Original Article

Transplantation of neural progenitors derived from embryonic stem cells brings about functional and electrophysiological recoveries of mice with spinal cord injury

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Cell transplantation is promising therapeutic strategy for spinal cord injury (SCI). Embryonic stem (ES) cells proliferate *in vitro* through many passages without losing their totipotentiality and are good sources for transplantable cells. However, whether the grafted cells further differentiate into mature motoneurons and directly contribute to the reconstruction of neuronal pathway in SCI remains largely unknown. In this study, mouse ES cells were treated with retinoic acid to induce neural progenitor cells, which were transplanted to the completely transected spinal cord at T7-T8 of mice. The mice transplanted with neural progenitor cells exhibited potent functional recoveries of their hind limbs and tail, showing significant higher scores of locomotor behavioral tests compared with SCI mice having vehicle injection, and electrophysiological assessment confirmed the re-emergence of motor evoked potential of injured spinal cord. Histological analysis of injured spinal cord demonstrated that engraftment of the neural progenitor cells into a vacant spinal column cavity made by removal of the transected spinal cord, reconnected the spinal cord and large numbers of grafted cells were found alive. The grafted cells expressed neurofilament middle chain and Synaptophysin, suggesting differentiation into mature neurons and synapse formation. RT-PCR and immunohistochemical analyses demonstrated that the differentiated neurons expressed markers for motoneurons, Islet1, HB9 and Lim1/2, suggesting their differentiation into spinal motoneurons. Taken together, neural progenitor cells derived from ES cells can be successfully transplanted to injured spinal cord, survive, further differentiate into motoneurons and reconstruct neuronal pathways *in vivo*, resulted in functional and electrophysiological recoveries of injured spinal cord.

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Introduction

SCI causes severe functional deficits due to axonal destruction, neuronal and glial cell death, and extensive demyelination. Although pathology of SCI is determined mainly by initial mechanical insults, secondary processes including ischemia, anoxia, free-radical formation, production of proinflammatory cytokines, and excitotoxicity that occur over hours and days following injury, also contribute to secondary damage of the spinal cord ¹⁾. There is considerable evidence that functional recovery after SCI is not a simple consequence of the initial mechanical destruction of tissue but is also the evolution of complex secondary events to contribute to early as well as delayed cell injury, suggesting the difficulties of complete functional recovery of SCI ²⁻⁹⁾.

It has been held that the capacity for intrinsic repair after injuries of central nervous system (CNS) is limited because the mature CNS can neither generate new neuron nor initiate functional axonal regeneration in response to the damages. Recently, adult CNS has been shown to be a regenerative system capable of incorporating exogenous stem, progenitor or fetal neuronal stem cells ¹⁰⁾. These implanted neural stem cells (NSCs) grew to specifically repair previously damaged and degenerated neuronal pathways. Indeed, transplantation of embryonic neural cells into injured spinal cord improved motor function of hind limbs ¹¹⁻¹³⁾. Thus, the cell transplantation has become the most promising candidate for treatment of SCI ^{10,14)}. NSCs can be isolated from embryonic and adult brain tissue, as well as from ES cells.

ES cells are derived from inner cell mass of blastocyst stage of embryos and harbor self-renewing potential and totipotential. ES cells may become an unlimited source of cells suitable for such transplantation therapy. They have been shown to differentiate preferentially into multiple neural cell types, when cultured with all-trans retinoic acid (RA) and a FCS depleted medium ¹⁵⁾. Recently, ES-derived oligodendrocytes have been shown to myelinate in the immature CNS and after transplantation to spinal cord ¹⁶⁻¹⁸⁾. Furthermore, we have reported that RA-treated ES cells differentiate into neural cell lineage including motoneurons and successfully transplanted to CNS, resulted in significant recovery of motor functions of a mouse model of hemiplegia ^{19,20)}.

In this study, we have transplanted the ES-derived neural cells to a mouse model of SCI having complete transection at T7-T8 as a recipient without usage of immunosuppressant because it may affect adaptation and subsequent survival of the graft. We studied recovery of motor function of SCI mice where transplanted

motoneurons could be identified by immunohistochemical analysis of the regenerated spinal cords.

Materials and Methods: Induction of neural differentiation of ES cells by RA treatment

E14.1 mouse ES cell line (Passage number 12-18, normal karyotype) was a kind gift from Dr. Klaus Rajewsky (Cologne University). Undifferentiated ES cells were maintained in the presence of mouse fetal fibroblasts treated with mitomycin C on gelatin-coated dishes in culture medium containing recombinant mouse leukemia inhibitory factor (LIF) (Life Technologies, Grand Island, NY) ^{19,20)}. Neural differentiation of ES cells was induced in the presence of RA (Sigma, Tokyo, Japan) as described ^{19,20)}. In brief, the ES cells were cultured in DMEM supplemented with 20 % FCS on bacterial grade petri dishes. 4 days later, floating cell aggregates, termed embryoid bodies (EBs), were transferred onto fresh petri dishes to which 1 μ M RA was added at day 4 and day 6. The RA treatment successfully induced neural differentiation of the ES cells of passages 12 to 18. For transplantation, the EB was recovered at day 8 and a single cell suspension of the EB was immediately used as a graft.

Labeling of the graft cells was achieved with the magnetic resonance contrast agent Feridex (Tanabe Seiyaku, Tokyo, Japan) consisting of ultrasmall superparamagnetic iron-oxide particles as described ²¹⁻²³⁾. In brief, the lipofection technique was applied using the lipofection reagent Dosper (Roche, Tokyo, Japan). Coincubation of Feridex with Dosper for 30 min led to the encapsulation of the agent, consequently facilitating the agent infusion into the cells. Labeling efficiency of the cells with the procedure was better than 70%. After harvesting, cells were washed several times, diluted, and used immediately.

Surgical procedures and animal care

Female C57BL/6 mice (6-8 weeks of age, 20-23 g) were used in following experiments. All subsequent procedures were received the approval of Animal Care Facility of St. Marianna University School of Medicine and conducted along with the guideline. We also followed the "Principles of laboratory animal care" (NIH publication No. 86-23, revised 1985). Animals were anesthetized with pentobarbital (20 mg/kg). Using a dissecting microscope, a laminectomy was made at the 7th to 8th thoracic (T7-T8) spinal vertebrae. It was followed by a complete spinal cord transection at the T7-T8 level by cutting with a surgical blade at the rostral and caudal ends that spanned a 1.5-

mm-long together with dura and removal of the resected spine. After hemostasis, an independent blinded observer confirmed the adequacy of the length and breadth of the lesion. The lesion was affirmed to be similar across all experimental groups of mice.

The mice were transplanted with ES-derived neural progenitor cells suspended in PBS into the vacant cavity between the cord stumps by injection using sterile glass needles (transplantation with neural cells; $n = 11$). Each site received 10 μ l of a suspension containing 5×10^6 ES-derived neural progenitor cells. As a negative control, mice were injected at the same site of the transected spinal cord with 10 μ l of PBS (SCI with vehicle injection; $n = 12$). The same surgical procedure without spinal cord transection was performed in the remaining mice (sham-operated; $n=10$) as positive controls. The surgeries for the cell transplantation or controls were performed on the same day to minimize the differences among each group arising from any refinement in surgical technique during the experiments. Following either the treatment, the musculature was sutured, skin was closed and the mice were returned to clean cages on a heating pad, allowed to recover from surgery, and given unlimited access to food and water. Ringer's lactate solution (5 ml) was given and bladders were evacuated twice daily until reflex bladder function was established. No antibiotic agents were required.

Analyses of motor function

To evaluate the functional consequence of SCI, we performed two different behavioral tests, Basso-Beattie-Bresnahan (BBB) score and vertical grid test. During the postsurgery period, beginning one day after injury and weekly thereafter, the mice were daily allowed to move freely in an open field. Well trained investigators blinded to the treatments performed behavioral analysis scoring the BBB score; the ability to maintain body position on an inclined plane, and contact righting reflex and spinal cord-mediated hind limb withdrawal to pain. The BBB score is a standard method for assessing the hind limb motor function after SCI in both rats and mice²⁴.

In addition, the climbing test on vertical grid was performed²⁵. Behavioral tests were performed at the same time each day and scaled by the same observers in a blinded manner. Hind limbs were assessed independently to analyze asymmetric recovery every week. Scores ranging from 0 to 2 (0, no response; 1, normal placing of one foot; 2, normal placing of both feet) were given to each mouse. To avoid false positive results due to reflexive movements, we only considered as positive responses real placement of the paw, and we did not consider attempts to place the

foot. Each foot was assessed independently three times, and a positive score for each hind limb was only given if there were at least two clear responses during at least two consecutive sessions. Three different examiners blind to the treatment participated in all of the behavioral tests. They were segregated into three teams of two observers each, and functional recovery was assessed every week by a different team. All tests were videotaped and two investigators blind to the treatment evaluated the functional outcome of each animal by reviewing the videotapes. The responses of mice were only scored when the conclusions obtained by "in situ" observers and from the videotapes were the same.

Electrophysiological evaluation of spinal cord

Spinal cord of the each group of mice was promptly recovered. The stimulus consisted of a square wave pulse of 5 mV and its duration was 5 msec. The stimulus was repeated at 5 Hz and delivered through an electrode placed at C7-C8. Motor evoked potentials (MEPs) were recorded at L4-L5. The evoked signal was amplified (gain 10,000 x; bandpass 10-500 Hz) and averaged over 500 pulses²⁶.

PCR

The injured site of spinal cord was cut into pieces with their length ~ 0.2 mm. A piece of the sliced tissue locating the exact center of the injured site was recovered. Total RNA extraction and reverse transcription of the tissue specimen were performed for RT-PCR. RT-PCR reaction was performed with a panel of primer combinations (Table 1). Cycling parameters were hot starting at 94 $^{\circ}$ C for 180 sec, denaturing at 94 $^{\circ}$ C for 30 sec, annealing at 60 $^{\circ}$ C for 30 sec, and elongation at 72 $^{\circ}$ C for 60 sec. The reaction was repeated 30 times and followed by elongation at 72 $^{\circ}$ C for 10 min.

Genomic PCR amplification was used to detect male-specific *sry* gene on Y chromosome of ES cells in DNA extracted from the spinal cord of female recipient mice using conventional method. The DNA obtained was amplified for 33 cycles using a set of primers specific for *sry* gene (Table 1). β -actin was used as a control. The 380 bp band (*sry*) obtained was identified with the male fetuses and the 440 bp product (β -actin) confirmed the correct amplification of the template DNA (data not shown). This method allowed the identification of the ES-derived neural cells in the female recipient^{27,28}.

Table 1 Neural markers used in RT-PCR

Name/ Abbreviation	Description	Forward primer	Reverse primer	The Length of amplified product (bp)
-actin	house keeping gene	GATGACGATATCGCTGCGCTG	GTACGACCAGAGGCATACAGG	450
Nestin	neural stem and progenitor cells	CTACCAGGAGCGCGTGCC	TCCAGAGCCAGCTGGAACCT	222
Islet1	motoneuron	ATGGGAGACATGGGCGAT	TGCCTAGCGGAGATGGGT	499
Lim1/2	motoneuron	ATGCCTGTCTGGGGCGGT	TAGGCCTGTGAGGTTGAG	300
HB9	motoneuron and interneuron	CCCTGCCGACCCCATCAA	GCCTTGTCCTCCCGAAACC	441
NT3 (neurotrophin3)	neurotrophic factor	GCAGAACATAAGAGTCACCG	CATCAATCCCCTGCAAC	211

Histological and immunochemical staining and antibodies

Spinal cord was dissected and fixed with 4% paraformaldehyde. For hematoxylin and eosin (HE) staining, the cryostat sections (5 μ m thick) mounted on slides were stained with HE. For the detection of intracellular colloidal iron introduced into donor cells, we performed Berline blue staining. For immunochemical staining, the sections were immunostained using M.O.M.TM Kit (Vector laboratory, Burlingame, CA) or HISTOMOUSETM-PLUS KITS (Zymed, South San Francisco, CA). Quenching of endogenous peroxidase and the opening of cell membranes were done with hydrogen peroxide (0.3%) and Triton X-100 (0.4%) in PBS for 15 min. After blocking with 5% bovine serum albumin, the sections were incubated with primary antibodies and followed by biotinylated second antibodies. The primary antibodies included anti-GFAP (Dako, Carpinteria, CA), anti- β -tubulin (Promega, Madison, WI), anti-NFM (Chemicon, Temecula, CA), anti-Synaptophysin (Dako), anti-Lim1/2 (Hybridoma bank, Iowa City, IA) and anti-Islet1 (Hybridoma bank). The reactivity was visualized with diaminobenzidine tetrahydrochloride solution.

To analyze the differentiation of ES cells into each neural phenotype *in vitro*, we stained RA-treated ES cells mounted on chamber slide using anti- β -tubulin, anti-NFM, anti-GFAP or anti-GalC antibody (Chemicon) individually, and counted the immunopositive cells for each staining under the microscopy.

Statistical analysis

Continuous variables subjected to repeated measurements over a period of time (BBB scores and vertical grid tests) were analyzed using one-way variance ANOVA with post hoc Tukey

test to determine statistical significance. A p value of < 0.05 was considered statistically significant.

Results:

Functional recovery of SCI by transplantation with ES-derived neural cells

After complete transection of the spinal cord at T7-T8, all injured mice were paralyzed in tail and both hind limbs. The functional recovery score was assessed for 8 weeks with BBB scoring system. At 2 weeks after injury, none of the mice were able to bear weight on the hind limbs. However, some of the mice transplanted with ES-derived neural cells showed incomplete movement of hip, knee and ankle joints, and the remaining presented slight movement of the knee and ankle joints and extensive movement of the hip. In contrast, SCI mice with vehicle injection, negative control mice, showed slight movements of just two joints and more frequently dragged their hind limbs during moving and the BBB scores were less than 2. After day 20, significant higher BBB scores were noted in mice transplanted with the neural cells compared with SCI mice with vehicle injection (Fig.1; upper panel). By 4 weeks after transplantation, more than half of the mice transplanted with the neural cells displayed recovery with up to 10 of 21 points of the BBB score, meaning that their hind limbs were coordinated with their fore limbs but did not obviously bear weight on the hind limbs. The sham-operated normal mice had a perfect score of 21 points at all times (Fig.1).

To confirm functional recovery of the SCI mice with neural cell transplantation, recovery of voluntary movements of hind limbs was further assessed using a vertical grid test. Using this test, significant functional improvement was not observed in any

Fig.1 Recovery of motor functions by transplantation of ES-derived neural cells after complete spinal cord transection

The spinal cords of C57BL/6 mice were completely transected and the mice were transplanted with ES-derived neural cells or injected with vehicle (PBS) alone at day 0. Sham-operation in other mice without SCI was simultaneously done. Motor function was evaluated by BBB open field test (upper panel) and vertical grid test (lower panel) at each time point. Significant improvements of both scores were observed in mice transplanted with the neural cells (; $n=11$) as compared with control mice injected with vehicle alone (; $n=12$) after day 30 (day 30-day 50, $p<0.01$ as indicated by asterisks). However, neural cell transplantation did not restore their motor function completely as compared with sham-operated mice (; $n=10$).

Fig.2 Electrophysiological evaluation of the transected spinal cord transplanted with ES-derived neural cells

MEP conducted in the spinal cords was measured. Spinal cord was promptly removed from mice with sham-operation (Normal), SCI mice with vehicle injection and SCI mice with neural cell transplantation (Transplantation at 7, 14 and 21 day). A square wave pulse of 5 mV intensity and 5 msec duration repeated at 5 Hz was delivered via an electrode placed at C7-C8 and recordings were made from L4-L5 with an amplification (gain 10,000 x; bandpass 10-500 Hz). Averaged MEP over 500 pulses was obtained. Arrows indicate the MEP.

of the SCI mice with vehicle injection after spinal cord transection. In contrast, the mice transplanted with the neural cells showed a significant recovery of motor function, meaning that some of them could grasp the grid with their hind limbs (Fig.1; lower panel). In our preliminary experiments, however, we found that transplantation of the undifferentiated ES cells and the dead

ES cells did not brought about the recovery of motor functions in the mice (data not shown).

Fig.3 Macroscopic and microscopic appearances of the completely transected spinal cord transplanted with ES-derived neural cells

A. The macroscopic view of the spinal cord transplanted with ES-derived neural cells after complete transection (upper panel). The spinal cord was removed from mice at day 50 after transplantation and immediately photographed. The transected spinal cord was completely connected and the connecting tissue showed a white matter-like appearance. HE staining of representative longitudinal sections of the spinal cord from each group of mice (lower panel). In a longitudinal section of the spinal cord in SCI mice with vehicle injection (SCI at day 0), cavity formation was observed (an arrow). Both white matter and gray matter were completely separated into their rostral and caudal sites by the cavity and the injured spinal cord exhibited severe atrophy in caudal, and a lesser degree in rostral portions. Thereafter, in the injured spinal cord injected with vehicle (SCI at day 7), the accumulation of inflammatory cells in the cavity was observed (an asterisk). Spinal cord with neural cell transplantation (Transplantation at day 7) showed condensed cellularity consisted of inflammatory cells and the ES-derived neural cells at the site of the injury (two asterisks). Original magnification of each panel is x40.

B. Higher magnification of HE sections from the spinal cord of each group of mice (left and middle panels) and double staining for GFAP and labeled cells with iron (right panel). Note that the transplanted cells were distinguished from recipient cells by their morphology, but no teratoma formation was observed. At day 7, severe gliosis was observed at SCI site with vehicle injection (right upper panel), but it was replaced by Fe-positive transplanted neural cells (right lower panel). Original magnification of each panel is x200.

Electrophysiological recovery of the injured spinal cord conduction in SCI by transplantation with ES-derived neural cells

In experimental models of SCI, electrophysiological evaluation using MEP as well as behavioral tests can provide objective assessments of the functional integrity of the spinal cord after injury and neural cell transplantation. In the present study, the base line for each group of mice was established using spinal cord of sham-operated mice. Fig.2 demonstrates the representatives of the recorded MEP data from each experimental group of mice whose BBB scores placed them near the mean of their respective groups. Sham-operated normal mice showed the normal pattern of MEP as shown in Fig.2 (an open arrow in the upper panel). At 3 weeks after complete transection of spinal cord, none of the SCI mice with vehicle injection showed the measurable MEP (SCI mice with vehicle injection of Fig.2). In contrast, the mice with neural cell transplantation showed gradual recovery of the MEP under the same condition (closed arrows in the lower panels of Fig.2). These findings suggested that the transplantation of the ES-derived neural cells to transected spinal cord reconnected the interrupted neural pathways, evidenced by the conducted MEP.

Macroscopic and microscopic appearances of the injured spinal cord transplanted with ES-derived neural cells

After SCI, spontaneous recovery of motor function was reported in rodents, especially in contusion models²⁹. Based on the fact, we made SCI model by complete spinal cord transection at the T7-T8 level cutting at the rostral and caudal ends that spanned a 1.5-mm-long. This rendered the spontaneous functional recovery impossible as confirmed by the locomotor behavioral tests and electrophysiological monitoring. In spite of that, the functional and electrophysiological recoveries were observed obviously in mice transplanted with ES-derived neural cells. So we next evaluated the anatomical recovery of neuronal pathways in injured spinal cord.

As shown in Fig.3A (upper panel), the macroscopic view of the spinal cord from the mice transplanted with neural cells at day 50 showed the marked anatomical recovery at a glance. Both rostral and caudal ends were completely connected each other and the site injured showed a white matter-like appearance. In contrast, spinal cord of SCI mice with vehicle injection was difficult to remove it from the vertebrae due to adhesion and the site injured was not filled with the white matter-like substance

observed in the mice with neural cell transplantation, but only a gelatinous string was observed (data not shown).

To further analyze the anatomical recovery in the mice with neural cell transplantation, we observed the spinal cord under microscopy. The images of the lesion presented in Fig.3A were representatives of each experimental group of mice whose BBB scores placed them near the mean of their respective groups. In a longitudinal section of a typical spinal cord at day 0 after SCI with vehicle injection, the cavity formation was observed and both white matter and gray matter were completely separated into their rostral and caudal sites by the cavity that encompassed the complete width of the spinal cord. Furthermore, injured spinal cord exhibited severe atrophy in caudal, and a lesser degree in rostral portions. It was demonstrated that the SCI with vehicle injection led to marked fibroblastic and inflammatory infiltrations from the surrounding tissue and scarring at day 7 (Fig.3A).

In the transplanted spinal cord with the neural cells at day 7, the injured site was fulfilled with a large number of cells. This higher cellularity observed at the site of transplantation as compared with the site of SCI with vehicle injection might due to the transplanted ES-derived neural cells. Even though there was also the infiltration of gliosis in the spinal cord with neural cell transplantation, vast majorities of the cells were different from the infiltrating gliosis observed in SCI with vehicle injection (Fig.3B). These findings were confirmed by double staining for GFAP and labeled iron, and the gliosis was inhibited by transplantation of neural cells (Fig.3B). This finding suggests that the transplanted ES-derived neural cells survived in the injured spinal cord, however, no teratoma formation was observed in all mice transplanted with neural cells even at day 14 after transplantation (Fig.3B).

In vivo survival and characterization of ES-derived neural cells transplanted in SCI mice

To further confirm the survival of ES-derived neural cells transplanted at the site of SCI, we performed PCR to detect male-specific *sry* gene on Y chromosome of ES cells in DNA extracted from the tissues at the site of SCI in female recipient mice^{27,28}. This method allowed the identification of the male genotype of ES-derived neural cells in the female recipient. Fig.4A demonstrated that the *sry* gene was expressed in the tissues at the site of SCI in mice with neural cell transplantation but not in SCI mice injected with vehicle. Furthermore, the *sry* gene was detected at both rostral and caudal sites > 2 mm from the both edges of the remaining spinal cord. These findings indicated that the trans-

Fig.4 Detection of *sry* gene and neural markers at the site of spinal cord transplanted with ES-derived neural cells

A. Detection of *sry* gene that indicates male genotype originated from ES cells as a marker of transplanted cells. DNA was extracted from the center portion of the injury, and >2 mm distant from both caudal and rostral edges of the transected spinal cord of female recipient mice with or without cell transplantation. The DNA obtained was amplified by genomic PCR using a set of primers specific for *sry* gene. Note the *sry* gene was detected at both rostral and caudal sites >2 mm distant from the both edges of transected recipient spinal cord, corresponding to T3-T4 and T11-T12, transplanted with ES-derived neural cells (Transplantation). However, SCI injected with vehicle (Spinal cord injury) failed to amplify the *sry* gene, indicating the absence of ES-derived transplanted cells. **B.** Detection of neural markers. Total RNA was extracted from an exactly center portion of the injured site of the spinal cord with transplantation of RA-treated ES-derived neural cells (RA), undifferentiated ES cells (ES) or injection of vehicle alone (SCI), then analyzed by RT-PCR. Normal embryonic (E13) mouse brain was used for positive control (PC) for each neural marker.

planted neural cells survived at the site of SCI, and a part of them migrated into the normal portions of transected spinal cord across the rostral and caudal cutting edges of SCI.

The functional, electrophysiological and anatomical recoveries of SCI mice with transplantation of ES-derived neural cells suggested that the transplanted cells survived and further differentiated into functional neural cells in the sites, and reconstructed the damaged neuronal pathways. To characterize the transplanted cells, we first investigated the expression of neural cell markers by RT-PCR (Fig.4B). From both mice with transplantation of ES-derived neural cells and mice with injection of vehicle alone at day 7 after SCI, small pieces of tissue in the center of the injured site of spinal cord were collected to avoid contamination of host spinal cord (Fig.3). RT-PCR demonstrated that Nestin, a neural stem/immature neural cell marker, was expressed as well as NFM, which was a marker for mature neuron, in the SCI sites of mice transplanted with neural cells but not in SCI mice with vehicle injection. In addition, the expression of motoneuron specific markers, Islet1, HB9 and Lim1/2 was detected in the SCI sites of mice transplanted with neural cells, and in a lesser degree in mice transplanted with undifferentiated ES cells. These results suggested the regeneration of spinal motoneurons specific

to the SCI sites transplanted with ES-derived neural cells. Furthermore, among the neuronal growth factors tested so far, only neurotrophin 3 (NT3) was successfully amplified in the SCI sites of mice transplanted with neural cells and undifferentiated ES cells, but not in SCI mice with vehicle injection. These findings suggest that the local expression of NT3 supported the neuronal plasticity of the transplanted ES-derived neural cells after SCI-induced denervation, though the phenotype of NT3-producing cells are unknown.

Differentiation of ES-derived neural cells into functional mature neurons and reconstruction of neuronal pathways at the site of SCI

To further investigate the neuronal regeneration at the SCI site transplanted with the neural cells and whether the neurons originate the ES-derived neural cells, we performed the double staining for neuronal markers and the colloidal iron that was used to label the ES-derived donor cells. Our labeling procedure with colloidal iron was simple and seemed essentially to make no change in characteristics and viability of the labeled cells. In addition, it was stable in the transfected cells and the leak of colloidal iron and the influence on neighbor cells were not

Fig.5 Immunohistochemical analyses for NFM positive mature neurons in the spinal cord transplanted with ES-derived neural cells

The transplanted cells were labeled with colloidal iron to trace them and detected by Berlin blue staining as blue spots. A longitudinal section (x 40) indicates the location of the ES-derive transplanted cells and positions of the following sections (x 400). A bold bar indicates the transected portion. Each section was stained with anti-NFM antibody (A, B, C, E, F) or anti- III tubulin antibody (D) and followed by Berline blue staining. Double stained mature neurons were observed at the site transplanted with ES-derived neural cells (panel C and D). Note that the transplanted neural cells were observed at the edges of remaining spinal cord (panel B; rostral edge and panel E; caudal edge), and several transplanted cells were also observed beyond the edges (panels A and F). Each alphabetical mark in the longitudinal section corresponds to the higher magnifications of NFM or III tubulin immunostaining in the lower panels.

Fig.6 Immunohistochemical analysis of Lim1/2 and Synaptophysin in the spinal cord transplanted with ES-derived neural cells

The sections were prepared from the spinal cord transplanted with neural cells labeled with colloidal iron at day 14 after transplantation. Double staining with Berlin blue and anti-Lim1/2 or anti-Synaptophysin antibody was performed and compared with the single staining with Berlin blue alone (x 1000). Note some of the Berlin blue positive transplanted cells were Lim1/2 (left upper panel) and looked like green cells in contrast to the blue cells that were Berlin blue single positive (right upper panel). Similarly, some of the Berlin blue positive cells were Synaptophysin immunopositive (left lower panel). Representatives of transplanted cells differentiated into Lim1/2 and Synaptophysin positive spinal motoneurons are shown.

observed²¹⁻²³). As shown in Fig.5, immunohistochemical staining demonstrated that the sites of SCI transplanted with Fe labeled ES-derived neural cells contained the NFM positive and III tubulin positive mature neurons (Fig.5C and 5D). These findings indicated that the NFM positive mature neurons may originate from the transplanted neural cells, though the possibility was not completely denied that the transplanted cells induced the mature neuron from host neural progenitors. In addition, the transplanted neural cells existed in the rostral and caudal edges of remaining spinal cord (Fig.5B and 5E). Several transplanted cells also existed far distant from the transplanted site beyond the edges (Fig.5A and 5F), confirming the results obtained by the detection of *sry* gene (Fig.4A). These findings suggest that the transplanted neural cells play important roles for the reconstruction of neuronal pathways interacting with neurons that exist at uninjured portions of the spinal cord. In addition, some of the Berlin blue positive transplanted cells were positive for Lim1/2 (Fig.6) and Islet1 (data not shown), demonstrating that some of the ES-derived neural cells differentiated into spinal motoneurons. Furthermore, Synaptophysin was positive in a part of Berlin blue positive transplanted cells at day 14 after transplantation (Fig.6), suggesting that the mature neurons differentiated from the transplanted neural cells induced functional synapse formation and reconstructed new neuronal pathways at the site of SCI.

Discussion

ES cells are potential therapeutic sources for cell transplantation because they are stable *in vitro* through multiple passages without loss of their totipotentiality. Moreover, it is possible to induce ES cells to differentiate into enriched populations of glial or neuronal precursors. It is possible to design the transplantation of ES cells customize to repair specific focal lesions in the damaged CNS^{11,19,20}.

In SCI, the trials for transplantation of endogenous NSCs originated from CNS and exogenous NSCs or precursor cells have been reported in recent years. The origins of NSCs used for transplantation to spinal cord were embryo-derived NSCs involved in E14 spinal cord³⁰) and E14-15 cerebral cortex of rats^{31,32}), adult rat spinal cord³³) and adult human cerebral cortex³⁴). However, in the transplantation of embryo-derived NSCs to spinal cord, vast majorities of the differentiated cells were astrocytes and/or oligodendrocytes rather than neurons³⁰⁻³²). Similarly, the stem cells from adult rat spinal cord and adult human cortex transplanted to rat spinal cord predominantly differentiated into astrocytes/oligodendrocytes and Schwann cells, respectively^{33,34}).

In addition, the induction of ES-derived neural cells has also been reported and transplanted to the injured spinal cord, where majorities of the differentiated cells were oligodendrocytes and lesser numbers of astrocytes¹⁶⁻¹⁸). In spite of these reports, we have induced neural cells from ES cells and *in vivo* differentiation into neurons, especially Islet1 or Lim1/2 positive spinal motoneurons, was observed when they were introduced to the injured spinal cord of mice. It has also been reported that the transplantation of fetal neural progenitor cells result in neurogenesis in SCI rats^{12,13}). One important possibility that differed our results from previous reports was that the total cell population treated with RA, but not oligodendrocyte or astrocyte enriched population, was used for transplantation. Our cell population used in this study had the differentiation capacity of the entire population to form neurons, astrocytes and oligodendrocytes as reported previously³⁵). Indeed, RA-treated ES cells differentiated into the NFM- or III tubulin-positive neurons, GFAP-positive astrocytes and GalC-positive oligodendrocytes (representative results of three independent experiments were 27-42%, 20% and 38%, respectively) though the percentages of neuronal differentiation were changed by the methods to evaluate it, and at least a part of RA-treated ES cells differentiated into the cells other than neural phenotypes. In addition to the cell population, we analyzed the functional and electrophysiological recoveries of transected spinal cord transplanted with ES-derived neural cells, focusing on the neuronal but not glial development in the site of SCI. In this regard, our study suggests that the neuronal reconstitutions are important coordinately with myelination by oligodendrocytes during the healing of SCI by cellular transplantation¹⁶⁻¹⁸).

We have attempted to obtain purified motoneurons by neuron specific transcription factor gene transfection (NS et al. manuscript in preparation). The motoneurons when transplanted to the SCI mice improved motor function of the hind limbs as well. Collectively, it seems that exogenous motoneurons as well as oligodendrocytes were important for motor functional recovery of the SCI mice.

RA induced all lineages of neural cell precursors from ES cells¹⁵). In our previous studies involving the transplantation of ES-derived neural cells in motor cortex injured mouse model of hemiplegia, we found significant recovery of motor functions and the *in vivo* differentiation of motoneurons that expressed mature neuronal markers including NFM^{19,20}). But they were Lim1/2 negative in the brain. These results suggested the neuronal differentiation of ES-derived neural cells in CNS. However, spinal cords of both newborn and adult rodents are special

regions that do not normally show neurogenesis *in vivo*³⁶. Recently, the important roles of astrocytes during neurogenesis have been reported, however, astrocytes of spinal cord from newborn had only small effects on neurogenesis from stem cells but those from adult spinal cord had no effect³⁷. One explanation for this finding is that newly generated astrocytes can increase the neuronal extensions, whereas older astrocytes that consist in spinal cord cannot³⁸. Thus, the renewals of astrocytes and the concordant regeneration of neurons may be important for the functional recovery of SCI. In addition to the characters of astrocytes in spinal cord, neurogenesis-inhibiting mechanisms including bone morphogenic proteins (BMPs) are involved in disadvantages of neurogenesis in spinal cord³⁹. Based on the previous findings, the factors controlling neurogenesis often controversially inhibit glial fates, making coordinated recoveries of both astrocytes and neurons in SCI difficult⁴⁰⁻⁴².

It has been reported that NT3 expressed in injured spinal cord supported the plasticity of intact axons⁴³. Our findings supported the facts and NT3 produced by ES-derived neural cells may help their neuronal regeneration in injured spinal cord. Taken together, ES-derived cells may further differentiate into neurons, astrocytes and oligodendrocytes *in vivo*, and may have advantages to reconstitute injured spinal cord by renewal of astrocytes and following neuronal differentiations and myelinatons, as previously reported, by differentiated oligodendrocytes¹⁶⁻¹⁸.

In this study, we used mice whose spinal cords were completely transected for SCI model. The fact was experientially known that spontaneous recovery of motor function after SCI was observed in rodents. The mouse has only recently been studied in greater detail for its response to SCI, and it has become clear that mice and rats differ in their responses to injury⁴⁴. There is considerable evidence that functional recovery after SCI is not a simple consequence of the initial mechanical destruction of tissue but is also the evolution of complex secondary events to contribute to early as well as delayed cell injury. Mice have only a minor secondary injury response to SCI, whereas the rat and human responses lead to the formation of large cavities that impede axonal regeneration in spinal cord. In mice, however, the cavitation was also observed in several degrees depend upon the severity of SCI, contusion, hemisection or total transection of spinal cord⁴⁵. In this study, anatomical observation demonstrated that a complete transection of 1.5 mm-length of mouse spinal cord led to the formation of large cavity, in which both rostral and caudal ends of the cord were completely separated, and large infiltrates and scarring into the injured site were

observed. Spontaneous functional recovery of the SCI mice was negligible in our model suggesting that endogenous NSCs locating near central canal were insufficient for the functional recovery in the complete transection model.

In this report, we transplanted ES-derived neural cells to congenic female mice of SCI without usage of immunosuppressants. Histological findings of spinal cord injury demonstrated the severe infiltration of inflammatory and fibroblastic cells as well as cavity formation and scarring in the sites of SCI¹⁸. Our previous report indicated that the infiltration of inflammatory cells and the gliosis were caused at the site of injured motor cortex and seemed to inhibit the improvement of neural network^{19,20}. These findings suggest the possibility that the usage of immunosuppressants may inhibit these inflammatory responses and have advantages in recovery of SCI. Indeed, immunosuppression by cyclosporine A induced tissue protection and enhanced recovery of motor function in injured rat spinal cord⁴⁶. In that sense, our experimental protocol without immunosuppression makes straightforward interpretation of the experimental results possible. We transplanted ES-derived neural cells at day 0 of SCI, however, the importance of timing to transplant neural cells has been shown because inflammatory responses interfere the recovery of SCI^{14,47}. These findings suggest that the optimal timing of transplantation of the ES-derived neural cells may result in further improvement of motor and electrophysiological functions in SCI mice.

In summary, our study demonstrated that ES-derived neural cells were successfully transplanted to the site of SCI in mice and differentiated into neurons without any difficulty such as teratoma formation during period observed. The functional, electrophysiological and anatomical improvements of SCI by transplantation with ES-derived neural cells provide a new therapeutic strategy for neural disorders and evidence for new interpretation to the cellular transplantation therapies.

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