan), frozen, and sectioned in the sagittal plane at a thickness of 16 µm using a cryostat (CM3050; Leica Microsystems, Wetzlar, Germany). The spinal cord sections were immunohistochemically stained and evaluated by quantitative analysis. For immunohistochemistry, tissue sections were stained with the following primary antibodies: anti-green fluorescent protein (GFP) (rabbit IgG, 1:500; Frontier Institute Co., Ltd., Hokkaido, Japan), anti-Hu (human IgG, 1:1000, a gift from Dr. Robert Darnell, The

Rockefeller University, New York, NY, USA), anti-GFAP (rat IgG, 1:500, Invitrogen), anti-adenomatous polyposis coli antigen (APC) (mouse IgG, 1:500; Calbiochem, San Diego, CA, USA), anti-neurofilament 200 kDa (NF-H) (mouse IgG1, 1:200; Chemicon, Millipore, Billerica, MA, USA) and anti-platelet endothelial cell adhesion molecule-1 (PECAM-1) (rat IgG, 1:200; BD Bioscience Pharmingen, San Diego, CA, USA). For differentiation efficiencies of grafted cells, the samples were incubated with secondary antibodies conjugated with Alexa488, Alexa555 (Invitrogen) appropriate for host species at 1:1000 dilution for 2 hours at room temperature after incubation with primary antibodies at 4°C overnight. Nuclei were stained with Hoechst 33258 (10 µg/ml; Sigma-Aldrich). For immunohistochemistry with anti-GFP, -NF-H, and -PECAM-1 antibodies, the sections were exposed to 0.3% H₂O₂ (Nacalai tesque Inc.) for 30 min at room temperature to inactivate endogenous peroxidases, and were then treated with a biotinylated secondary antibody (Jackson Immunoresearch Laboratory Inc., West Grove, PA, USA). Signals were enhanced with the VECTASTAIN® ABC kit (Vector Laboratories, Inc., Burlingame, CA, USA). All images were obtained with a fluorescence microscope (BZ 9000; Keyence Co., Osaka, Japan) or a confocal laser scanning microscope (LSM 700; Carl Zeiss).

RNA isolation and RT-PCR

To compare differential mRNA expression in the transplantation site of the E and RC segments, a 4.5 mm segment of the injured spinal cord was removed from wild-type mice 9 dpi and divided into the E segment and the adjacent rostral and caudal segments, each 1.5 mm wide. mRNA expression of growth factors, inflammatory cytokines, markers of inflammatory cells and bone morphogenetic proteins (BMPs) was analyzed in each of these three segments. This revealed the profile of the spinal cord prior to NS/PC transplantation (n=3). mRNA levels were analyzed by quantitative RT-PCR (qRT-PCR) arrays using TaqMan® Array Fast (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer's instructions. Dissected spinal cords were rapidly frozen and placed in TRIzol® (Invitrogen). Subsequent RNA isolation was performed according to the

manufacturer's instructions. Total RNA was treated with TURBO™ DNase (Ambion, Austin, TX, USA) and then reverse transcribed with oligo (dT) primers and SuperScript III® reverse transcriptase (Invitrogen). Quantitative mRNA expression of trophic factor-related genes was simultaneously analyzed (n=3). The amount of mRNA was normalized against the amount of 18s ribosomal RNA. The following manufactured primers (Applied Biosystems; <http://www.appliedbiosystems.com>) against mouse DNA sequences were used: Bdnf-Mm01334044_m1, Cntf Zfp91 U05342-Mm00446373_m1, Ngf-Mm00443039_m1, Gdnf-Mm00599849_m1, Hgf-Mm01135183_m1, Fgf2-Mm01285715_m1, Vegfa-Mm01281449_m1, Vegfb-Mm00442102_m1, Il1b-Mm01336189_m1, Il10-Mm01288386_m1, Il16-Mm00516039_m1, Ifng-Mm01168133_m1, Tgfb1-Mm01178819_m1, Tgfb2-Mm03024009_m1, Ccl2-Mm99999056_m1, Cd68-Mm03047343_m1, Mmp9-Mm00600164_g1, Bmp2-Mm01962382_s1, Bmp4-Mm00432087_m1, 18s ribosomal RNA-Hs99999901_s1. After normalization against the level of 18s ribosomal RNA, the amount of mRNA in the RC segment was determined by calculating the mean of the levels in the rostral segment and the caudal segment, and this was compared to the level of mRNA in the E segment.

Quantitative analyses

Quantitative analyses of the histological findings (immunostaining for NF-H and PECAM-1) were performed using a BZ-9000 microscope and Dynamic Cell Count BZ-HIC software (Keyence Co.). The threshold values were the same for all analyses. To quantify NF-H-positive fibers and PECAM-1-positive vessels, 0.75 mm wide segments of the epicenter and the region 1 mm rostral and caudal of the epicenter were imaged in the midsagittal sections of mice in the E, RC and PBS groups (n = 3 per group) (16). To quantify the proportion of grafted cells with each phenotype in vivo, five regions were captured in the sagittal sections at 200× magnification using an LSM 700 confocal laser scanning microscope. The number of cells that were both GFP- and phenotypic marker-positive were counted in each section (n = 3 per group).

Statistical analysis

All data are reported as the mean \pm SEM. An unpaired two-tailed Student's t-test was used to evaluate differences between the E and RC groups in the *in vivo* differentiation assays. One-way ANOVA followed by Tukey's test for multiple comparisons was used for the analyses of BMS scores, NF-H-positive areas and PECAM-1-positive areas. A paired two-tailed Student's t-test was used to evaluate the differences in mRNA levels between the E and RC segments. P values < 0.05 were considered statistically significant.

RESULTS

In vitro imaging and differentiation assay of NS/PCs

NS/PCs derived from CAG-*ffLuc*-cp156 mice were used in this study to identify and quantitatively monitor the survival of the grafted cells (13). This mouse was generated by cross-breeding the reporter mouse CAG-*neo*^{loxP/loxP}-*ffLuc*-cp156 (Fig. 1A) with the CAG-Cre mouse (a gift from Dr. Okabe, Osaka University, Osaka, Japan). NS/PCs derived from these transgenic mouse embryos express *ffLuc*-cp156, which is a fusion protein of a variant of the fluorescent protein Venus and luciferase; therefore, these NS/PCs are fluorescent and bioluminescent (Fig. 1B). Transgenic NS/PCs expressed *ffLuc*-cp156 more uniformly than NS/PCs transfected with *ffLuc*-cp156 using a lentivirus, and there was a strong linear relationship between cell number and photon count (Fig. 1C). The *in vitro* differentiation assay revealed that NS/PCs-derived from the transgenic mice differentiated into Tuj-1-positive neurons (12.2 \pm 1.5%), GFAP-positive astrocytes (55.0 \pm 2.0%) and CNPase-positive oligodendrocytes (9.8 \pm 1.0%) (Fig. 1D–G).

Survival of NS/PCs grafted into the injured spinal cord

To monitor the survival of grafted NS/PCs, photon counts were measured 0, 7, 21 and 35 dpt. Representative bioluminescence images of mice in the E and RC groups are shown in Fig. 2A and B, respectively, at 7 and 35 dpt. Quantitative analysis revealed that the photon counts of the grafted cells sharply decreased by 7 dpt and then plateaued at approximately 5×10^6 photons/s/cm²/steradian in both groups. Mice in the RC-High and E-High groups received more NS/PCs than mice in the RC-Low and E-Low groups. As expected, the initial photon counts were higher in the former groups than in the latter groups. Despite this initial difference, the photon count was similar in the E-Low and E-High groups (Fig. 2D) and in the RC-Low and RC-High groups (Fig. 2E) by 21 dpt and remained similar throughout the rest of the time-course. There were no significant differences in the photon counts among the all groups at 35 dpt (Fig. 2D, E).

Distribution and differentiation of the grafted NS/PCs in the injured spinal cord

Anti-GFP immunohistochemistry with DAB staining, which detects the *ffLuc-cp156* protein, was performed to investigate the survival and distribution of the grafted NS/PCs within the injured spinal cord. Representative staining of samples from the E and RC groups showed that the grafted cells were located outside the lesion epicenter and there was no apparent difference in the distribution of the grafted cells between these groups (Fig. 3A, B).

Immunohistochemical analysis revealed that the grafted NS/PCs differentiated into Hu-positive neurons, GFAP-positive astrocytes and APC-positive oligodendrocytes in both the E and RC groups (Fig. 3C, D). Quantitative analysis demonstrated that the percentage of neurons was significantly higher in the RC group than in the E group, and that the percentage of astrocytes was significantly lower in the RC group than in the E group. There was no significant difference in the percentage of oligodendrocytes between the two groups (Fig. 3E).

Motor functional recovery

We evaluated the locomotor function of the hind limbs using the BMS. The contusive SCI initially caused complete paralysis, followed by gradual recovery that plateaued at a BMS score of around 2 in the PBS group. The BMS score was significantly higher, indicative of better motor functional recovery, in the E and RC groups than in the PBS group from 21 dpi until the end of the time-course (Fig. 4A). Furthermore, the BMS score was significantly higher in the E-High, E-Low, RC-High and RC-Low groups than in the PBS group at 49 dpi (Fig. 4B). The BMS scores did not significantly differ between the E and RC groups (Fig. 4A), or among the E-High, E-Low, RC-High and RC-Low groups (Fig. 4B).

Evaluation of spared fibers and angiogenesis

To evaluate the effects of NS/PC transplantation on spared neuronal fibers after SCI, immunohistochemical analysis of NF-H was performed at 42 dpt. NF-H-positive areas were quantified in rostral, epicenter and caudal regions (Fig. 5A–C). The NF-H-positive areas in each of these regions were significantly larger in the E and RC groups than in the PBS group, whereas there was no significant difference between the E and RC groups (Fig. 5D).

To assess the effects of NS/PC transplantation on angiogenesis after SCI, anti-PECAM-1 DAB staining was performed at 42 dpt (Fig. 6A–C). The PECAM-1-positive areas in the rostral, epicenter and caudal regions were significantly larger in the E and RC groups than in the PBS group, whereas there was no difference between the E and RC groups (Fig. 6D).

mRNA expression in the E and RC segments

To compare the microenvironments of the E and RC segments for NS/PC transplantation, the mRNA expression of growth factors, inflammatory cytokines, markers of inflammatory cells and BMPs was examined by qRT-PCR (Fig. 7). Brain-derived neurotrophic factor (BDNF) mRNA expression was

higher in the RC region than in the E region. However, the expression levels of none of the other mRNAs examined significantly differed between the E and RC segments.

DISCUSSION

***ffLuc-CP156* transgenic mouse**

When investigating cell transplantation into the injured spinal cord, many researchers including ourselves have used cells directly transfected with luciferase (35) and/or GFP (20,36) to trace and detect the grafted cells. Abematsu *et al* reported the transplantation of NS/PCs derived from a transgenic mouse ubiquitously expressing GFP and luciferase into SCI model. They found the survival of these transplanted cells drastically diminished after the ablation and also characterized the temporal changes of the survival of the transplanted cells without the ablation using bioluminescence imaging system. (1). In this study, we used NS/PCs derived from a transgenic mouse that expressed a novel reporter gene, *ffLuc-CP156* (13). These transgenic NS/PCs expressed *ffLuc-cp156* more uniformly than NS/PCs directly infected with lentivirus expressing *ffLuc-cp156*. Consequently, a strong linear relationship between photon count and cell number was detected by *in vivo* BLI and the number of surviving grafted cells could be accurately quantified. The luciferin-luciferase reaction requires ATP and only occurs in living cells; therefore, the number of surviving grafted cells can be quantified more accurately by measuring the photon count than by using histological methods. In addition, NS/PCs derived from the ganglionic eminence of this transgenic mouse at embryonic day 14.5 differentiate into three neural lineages and the differentiation efficiency did not significantly differ between the transgenic NS/PCs and wild-type NS/PCs (35).

Photon counts of the transplanted cells

Previously, measurement of the photon count by *in vivo* BLI estimated that 10–20% of grafted cells survive when 500,000 mouse NS/PCs are transplanted into the lesion epicenter (35,46). In the current study, 500,000 grafted cells was equivalent to a photon count of approximately 60×10^6 photons/s/cm²/steradian at the time of transplantation, and the mean final photon count was 5×10^6 photons/s/cm²/steradian at 35 dpt. Therefore, approximately 8% of grafted cells survived when 500,000 cells were initially transplanted. There is no significant difference between this survival rate and those previously reported.

Interestingly, the final photon count at 35 dpt was similar regardless of the initial photon count. Furthermore, there was no difference in photon counts between the E and RC groups. This suggests that the number of NS/PCs that survive is independent of the original number of cells that are transplanted as long as a certain threshold number of NS/PCs are initially delivered into the injured spinal cord. This is probably because the survival of grafted cells were affected by the microenvironment of the injured spinal cord as well as the size of available physical spaces for grafted cells within the injured spinal cord.

Survival and distribution of grafted NS/PCs

Many researchers have transplanted various neural cells into the lesion epicenter of injured spinal cords or into sites rostral and/or caudal of the epicenter. Schwann cells and olfactory ensheathing cells, which might contribute to remyelination of demyelinated axons after SCI, have tended to be grafted into the rostral and/or caudal regions rather than into the lesion epicenter (18,24,37,42). On the other hand, NS/PCs are more suitable for transplantation into the lesion epicenter than into rostral and/or caudal regions (12,26,45,47,48). These grafted cells are thought to contribute to motor functional recovery by secreting trophic factors, regenerating axons, constructing remodeled neural circuits, remyelinating neurons and promoting angiogenesis (4,5,21,27,29,51). These potential mechanisms are more applicable in the lesion epicenter than in the adjacent injured spinal cord areas.

A few reports have examined sagittal sections following transplantation of NS/PCs into the lesion epicenter or into rostral and caudal sites during the sub-acute phase of contusive SCI. In all cases, the grafted cells migrate away from the lesion epicenter (16,20,35,49). This indicates that cell migration is unaffected by the transplantation site. Parr et al. investigated the effects of NS/PC transplantation at different sites and at various dpt on SCI (36). The survival of grafted NS/PCs cells was significantly higher when cells were transplanted into an area 1 mm rostral and caudal to the injury site than when they were transplanted into the lesion site. However, this difference was only detected 7 dpt, and was not detected 14 or 28 dpt. The grafted cells were found in the less damaged tissue and cell survival was associated with the microenvironment in the injured spinal cord, such as the extent of the inflammatory response and the levels of secreted factors. However, this report did not address why the level of cell survival rate in the two transplantation procedures only differed at 7 dpt. In addition, Piltti et al. compared transplantation sites between epicenter and intact parenchyma (38), in which contusion injuries were induced at T9 level in the athymic nude rats and they transplanted human NS/PCs into lesion epicenter (EPI) or two bilateral rostral (T7/8) and caudal (T10/11) site (R/C) nine days after injury. In their report, cell survival was higher in the R/C group compared to the EPI group though transplantation site did not affect grafted cell migration normalized by distance from the injection site. In our present study, on the other hand, there was no significant difference in cell survival regardless of whether NS/PCs were grafted into the E or RC segments during the sub-acute phase of SCI. This difference can be interpreted by that of transplantation site in the R/C group between two studies. While Piltti et al. (38) transplanted cells at distant sites from the epicenter in their R/C group, we transplanted cells in RC region adjacent to the epicenter. Thus, microenvironments in the E region and the adjacent RC regions might have supported the survival of transplanted NS/PCs similarly in our present study. This idea is supported by qRT-PCR analyses, which showed that the expression levels of inflammatory cytokines, markers of inflammatory cells, and many growth factors apart from BDNF did not significantly differ between the E and RC segments. The up-regulation of BDNF mRNA expression in RC segment after SCI was supposed to originate in reactive astrocytes around the lesion epicenter, which are known to exhibit higher BDNF expression (11,15). On the other hand, in the

report by Piltti et al., microenvironments in E region and transplanted sites in R/C group, which is distal from the epicenter, are likely to be different from each other. Furthermore, it is well known that microenvironment within the injured spinal cord changes from the sub-acute phase to the chronic phase (32). Thus, it is important to determine the survival of the NS/PCs transplanted in E and RC regions at the chronic phase of SCI in the future.

Differentiation efficiencies of transplanted NS/PCs

The grafted NS/PCs differentiated into neurons, astrocytes and oligodendrocytes in the E and RC groups. There were differences in the differentiation efficiencies of NS/PCs into neurons and astrocytes, but not into oligodendrocytes, between these two groups. Okada et al. reported that the differentiation of NS/PCs grafted into the lesion site into astroglia is more prominent when they are transplanted during the acute phase of SCI than during the sub-acute phase (35). This is because the levels of ciliary neurotrophic factor (CNTF) (6,39) and BMPs (40,52) mRNAs increase dramatically during the acute phase of SCI (30) and induce NS/PCs to differentiate into astrocytes. Therefore, we predicted that the expression of these mediators and cytokines would be higher in the lesion epicenter than in adjacent sites during the sub-acute phase. However, qRT-PCR analyses did not detect any significant differences between the E and RC segments in the expression of many mediators that are reported to influence the differentiation of grafted NS/PCs including CNTF, BMPs and hepatocyte growth factor (HGF). However, the expression of BDNF, which stimulates NS/PCs to differentiate into neurons (17,53), was higher in the RC segment than in the E segment. This may underlie, at least in part, why the proportion of neurons was higher in the RC group than in the E group.

Efficacy of grafted NS/PCs

Previous studies demonstrated that transplantation of NS/PCs promotes the regrowth of NF-H-positive fibers after SCI (5,27,51). Consistently, the cross-sectional areas of NF-H-positive fibers were

significantly larger in the E and RC groups than in the PBS group. Furthermore, the PECAM-1-positive areas were significantly larger in the E and RC groups than in the PBS group. Angiogenesis after SCI improves endogenous repair and supports axonal outgrowth (4,21). The size of the NF-H-positive fibers areas and the PECAM-1-positive areas were not affected by the site of NS/PC transplantation.

Eventually, motor functional recovery was significantly improved in both the E and RC groups compared to the PBS group. There was no significant difference in recovery from SCI between the E and RC groups, although the differentiation efficiency of the grafted cells did differ between the two groups. Consistently, Watanabe et al. reported that transplantation of NS/PCs derived from the forebrain or from the spinal cord into an injured spinal cord promotes motor functional recovery after SCI equally, although the differentiation efficiencies of these NS/PCs differ *in vivo* (50). These results indicate that the differentiation efficiency of grafted NS/PCs varies depending on the site at which they are transplanted; however, this does not influence motor functional recovery.

The differentiation efficiency of NS/PCs into three neural lineages was the only attribute examined that significantly differed between the E and RC groups. These findings and the qRT-PCR results suggest that there is no marked difference in the abilities of the microenvironments of the lesion epicenter and the adjacent areas to support NS/PCs that are transplanted 9 dpi. Therefore, we conclude that it is optimal to graft a certain threshold number of NS/PCs into the epicenter lesion during the sub-acute phase of SCI, and thereby avoid causing further iatrogenic injury to the intact RC regions of the spinal cord.

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