Effects of Glial Transplantation on Functional Recovery Following Acute Spinal Cord Injury

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Effects of glial transplantation on functional recovery following acute spinal cord injury

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The Doctor's Thesis submitted to the Department of Medical Science, the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Doctor of Medical Science

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December 2003

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December 2003

ACKNOWLEDGEMENTS

본 학위과정을 위하여 늘 부족한 저를 따뜻한 격려와 배려를 아끼지 않고 부모님처럼 보살펴 주시고 지도 편달해 주신 이 배환 교수님께 먼저 진심으로 감사 드립니다. 그리고 많은 격려와 조언으로 늘 도와주신 박경아 교수님, 윤도흠 교수님, 김동구 교수님, 정광철 교수님, 또한 많은 도움을 주신 정상섭 교수님, 박용구 교수님, 그리고 장진우 교수님께도 감사드립니다.

실험실에서 같이 생활하면서 항상 격려와 용기를 주셨던 원란 선생님과 전기생리학연구실의 김은주, 김은정, 김은신 그리고 함께 노력하며 대학원 생활의 고락을 같이 나눈 정세정 후배에게도 감사의 마음을 전합니다.

힘들때마다 따뜻한 위로와 함께 용기를 준 김설, 윤신영, 조윤희, 김명희 선생님과 15년의 우정을 같이하면서 정신적 위안을 준 은희, 현주, 정애, 민혜 그리고 혜경이에게도 감사의 마음을 전하며 이 조그만 노력의 결실을 나누고 싶습니다.

끝으로 항상 사랑으로 지켜봐 주시고 심적인 위로와 용기를 주신 시어머님과 시누이 그리고 대학원 생활을 잘 이해해주고 공부하도록 도와준 오빠와 남동생에게도 감사드리며, 항상 가장 가까운 곳에서 묵묵히 지켜보며 늘 말없이 그늘이 되어주어 학업에 전념하도록 도와주신 남편께 감사함과 고마움을 전합니다. 그 누구보다도 가장 이 결실을 자랑스러워하시고 기뻐해 주셨을 부모님께 가슴저리도록 감사드립니다.

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ABSTRACT

Effect of glial transplantation on functional recovery following acute spinal cord injury

There have been efforts to maximize the therapeutic effects for the spinal cord injury (SCI). Treatment of SCI is difficult not only because of the focal cell death caused by the initial insult but also from penumbral cell death caused by the secondary biochemical and pathological changes in the spinal cord. Over the past few years, many attempts have been made in animals to produce cellular regeneration in the spinal cord using transplantation of different cell types. Recently oligodendrocyte-type 2 astrocyte (O-2A) progenitor cells were known to remyelinate the focal areas of the demyelinated adult rat spinal cord. The aim of this study, therefore, was to investigate the therapeutic potential of O-2A cells when transplanted into the acute SCI model.

Under pentobarbital anesthesia, male Sprague-Dawley rats were subjected to SCI. Rats were laminectomized and SCI was induced using NYU impactor at T9 spinal segment. O-2A progenitor cells, which were labeled with BrdU were transplanted 1 week after the induction of SCI. Behavioral, electrophysiological (motor evoked potential: MEP and somatosensory evoked potential: SSEP), immunohistochemical (double staining of BrdU and CNPase, NeuN, or GFAP) and tract tracing studies were performed to observe the improvement of functional recovery.

The behavioral test showed significant improvement in O-2A transplanted group compared with control group since 6 weeks after the transplantation. From the electrophysiological study, SSEPs have not shown any implications in amplitudes and latencies when O-2A transplanted group was compared with media treated group. However, from the MEP recording, the initial, N1, and P1 latencies in O-2A group were significantly shorter than control. Morphologically, the BrdU-positive cells were located in the injured and the

adjacent sites after O-2A transplantation. Double staining of BrdU and CNPase showed that the cells have been differentiated into oligodendrocytes, after 2 months of transplantation. On the contrary double staining of BrdU and GFAP, or NeuN have shown to be negative, indicating that astrocytes and neurons have not been produced by the transplantation of O-2A cells in vivo. According to the tract tracing study, anterogradely labeled processes have been observed to cross the cavity of the injured site of the spinal cord. Retrogradly labeled neurons in the reticular formation, raphe nucleus and red nucleus have shown to increase after O-2A transplantation.

Therefore, it can be concluded that O-2A cells have shown to migrate to the adjacent sites of the injured site, leading to the differentiation into oligodendrocytes when transplanted to the animals with SCI. Behavioral test, electrophysiological and morphological studies showed that the transplantation of O-2A cells may play an important role in functional recovery and axonal regeneration. These results may have an implication in the

development of potential therapeutic strategies for SCI based on glial precursor cell transplantation.

Key Words: spinal cord injury, O-2A cell, transplantation, behavioral test, electrophysiology, morphology

Effect of glial transplantation on functional recovery following acute spinal cord injury

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I. INTRODUCTION

The causes of spinal cord injury (SCI) can vary from fall to disastrous accidents. According to the Spinal Cord Injury Association of Illinois, 183,000 to 230,000 people are affected by SCI in the US and about 11,000 new cases are arising each year. Majority of them are male (81.6%) and falls between 16-30 years of age.[1] The primary cause of malfunction in SCI are complete or

incomplete loss of supraspinal motor, sensory and autonomic drive below the lesion that leads to complete or incomplete loss of functions depending on the severity. [2] The severity of SCI on the whole is determined not only by its primary insult but also by the secondary processes including ischemia, anoxia, free-radical formation, and excitotoxicity that occur over the hours and days following injury that leads to penumbral cell death.[3] The resultants of all of these processes are axonal damage, loss of neurons and glial cells, and demyelination.

The contusive experimental model of SCI offers the advantage of being clinically pertinent. Contusion of spinal cord in rats has been shown to provide a reliable and reproducible injury,[4] and some studies have shown to produce similar histopathological features of SCI in different species.[5,6] One of the method to produce contusion in the spinal cord is weight drop. The weight drop method involves dropping down a guide rod with calibrated weight from a specified height in order to strike the surgically exposed spinal cord. NYU

weight drop model has been adopted widely for this study. Although SCI still have poor prognosis, many researchers have worked on finding effective ways for the functional recovery after acute SCI using pharmacological agents and various types of neurotrophic factors. However, a potentially effective repair strategy that is raising researchers' interest today is surgical transplantation of axonal growth-promoting and –guiding cells into the injured spinal cord segment.

Promotion of axonal regeneration and recovery from functional deficit are the major target of SCI treatment. A variety of cell transplantation has been approached as a source of treatment to replace tissues that has been lost through cell death. Especially huge efforts have been admitted to replace damaged myelin or to supply a tissue bridge for nerve fiber growth. Several studies have reported various cell types transplantation that have been shown to have efficiency in the locomotion improvement in different SCI models.[7,8,9,10,11,12,13,14] These include Schwann cells (SCs),[15,16,17] olfactory

ensheathing glial (OEG) cells,[18,19] fetal neural tissues,[7,20,21,22] peripheral nerves,[23,24,25,26] and stem cells,[12,27,28] which can differentiate into multiple classes of neurons or glia. Although varieties of cells were found to be useful in the treatment of SCI, glial cell lineage is found to be the most efficient cell line for the axonal regeneration and remyelination. Glial cells are classified into two types according to their origins: peripheral nervous system (PNS) and central nervous system (CNS). SCs which is PNS myelin forming cell, have been well known and studied in the past.[29,30] However, there are many different types of CNS glial progenitors and they are newly arising cell lines for the source of the treatment of SCI. When SC graft was transplanted to a transected adult rat thoracic spinal cord, it has been known that the SC graft has played a major role in the promotion of regeneration and remyelination of propriospinal axon.[31,32,33] The combination of SC grafts and methylprednisolone[7] or neurotrophic factors[34,35] is found to be effective in the promotion of axonal regeneration from brainstem neurons. Although CNS

axons can be regenerated by grafting SCs (PNS cells) into CNS, it was found that the cells could not migrate to the adjacent area and therefore the synaptic contacts could not be made even though it is crucial for the functional recovery. To substitute these problems, CNS glial cell types have been chosen. One of the most promising CNS glial cell types is OEG cells.[36,37,38,39,40] OEG grafts were found to promote axonal growth and functional improvements in different SCI model.[10,13,41,42] Transplanted OEG cells appear to migrate further within the CNS than transplanted SCs.[13] Moreover, when a combination of SC grafts and OEG cells were transplanted into the transected or contused spinal cord region, it has been found to promote regeneration of descending and ascending axons into the caudal and rostral spinal cord, respectively.[43]

Although it is known that compact myelin may be formed after transplantation of exogenous glial cells into demyelinated region of adult CNS,[44] assessment of the electrophysiological properties of the remyelinated

axons has not been undertaken. Such electrophysiological study is important because reliable impulse conduction in remyelinated axons require not only the formation of compact myelin sheath but also establishment of appropriate myelin segment length,[45,46] and ion channel organization at the newly formed nodes of Ranvier.[47,48,49,50] It is not known whether remyelination by transplanted exogenous OEGs and SCs, especially in the adult CNS, meet these criteria for the reestablishment of normal conduction. However, since the cells from CNS like OEG cells were found to be effective in the treatment of SCI, it is tempting to study the effects of progenitor cells from the brain in the recovery from the functional deficit.

O-2A cells are named after the two types of progeny that differentiate to oligodendrocytes and type 2 astrocyte in vitro, which were first described by Raff et al.[51] These cells can be isolated from the postnatal rat optic nerve, cerebellar cortex, brain stem and spinal cord. O-2A cells are known to differentiate predominantly into myelinating oligodendrocytes upon

transplantation, but differentiation into neuronal cells has not been observed.[52] Differentiation into astrocytes in vivo has not been consistently observed.[53,54,55] Therefore some investigators have suggested naming O-2A cells as oligodendrocyte precursor. Oligodendroglial precursor cells undergo migration and proliferation stages before the cell differentiation into myelin forming oligodendrocyte.[56] O-2A lineages express characteristic developmental marker which is anti-ganglioside antibodies (A2B5).[51] This antibody has been used widely to identify progenitors of O-2A lineage. Neonatal rat brain displays the most enriched ganglioside compositions compared with cells from other sites.[57,58] Furthermore, O-2A cells isolated from the cerebral cortex appear to generate oligodendrocytes at a later stage and over a longer time period than cells isolated form the optic nerve and chiasm.[59] Power et al.[59] have shown that O-2A cells isolated from the cortex shows higher purity of O-2A cells even after long culture period.

A number of studies have shown that neonatal oligodendrocye precursors

were able to survive, proliferate, migrate, and myelinate after the transplantation into the CNS of myelin mutant neonates which lack myelination.[52,60,61] These studies highlighted the potential use of oligodendrocyte precursor as a therapy for the diseases that has been caused by demyelination such as multiple sclerosis (MS). Very similar characteristics have been observed from the cells that have been isolated from adult CNS. When transplanted into the CNS of hypomyelinated hosts, oligodendrocyte precursors migrate over considerable distances, and give rise to large numbers of myelinating oligodendrocytes.[60,62]

In the present study, the effect of O-2A cells transplantation into the injured spinal cord was studied. To support this study, behavioral test for the locomotion recovery, electrophysiological study for the axonal conduction, immunohistochemical study to observe the trends of cells that have undergone migration and differentiation and tract tracing study to observe if the axons have established synaptic contact were performed.

II. MATERIALS AND METHODS

1. Primary O-2A Progenitor Cell Culture

Primary O-2A progenitor cultures were prepared from 2 day postnatal rat pups by the method of Juurlink et al.[63] Cerebral hemispheres were removed, mechanically dissociated, suspended in primary culture growth medium and plated on four 100-mm tissue culture Petri dishes for each rat pup (Falcon no. 3003). After 5 days, primary cultures were placed on OPM-G (Working DMEM with 1 M glucose, 50 mM lactate, transferring-biotin-selenium, 30% B104 conditioned medium, 5 μg/ml insulin). The majority of cells were oligodendroglial precursors after 10-14 days of culture then the culture was ready for harvest. Cell suspension was filtered sequentially through nylon filters and was centrifuged at 180 g for 15 min. The cells were plated on polylysine-coated dishes and grown in OPM without glucose. To maintain

proliferation of O-2A progenitors, the cells were grown in defined medium supplemented with conditioned medium from B104 neuroblastoma cells according to the procedure described by Louis et al.[64] Cell morphology and density was monitored daily under a phase contrast microscope. The characteristics of culture and its purity was determined by performing Immunocytochemistry with primary antibodies such as A2B5 for O-2A progenitor (1:100, Chemicon, Temecula, CA, USA), GFAP for astrocyte (1: 500, BD, San Jose, CA, USA), O4 for immature oligodendrocyte (1:40, Chemicon), and CNPase for oligodendrocyte (1:100, Chemicon). Cells were labeled by incubating them in the presence of 2 μM bromodeoxyuridine (BrdU: Sigma, Saint Louis, Missouri, USA) further for a week before the transplantation.

2. Spinal Cord Injury

Fifty male Sprague-Dawley rats (Daehan Biolink, Chungbuk, Korea) weighing 300-350 g at the time of surgery were used. They were housed in groups of four and had free access to food and water. All animal experiments were approved by the Institutional Animal Care and Use Committee of Yonsei University College of Medicine.

Acute spinal cord injury was induced using NYU weight-drop device. Adult male Sprague-Dawley rats were anesthetized with pentobarbital (50 mg/kg, i.p.) and a laminectomy was performed at the T9 spinal level. The exposed dorsal surface of the cord was then subjected to a weight-drop impact, 10 g weight impact rod was dropped from a height of 25 mm to produce moderately contused SCI model. During recovery, rectal temperature was maintained at 37℃ by a feedback regulated heating pad. Postoperative nursing care included bladder expression two times a day. Prophylactic

gentamycin sulfate (1 mg/kg) was regularly administered for a week.

3. Behavioral Assessment after SCI

Behavioral test was performed to measure functional recovery of hindlimb. Open field testing procedures have been described by Basso et al.[65] This scale measures hindlimb movements with a score of 0 indicating no spontaneous movement, with increasing score for use of individual joints, coordinated joint movement, coordinated limb movement, weight- bearing and so on to a maximum score of 21.

Rats were gently adapted to the open field. Once a rat walked continuously in the open field, two examiners conducted a 5 min testing sessions in each leg, postoperative (p.o.) open field testing was performed at least once a week from day 1 p.o. to 9 weeks for all the animals.

4. Transplantation of O-2A Cells

According to behavioral test for one week, rats were assigned, without bias, as media-treated group (n=19) and O-2A groups (n=21). At 7 days after the induction of injury, rats were anesthetized with halothane (1:2, N_2O:O_2). Using a capillary glass tube, transplantation of cultured 5 μl O-2A ($1x10^5$/ μl) cells was performed in the O-2A group, which was labeled with BrdU into the epicenter of injury. The media-teated group received 5 μl of media injection within the epicenter of injury. Cyclosporine A (1 mg/100 gm) was injected daily since 2 days before the transplantation.

5. Electrophysiological Assessment

1) Animal preparation

Eight weeks after the transplantation, the animals were anesthetized with

urethane (1.25 g/kg). Each animal was also given atropine sulfate (0.8 mg/kg) to reduce tracheal secretions. Pancuronium bromide (0.4 ml) was then perfused through the tail vein to induce muscle relaxation. The rat was then artificially respired using a small animal respirator (Model 683, Rodent Ventilator, Harvard, Holliston, MA, USA) and maintained within the physiological range using a capnometer (Model 2200, Traverse Medical Monitors, Saline, Michigan, USA). The animal was placed on the stereotaxic device (Narishige Scientific instrument laboratory, Setagaya-ku, Tokyo, Japan) and the rectal temperature was maintained between 36.5-37.5℃.

2) Somatosensory evoked potentials (SSEPs)

SSEPs were recorded to measure the conduction recovery of the sensory system. A special electrode (NE-120, Rhodes Medical Instruments, Inc., Woodland Hills, CA, USA) was used for SSEP recording. The pointed tip of the electrode was used as active and the rounded part of the electrode was

used as a reference. For the recording, the electrode was placed in the sensorimotor cortex (bregma: -2 mm, lateral: 2 mm). Bipolar platinum wire electrode placed in contralateral sciatic nerve was used as a stimulating electrode. A single square pulse (0.1 ms duration) of electrical stimulus was delivered by a stimulus isolator (A365D or A 365, World precision Instruments, Inc., New heaven, Connecticut, USA), which was driven by a pulse generator (Pulsemaster A300, World Precision Instruments, Inc., New Heaven, Connecticut, USA). The analog signals of the evoked potentials were amplified (x10000), filtered (bandpass 300-1000 Hz), and fed to IBM-compatible PC through AD/DA converter (CED, Cambridge, UK) to be averaged using Spike 2 software. Each SSEP consisted of an average of 100-300 single sweep epochs. The threshold of electrical stimulation was first determined in each experiment. The effect of the stimulation intensity on SSEPs was analyzed in the wave forms by latencies and amplitude.

3) Motor evoked potentials (MEPs)

MEPs were recorded to measure the conduction recovery of the motor system. It was necessary to increase the area of stimulation in the motor cortex in order to properly monitor the MEPs with low stimulus intensity. For this purpose, the special electrodes that were identical to the recording electrodes to record SSEP were used as stimulating and recording electrodes. Same specifications for the recording and stimulating electrodes were applied. In cortical stimulation, a single square pulse (0.1 ms duration) of electrical stimulus was delivered by a stimulus isolator, which was driven by a pulse generator. Laminectomy was performed at L1 of the spinal cord for the placement of the epidural recording electrodes. Following the laminectomy, the electrode was inserted into the contralateral gray matter of the L1 spinal cord. The analog signals of the evoked potentials were amplified (x10000), filtered (bandpass 300-1000 Hz), and fed to IBM-compatible PC through AD/DA converter to be averaged using Spike 2 software. Each MEP consisted

of an average of 100-300 single sweep epochs. The threshold of electrical stimulation was determined in each experiment. The effect of the stimulation intensity on MEPs was analyzed in the wave forms by latencies and amplitude.

6. Immunohistochemical Assessment

Immunohistochemistry was performed to evaluate the morphological features of transplanted cells in vivo. Nine weeks after the induction of SCI, the rats were perfused, by PBS and 4 % paraformaldehyde in pipe solution (1 M, pH 6.8). The spinal cords and brains were removed and post-fixed for 24 h in 4 % paraformaldehyde followed by 30 % sucrose in PBS overnight. Serial longitudinal sections of the spinal cord and coronal sections of the brain were made by the cryostat (12um thick: Microm/HM500V, CE, Germany) and the specimens were stored at -20℃.

The sections were rinsed in PBS for 3 min. Then the sections were incubated in 2 N HCl at 37℃ for 35 min followed by 0.1 M borated buffer. Blocking solution was used to treat the sections. The sections were incubated for two days with primary antibodies at 4℃. Secondary antibodies were applied to the sections for three hours and then the anti BrdU -FITC (1:50, Roche, Indianapolis, IN, USA) was used for double staining. Following primary antibodies were used: anti-glial fibrillary acidic protein (anti-GFAP, 1: 500, BD), anti-CNPase (1:100, Chemicon), anti-neuronal nuclei (NeuN, 1:500, Chemicon). The sections were mounted on slide glasses with fluorescent mounting medium (DAKO, Carpinteria, CA, USA) and observed by florescence microscope (BX51, Olympus, Tokyo, Japans).

7. Tract tracing study

1) Anterograde labeling of ascending axons

Anterograde tract tracing study was performed to determine the extent of corticospinal neurons with axon projecting to the spinal cord. Eight weeks after the transplantation, the anterograde tracer, biotinylated dextran amine fluorescein (BDA, 1 μl: Vector, Burlingame, CA, USA), was stereotactically injected into the sensorimotor cortex. Rats were resuscitated and allowed to survive for 10 days to permit transport of the tracer. Then rats were perfused as described above. For the tissue preparation from the injured site up to the T13 spine was removed and sectioned sequentially. Every third sections were used to examine processes that contain BDA. Sections were preincubated in 3 % H_2O_2 in methanol to block endogeneous peroxidase (10 min, RT) and then allowed to be incubated in avidin-biotinylated peroxidiase fluorecein (Vector).

2) Retrograde labeling of descending axons

Retrograde tract tracing study was performed to determine the extent of

supraspinal neurons with axons projecting to the spinal cord across the injured site. Eight weeks after the transplantation, rats were anaesthetized as described above. Rats were assigned into O-2A cell transplanted group, media transplanted group and normal group for the quantifications. The spine (L1) was exposed and microinjections of the retrograde tracer, fluorogold (FG: Fluorochrome, Denver, CO, USA), were made into the spinal cord caudal to the injury site using a glass needle attached to a 1 μl Hamilton syringe in all the groups. Two injections (2 x 0.5 μl, FG) were bilaterally made at 0.5 mm lateral to the postero-median vein and 1.2 mm depth. One week after the injection of the tracer, the rats were perfused and the fixed brains were removed and then sectioned. Every third sections were mounted onto gelatin-coated glass slides for the quantification of the number of FG-labeled cells. The retrograde FG label was visualized under UV excitation with 10x objective on fluorescence microscope. The sections were pictured and modified using Meta Morph computer software (Meta Morph version46r5,

Downingtown, PA, USA). For the quantification of the slides, the labeled cells in the reticular formation, raphe nucleus and red nucleus were manually counted and averaged for each groups.

8. Statistical analysis

The independent t-test was used to determine statistical differences between O-2A and media-treated groups for BBB scores at each time point. One-way ANOVA followed by Dunnett's post-hoc multiple comparisons was conducted in electrophysiological and morphological studies in order to compare the media-treated group with O-2A or normal group.

III. RESULTS

1. O-2A progenitor cell culture

Primary O-2A progenitor cell cultures have bipolar morphology (Fig. 1). O-2A progenitor goes through a series of maturational stages, which can be identified by the sequential expression of cell surface epitopes. Discrete characteristics of the cell can be recognized by the antibodies A2B5, O4, GFAP, and CNPase. The composition of the transplanted O-2A progenitor cells show almost 80% of A2B5-positive, 10 % of O4-positive, 7 % of GFAP-positive, and 3 % of CNPase-positive immunoreactivity. The total number of immunopositive cells adds up to >90 %, which may be accounted for by the presence of A2B5-positive, O4-positive cells, which are intermediate in maturity between O-2A progenitor and pro-oligodendrocytes. Thus, the suspension was highly enriched in oligodendrocyte progenitors (Fig. 2).

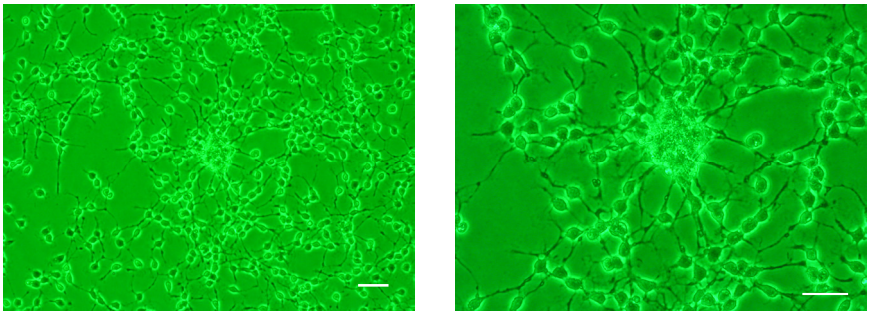

Fig. 1. O-2A cell culture. The phase contrast fields show the bipolar morphology of the O-2A cell at magnification of × 40 (left) and × 100 (right) (Scale bar: 100 μm).

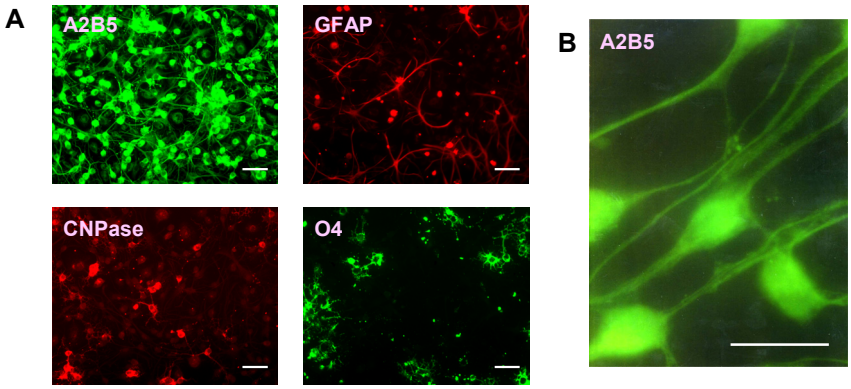

Fig. 2. Immunocytochemistry by primary antibodies of A2B5, GFAP, CNPase and O4 after primary culture. A: Progenitor cell stained positively in all antibodies. Especially, strong staining was seen in A2B5 at magnification of ×

100 (scale bar: 100 μm). B: A2B5-positive stained cells in a higher magnification of × 400.

2. Behavioral Assessment

Hindlimb locomotor performance was tested in all rats using the BBB open-field scaling.[65] Before the transplantation, BBB tests were performed in all animals at 1, 4, 7 days after SCI and the ones with low score and the ones with equally malfunctional hindlimbs were specially selected for the experiment. The media-treated group (n=14) has scored 0 in both legs at 1 day post injury then gradually increased to a final score of 9.5 ± 0.4 in left leg and 10.1 ± 0.39 in right leg at 9 weeks after the injury (Fig. 3). The O-2A transplanted group showed significantly improved hindlimb performance since 6 weeks after the transplantation compared to media-treated group ($p < 0.05$). The final BBB scores of the O-2A cell transplanted group (n=17) were 12.25 ±

0.37 (L) and 12.17 ± 0.36 (R) in both legs.

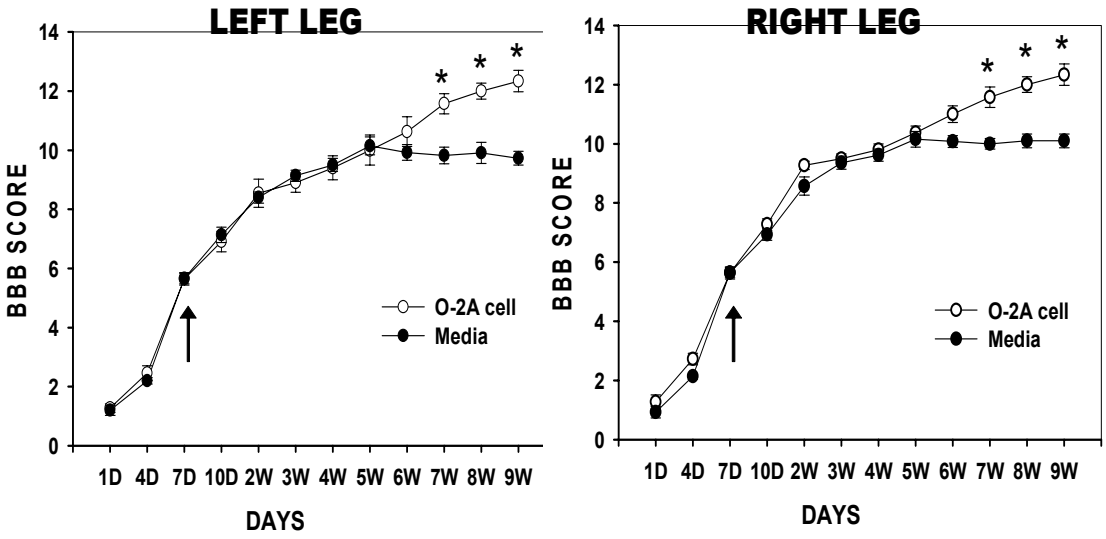

Fig. 3. BBB scores of SCI rats before and after the transplantation. O-2A transplantation at 7 days p.o. significantly improved hindlimb performance in both legs since 6 weeks after transplantation (↑: transplantation time, *: $p < 0.05$).

3. Electrophysiological Assessment

1) Somatosensory evoked potentials (SSEPs)

The SSEPs were recorded in the sensorimotor cortex following the stimulation of sciatic nerve. When the sciatic nerve was stimulated, a negative-positive-negative deflection with a short latency was observed at the sensorimotor cortex. The latencies of SSEPs were classified as initial, N1- and P1- peak latencies. Fig. 4 shows representative wave forms of SSEPs by different intensity stimulations. Table 1 and 2 shows the numerical data of the SSEP recording. The initial, N1, and P1 latencies in O-2A group were tend to lengthen compared to media-treated group. However, when it was statistically analyzed, no significance was observed. Amplitudes of experimental groups (media and O-2A cell group) were significantly lower than normal, whereas when media-treated and O-2A group were compared, no differences were observed in both N1 and P1 (Fig. 5).

Table 1. Latencies of SSEPs.

(unit: msec)

LATENCY	Initial	N1	P1
Normal (n=7)	7.3 ± 0.19*	18.7 ± 0.51*	35.3 ± 0.94*
Media (n=12)	9.7 ± 0.68	27.2 ± 1.22	54.0 ± 2.03
O-2A (n=19)	8.8 ± 0.32	24.1 ± 0.69*	49.9 ± 1.34

Numerical data of SSEPs showed significant difference in N1-peak indicating the recovery of conduction velocity in sensory system. Asterisks indicate statistically significant difference compared to media-treated group.

Table 2. Amplitudes of SSEPs.

(unit: μV)

AMPLITUDE	N1	P1
Normal (n=7)	22.41 ± 3.58*	46.75 ± 8.88*
Media (n=12)	6.17 ± 1.18	10.36 ± 1.90
O-2A (n=19)	4.05 ± 1.31	7.13 ± 2.24

Numerical data of SSEPs showed no significant difference between media and O-2A group.

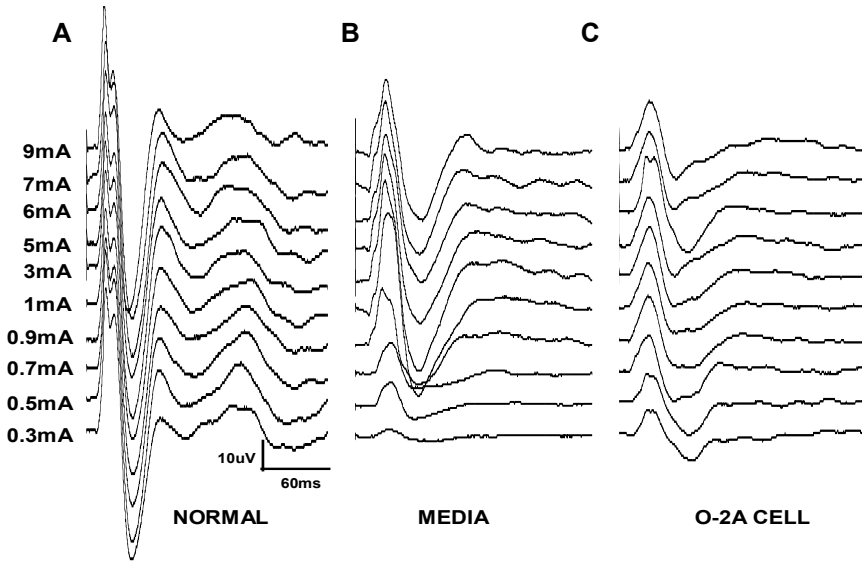

Fig. 4. Representative wave forms of somatosensory evoked potentials by different intensity stimulations. Although lengthened latency and reduced in amplitude were seen in both media and O-2A groups compared to normal, similar patterns were observed in media and O-2A cell groups. A: Normal group, B: Media group, C: O-2A group.

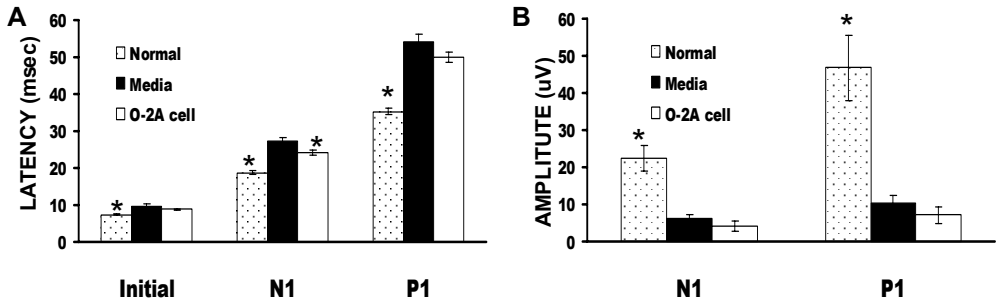

Fig. 5. Latencies and amplitudes of SSEP. A: Comparison of initial, N1- and P1-peaks latencies in different groups. B: Comparison of N1- and P1-peaks amplitudes in media-treated and O-2A groups were shorter than normal group. Asterisks (*) indicate statistically significant differences between media-treated group and normal or O-2A group by Dunnett's post-hoc multiple comparisons.

2) Motor evoked potentials (MEPs)

The MEPs were recorded using a bipolar disk electrode in the L1 spinal cord after hindlimb area of the sensorimotor cortex was stimulated. The wave forms were very similar to SSEPs with negative-positive-negative deflection.

Table 3 and 4 shows the numerical data of the MEP recording. After spinal injury, the animals showed lengthened MEP latencies and reduced amplitudes. Fig. 6 shows representative wave forms of MEPs recorded in normal, media-treated, and cell transplanted animals. Note the different amplitude scales. The latencies and amplitudes of MEPs were significantly lengthened and reduced, respectively. In MEPs, the latencies of the initial, N1- and P1- peak of the media-teated group were significantly lengthened ($p < 0.05$) compared to normal group. Latencies of O-2A group were shorter than the media-treated group. Although the amplitudes of the media-treated and O-2A groups have reduced than normal group, statistical analysis have shown no significant differences between media-treated and O-2A group (Fig. 7).

Table 3. Latencies of MEPs.

(unit: msec)

LATENCY	Initial	N1	P1
Normal (n=7)	9.3 ± 0.52*	22.3 ± 0.87*	43.1 ± 1.54*
Media (n=11)	28.6 ± 2.05*	40.5 ± 2.55*	59.0 ± 1.64*
O-2A (n=13)	16.4 ± 1.26	28.8 ± 2.04	48.4 ± 2.71

Numerical data of MEPs showed significant differences in initial, N1- and P1-peak latencies, indicating the recovery of conduction velocity in motor system. Asterisks (*) indicate statistically significant difference compared to media-treated group.

Table 4. Amplitudes of MEPs.

(unit: μV)

AMPLITUDE	N1	P1
Normal (n=7)	9.26±2.54*	18.65±5.39*
Media (n=11)	0.35±0.18	0.57±0.11
O-2A (n=13)	0.52±0.09	0.91±0.15

Numerical data of measured MEPs showed no significant difference between media and O-2A groups.

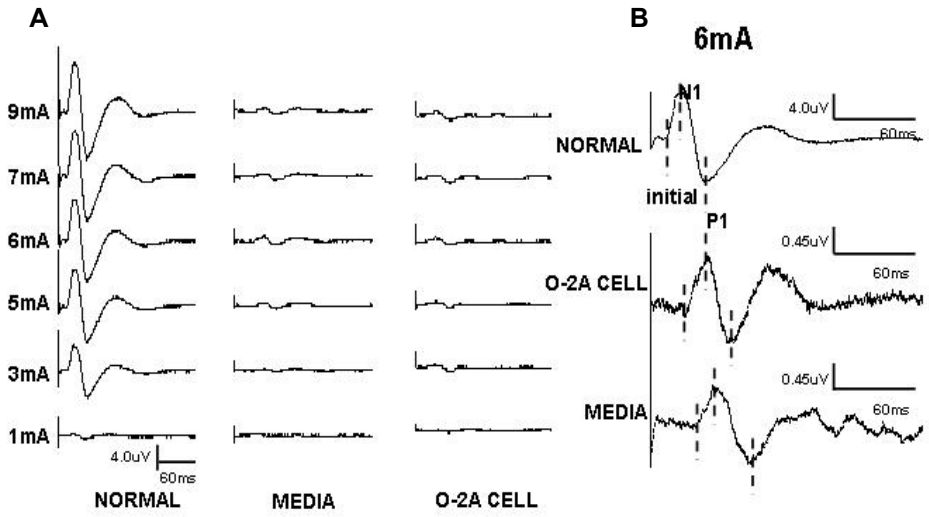

Fig. 6. Representative wave forms of motor evoked potentials by different intensity stimulations. Lengthened latencies and reduced amplitudes were seen in both media-treated and O-2A cell compared to normal. In 6mA, O-2A cell group displayed shorter latencies compared to media indicating the recovery of conduction velocity in motor system. A: Representative wave forms of MEPs for normal, media, and O-2A groups by different stimulation intensity, B: Representatives wave forms of MEPs by 6mA stimulation. Note different scaling in different groups.

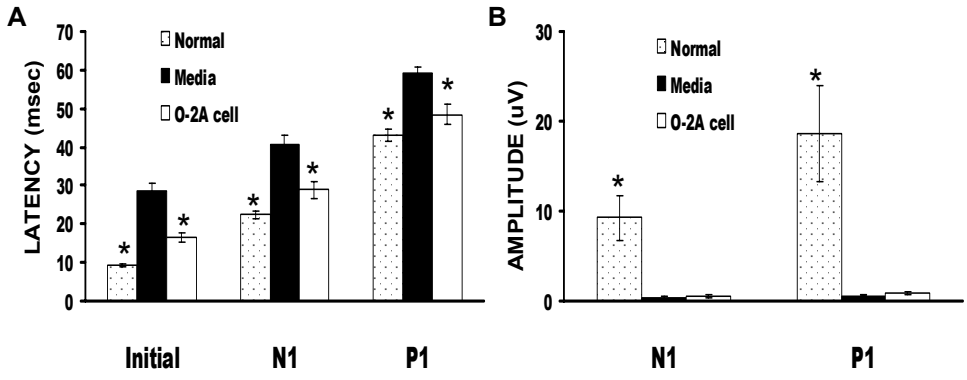

Fig. 7. Latencies and amplitudes of MEPs. A: Comparison of initial, N1- and P1-peaks latencies in different groups. All latencies in O-2A transplanted group were significantly shorter than media-treated group. B: No differences were observed in amplitude between media-treated and O-2A groups. Asterisks (*) indicate statistically significant differences between media-treated group and normal or O-2A group by Dunnett's post-hoc multiple comparisons.

4. Immunohistochemical Assessment

The amount of cell that have settled and survived after the transplantation

of cells was determined by BrdU immunoreactivity in longitudinal sections including epicenter of the spinal cord 8 weeks after the transplantation. The BrdU-positive cells appeared in injured site and upper and lower site of the spinal cord (Fig. 8). The double staining of BrdU and CNPase-positive cell was present in transplanted site and the adjacent sites, indicating that the transplanted cells have well been settled and differentiated as oligodendrocytes (Fig. 9). However, the double staining of BrdU and GFAP, or NeuN was not observed. Here, it may be supposed that the transplanted cells have migrated to the adjacent sites of the injury and mostly differentiated to oligodendrocytes.

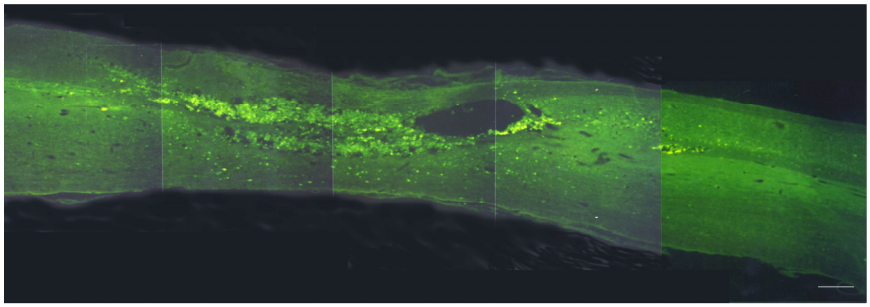

Fig. 8. Immunohistochemistry with BrdU. BrdU-positive cells were revealed

around the injury site and adjacent upper (left) and lower (right) region, which shows well migrated and evenly spread O-2A cells (scale bar: 100 μm).

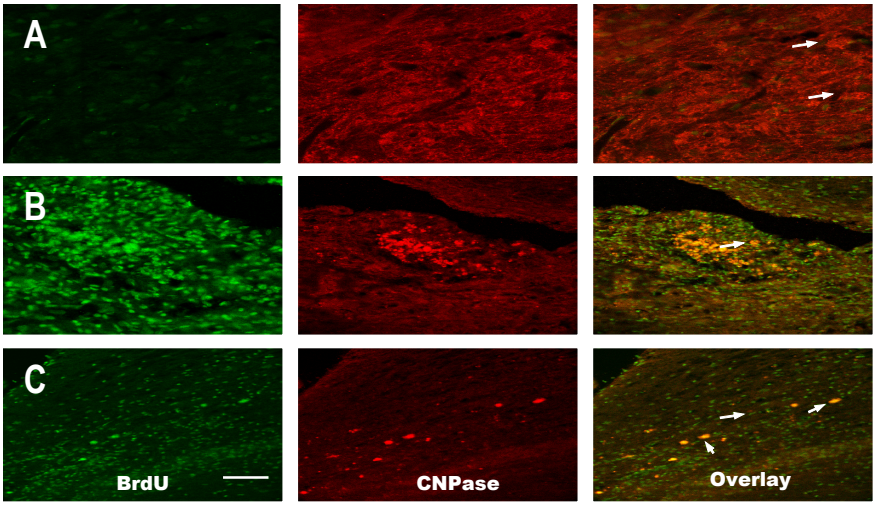

Fig. 9. Double staining of BrdU and CNPase analyzed by confocal microscope. A: Media-treated control group. BrdU and CNPase were seen negative. B: The injection site of O-2A group. Double-stained BrdU-positive cells and also CNPase was seen positive (arrow), indicating that the cells have fell the lesion. C: The adjacent site of injury site of O-2A group. O-2A cell transplantation promotes cell migration and differentiation in oligodendrocytes around adjacent upper and lower sites revealed by double-staining of BrdU

and CNPase.

5. Tract tracing study

1) Anterograde tract tracing

BDA, an anterograde tracer, was used to determine the extent of corticospinal neurons with axon projecting to the spinal cord. The amount of anterogradly labeled process that have absorbed BDA molecules were observed more towards the uninjured site caudal to the injured spinal cord, indicating that the tracer have traversed the demyelinated axons somehow. The amount of BDA-positive processes was observed more from the O-2A cell transplanted group than media treated group (Fig. 10).

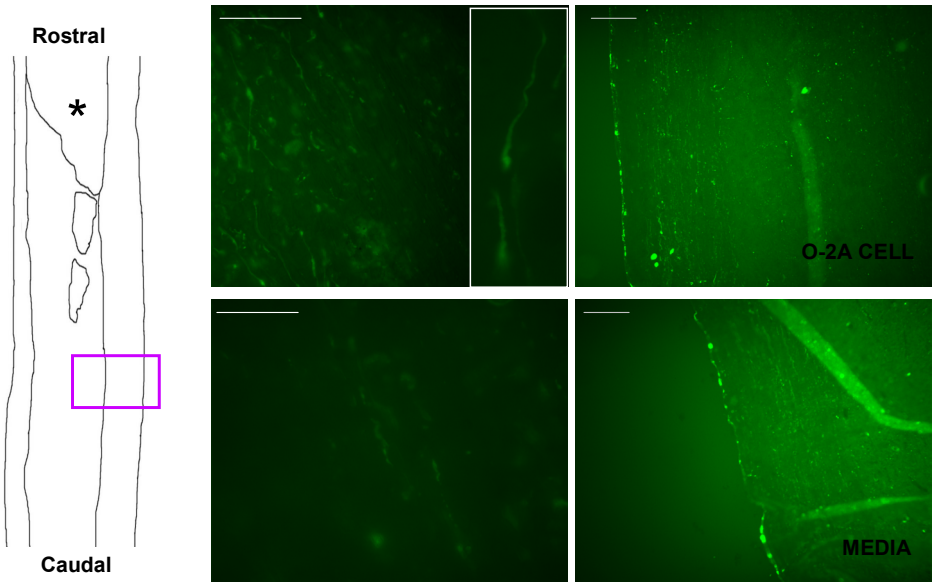

Fig. 10. Anterograde labeling of corticospinal neurons by BDA. Anterogradely labeled processes were observed more towards the uninjured site caudal to the injured spinal cord (* : cavity, scale bar: 100 μm).

2) Retrograde tract tracing

FG was used to determine the extent of supraspinal neurons with axons projecting to the spinal cord across the injured site. The number of retrogradely labeled neurons was determined from the red nuclei and two brainstem nuclei such as the reticular formation and raphe nuclei. Fig. 11

shows representative images of FG-labeled neurons in the nucleis in both experimental (media and O-2A treated) and normal groups. O-2A transplanted group showed an increased amount of FG-positive cells in those three areas of the brain than media-treated group. Fig. 12 shows the quantified results of FG-positive cells in different regions of the brain.

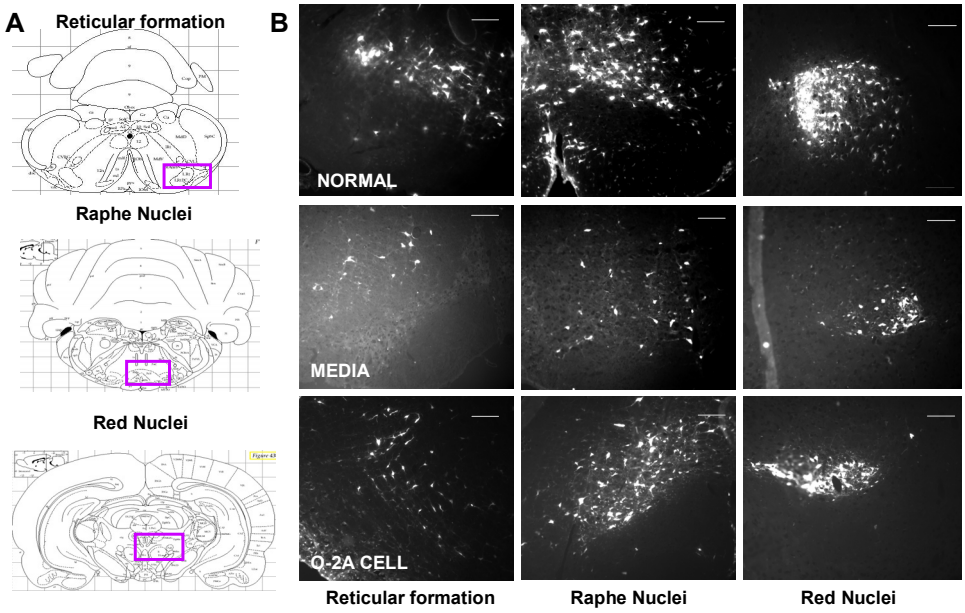

Fig. 11. Retrograde labeling of brain stem neurons by FG. O-2A transplantation promoted axonal regeneration in the different brain stem

regions. The increased amount of FG-positive neurons was seen in O-2A group (scale bar: 100 μm). A: Maps of different parts of brain, B: morphological view of FG stainings for different groups from its corresponding site of the brain.

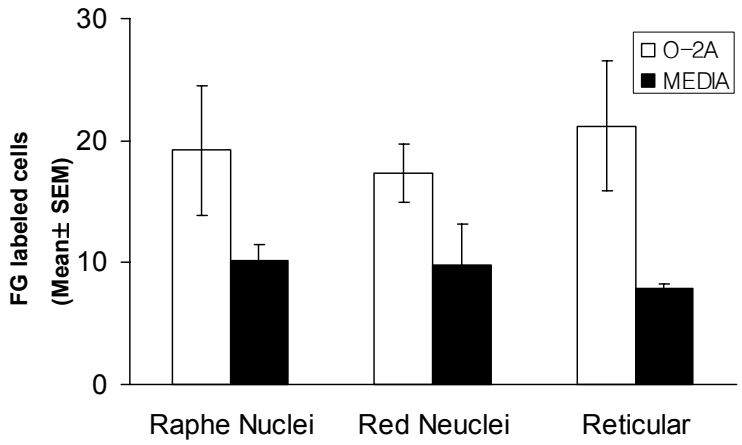

Fig. 12. Quantified FG-positive neurons in different brain stem areas. O-2A cell transplantation after SCI increased FG-labeled neurons in the raphe nucleus, red nucleus and reticular formation.

Ⅳ. DISCUSSION

In the present study, after O-2A progenitor when transplantation into the acute SCI, locomotion recovery by BBB scale was significantly improved compared with media-treated group. The electrophysiological study has shown no significant difference in SSEPs between O-2A and control groups. However, from the MEP recording, the initial, N1- and P1-peak latencies in O-2A group were significantly shorter than media-treated group. The BrdU positive cells were located at the injured and the adjacent sites after O-2A transplantation. Double staining of BrdU and CNPase showed positive results after 2 months of transplantation, whereas double staining of BrdU and GFAP or NeuN was negative. When BDA, an anterograde transport, was injected into the sensorimotor cortex, anterogradly labeled processes were observed in the lower part caudal to the injured site, indicating that BDA traversed the injured spinal cord. Retrogradly labeled neurons in the reticular formation, raphe nucleus and red nucleus increased after O-2A transplantation

compared to media-treated group.

1. Measurement of Locomotion Improvement by BBB

Groves et al.[52] has examined the ability of O-2A cells to promote remyelination in demyelinated spinal cord in x-irradiated model. The present data support the evidence that has been reported by Groves et al.[52] using contused SCI models. A modest but significant improvement in hindlimb performance in the later period of the 9 week endurance in the O-2A transplanted group was observed compared with the media-treated group. It has been shown that it generally takes 5 weeks for the human embryonic stem cells to survive and differentiate into oligodendrocytes, astrocytes and neurons in rat.[12] Similarly the O-2A transplanted group has shown significantly improved hindlimb performance since 6 weeks after the transplantation. It can be assumed that the transplanted cells require 5 to 6 weeks for the migration,

proliferation, and differentiation when it has been transplanted into the right site in vivo. Transplanted O-2A cells may have either newly formed axonal connections or repaired existing connections of the supraspinal neurons with spinal motor neurons that innervate hindlimb muscles.

2. Recovery of Neural Conduction by Axonal Regeneration and Remyelination

Many studies have reported for axonal regeneration and remyelination following various cells transplantation. However, the functional recovery of newly regenerated axons and reestablished myelins could hardly be observed. In the present study, the SSEPs and MEPs were observed by performing electrophysiological study. The results indicate that the latencies of SSEPs, which has been recorded from the sensorimotor cortex following sciatic nerve stimulation, were tend to be lengthened compared to the media-treated group

but the difference was not significant.

In the present study, NYU Impactor was used to produce SCI models. The contusion of spinal cord was performed at dorsal part of the spinal cord (T9). Numerous cell deaths have been shown to occur at the dorsal part of the lesion site. One of the main reasons that SSEPs have not shown significant result is because exogenous oligodendrocyes only assist remyelination of damaged axons. In the injured spinal cord, not only demyelination of neurons have occurred, but neuronal cell death have also occurred, therefore, the improvement of SSEPs have not shown because neurons and axons enough to meet the required amount of myelin sheaths to deliver the action potentials are not present. However, when MEPs were recorded from L1 spinal cord following the motor cortex stimulation, the latencies of the MEPs in O-2A transplanted group were statistically shortened compared with media-treated group. From the results that have been obtained, it can be assumed that the ventral part of the lesion site may have more survived cells and axons even

though they might have been demyelinated. Consequently, regeneration and remyelination of axons in the ventral part of the injured spinal cord might be easier to take place after the transplantation of O-2A cell. Furthermore, transplanted cells, which are BrdU-labeled, have migrated more towards the ventral part of the lesion site in the present study.

According to the rat anatomy, the anterior part of the motor cortex projects to the cervical spinal cord controlling the forelimbs, and the posterior part of the motor cortex projects to the lumbar spinal cord controlling the hindlimbs.[66,67] These electrophysiological findings using MEPs seem to correspond to behavioral data described above.

3. Immunohistochemical Study to Support Survival, Migration, and Differentiation Patterns of Transplanted Cells

The present study has used BrdU to observe migration and differentiation

patterns of transplanted cells. The extent of cells that have settled and survived after the transplantation was determined by observing BrdU-positive neurons, which stains nuclei, in longitudinal sections of spinal cord including the epicenter of injury site 8 weeks after the transplantation. The BrdU-positive cells were observed in injury site and its adjacent sites of the spinal cord. Thus this finding that the BrdU-positive cells have been observed around the spinal cord in the present study may be related to the ability of CNS-derived glial progenitor cells to migrate from the injury site into the adjacent tissue. SCs do not have such migratory ability.[31,61] SCs and OEG cells produce various growth factors such as nerve growth factor,[68] brain-derived neurotrophic factor,[69,70] ciliary neurotrophic factor (CNTF).[70,71,72] and glial cell line-derived neurotrophic factor.[73] Particularly CNTF is a molecule of increasing interest in developmental neurobiology due to its ability to function as a modulator of survival and differentiation of a variety of neurons and of glial cells in the CNS.[74,75,76,77,78,79,80,81,82] The CNTF and a CNTF-like protein

are present in the environment of O-2A progenitor cells isolated from the optic nerve.[75,76] During development of the rat optic nerve, oligodendrocyte precursors appear to originate from the brain and subsequently migrate into the nerve.[74] Therefore in the present study, O-2A cells that has been obtained from the brain might produce supportive factors which may support cell survival and differentiation.

The double staining of BrdU and CNPase was observed from the transplanted and the adjacent sites, indicating that the cells that have been transplanted have well settled and differentiated into oligodendrocytes. Identification of type-2 astrocyte in vivo was not possible to be performed. Kondo et al.[82] shows that oligodendrocyte progenitors isolated from the optic nerve may give rise to neurons. However, the double staining of BrdU and GFAP or NeuN was seen to be negative, indicating that the transplanted cells have not been differentiated into neurons or astrocytes in the present study. Therefore, it can be confirmed that O-2A cells have been differentiated to

oligodendrocytes and migrated to remyelinate the demyelinated axons. At this point, the transplanted cells might have migrated to the adjacent sites of the injury and mostly differentiated into oligodendrocytes.

4. Reestablishment of Ascending or Descending Pathway

According to the present retrograde tracing study, it could be observed that the O-2A cells transplanted animals had more retrogradely labeled neurons in the brain than media-treated control, indicating that more supraspinal axons were survived in the uninjured site caudal to the transplanted area. The regeneration of descending brain stem axons into the spinal cord caudal to the graft site is consistent with other finding.[38] Brain stem neurons in the raphe magnus, reticular formation and red nucleus normally project their axons to the spinal cord.[84] An analysis of the retrograde tracing was performed in more detail and the number of labeled neurons was quantified in

such brain stem nuclei relevant to the initiation and modulation of locomotion. It has been shown previously that hindlimb recovery after an incomplete SCI depends on the number of spared and regenerated descending axons from brain stem nuclei and cerebral cortex[85,86] and from local propriospinal axons.[87,88,89] Interestingly, it appears that only a small percentage of the descending brainstem axons is required to drive the segmental circuits involved in the generation of basic locomotion.[86,89,90] Thus improvement in functional recovery can be explained by the increased amount of descending axons and local propriospinal axons seen in the present study with the evidence of electrophysiological study. Consequently, the improvement in behavioral outcome may be explained, at least in part, by remyelination of the spared axons by the transplanted O-2A cells.

In conclusion, O-2A cells have been shown to migrate and differentiate into oligodendrocytes when transplanted to the animal with SCI. The data suggest that the transplantation of O-2A cells play an important role in functional

recovery and axonal regeneration. The transplantation of O-2A cells with properties to promote remyelination may emerge as one of important candidates for the future cell transplantation strategies in the injured adult spinal cord.

REFERENCES

1. http://www.sci-illinois.org/, Spinal Cord Injury Association of Illinois.
2. Tator CH, Fehlings MG. Review of the secondary injury theory of acute spinal cord trauma with emphasis on vascular mechanisms. J Neurosurg 1991;75:15-26.
3. Beattie MS, Farooqui AA, Bresnahan JC. Review of current evidence for apoptosis after spinal cord injury. J Neurotrauma 2000;17:915-25.
4. Black P, Markowitz RS, Damjanov I, Finkelstein SD, Kushner H, Gillespie J and Feldman M. Models of spinal cord injury: Part 3. Dynamic load technique. Neurosurg 1988;22:51-60.
5. Bresnahan JC, King JS, Martin GF, Yashon D. A neuroanatomical analysis of spinal cord injury in the rhesus monkey. J Neurol Sci 1976;28:521-42.
6. Noble LJ, Wrathall JR. Spinal cord contusion in the rat: morphometric analyses of alterations in the spinal cord. Exp Neurol 1985;88:135-49.
7. Iwashita Y, Kawaguchi S, Murata M. Restoration of function by replacement of spinalcord segments in the rat. Nature 1994;367:167-70.
8. Chen A, Xu XM, Kleitman N, Bunge MB. Methylprednisolone administration improves axonal regeneration into Schwann cell grafts in transected adult rat thoracic spinal cord. Exp Neurol 1996;138:261-76.
9. Grill R, Murai K, Blesch A, Gage FH, Tuszynski MH. Cellular delivery of

neurotrophin-3 promotes corticospinal axonal growth and partial functional recovery after spinal cord injury. J Neurosci 1997;17:5560-72.

10. Li Y, Field PM, Raisman G. Repair of adult rat corticospinal tract by transplants of olfactory ensheating cells. Science 1997;277:2000-2.
11. Liu Y, Kim D, Himes BT, Chow SY, Schallert T, Murray M, Tessler A, Fischer I. Transplants of fibroblasts genetically modified to express BDNF promote regeneration of adult rat rubrospinal axons and recovery of forelimb function. J Neurosci 1999;19:4370-87.
12. McDonald JW, Liu XZ, Qu Y, Liu S, Mickey SK, Turetsky D, Gottlieb DI, Choi DW. Transplanted embryonic stem cells survive, differentiate and promote recovery in injured rat spinal cord. Nat Med 1999;5:1410-12.
13. Ramon-Cueto A. Olfactory ensheathing glia transplantation into the injured spinal cord. Prog Brain Res 2000;128:265-72.
14. Coumans JV, Lin TT, Dai HN, MacArthur L, McAtee M, Nash C, Bregman BS. Axonal regeneration and recovery after complete spinal cord transaction in rats by delayed treatment with transplants and neurotrophins. J Neurosci 2001;21:9334-44.
15. Martin D, Schoenen J, Delree P, Leprince P, Rogister B, Moonen G. Grafts of syngenic cultured, adult dorsal root ganglion-derived Schwann cells to the injured spinal cord of adult rats: preliminary morphological studies. Neurosci Lett 1991;124:44-8.
16. Paino CL, Bunge MB. Induction of axon growth into Schwann cell implants

grafted into lesioned adult rat spinal cord. Exp Neurol 1991;114:254-7.

17. Paino CL, Fernandez-Valle C, Bates ML, Bunge MB. Regrowth of axons in lesioned adult rat spinal cord: promotion by implants of cultured Schwann Cells. J Neurocytol 1994;23:433-52.
18. Blakemore WF, Olby NJ, Franklin RJ. The use of transplanted glial cells to reconstruct glial environments in the CNS. Brain Pathol 1995;5:433-50.
19. Rosenbluth J, Schiff R, Liang WL, Menna G, Young W. Xenotransplantation of transgenic oligodendrocyte-linge cells into spinal cord-injured adult rats. Exp Neurol 1997;147:172-82.
20. Kunkel-Bagden E, Bregman BS. Spinal cord transplants enhance the recovery of locomotor function after spinal cord injury at birth. Exp Brain Res 1990;81:25-34.
21. Bregman BS, Bernstein-Goral H. Both regenerating and late-developing pathways contribute to transplant-induced anatomical plasticity after spinal cord lesions at birth. Exp Neurol 1991;112:49-63.
22. Bernstein-Goral H, Bregman BS. Spinal cord transplants support the regeneration of axotomized neurons after spinal cord lesions at birth: a quantitative double-labeling study. Exp Neurol 1993;123:118-32.
23. David S, Aguayo AJ. Axonal elongation into peripheral nervous system ‘briges’ after central nervous system injury in adult rats. Science 1981;214:931-3.
24. Richardson PM, Issa VM, Aguayo AJ. Regeneration of long spinal axons

in the rats. J Neurocytol 1984;13:165-82.

25. Houle JD. Demonstration of the potential for chronically injured neurons to regenerate axons into intraspinal peripheral ner grafts. Exp Neurol 1991;113:1-9.
26. Cheng H, Cao Y, Olson L. Spinal cord repair in adult paraplegic rats: partial restoration of hind limb function. Science 1996;273:510-3.
27. Kalyani AJ, Piper D, Mujtaba U, Lucero MT, Rao MS. Spinal cord neuronal precursors generate multiple neuronal phenotypes in culture. J Neurosci 1998;18:7856-68.
28. Liu S, Qu Y, Stewart TJ, Howard MJ, Chakrabortty S, Holekamp TF. Embronic stem cells differentiate into oligodendrocytes and myelinate in culture and after spinal cord transplantation. Proc Natl Acad Sci USA 2000;889:344-57.
29. Bunge RP. The role of the Schwann cell in trophic support and regeneration. J Neurol 1994;242:S19-21.
30. Bunge RP, Puckett WR, Hiester ED. Observations on the pathology of several types of human spinal cord injury, with emphasis on the astrocyte response to penetrating injuries. Adv Neurol 1997;72:305-15.
31. Xu XM, Guenard V, Kleitman N, Bunge MB. Axonal regeneration into Schwann cell-seeded guidance channels grafted into transected adult rat spinal cord. J.Comp Neurol 1995;351:145-60.
32. Xu XM, Chen A, Guenard V, Kleitman N, Bunge MB. Bridging Schwann

cell transplants promote axonal regeneration from both the rostral and caudal stumps of transected adult rat spinal cord. J Neurocytol 1997;26:1-16.

33. Xu XM, Zhang SX, Li H, Aebischer P, Bunge MB. Regrowth of axons into the distal spinal cord through a Schwann cell-seeded minichannel implanted into hemisected adult rat spinal cord. Eur J Neurosci 1999;11:1723-40.
34. Xu XM, Guenard V, Kleitman N, Aebischer P, Bunge MB. A combination of BDNF and NT-3 promotes supraspinal axonal regeneration into Schwann cell grafts in adult rat thoracic spinal cord. Exp Neurol 1995;134:261-72.
35. Menei P, Montero-Menei C, Whittemore SR, Bunge RP, Bunge MB. Schwann cells genetically modified to secrete human BDNF promote enhanced axonal regrowth across transected adult rat spinal cord. Eur J Neurosci 1998;10:607-21.
36. Franklin RJM, Barnett SC. Olfactory ensheathing cells and CNS regeneration: the sweet smell of success? Neuron 2000;28:15-8.
37. Kleitman N, Bunge MB. Olfactory ensheathing glia: their application to spinal cord regeneration and remyelination strategies. Topics Spinal Cord Inj Rehab 2000;6:65-81.
38. Ramon-Cueto A, Cordero MI, Santos-Benito FF, Avila J. Functional revovery of paraplegic rats and motor axon regeneration in their spinal cords by olfactory ensheathing glia. Neuron 2000;25:425-35.

39. Plant GW, Ramon-Cueto A, Bunge MB. In: Ingoglia NA, Murray M, editors. Axonal regeneration in the central nervous system. Transplantation of Schwann cells and ensheathing glia to improve regeneration in adult spinal cord. New York: Marcel Dekker;1998. p.529-61.
40. Raisman G. Olfactory ensheathing cells: another miracle cure for spinal cord injury? Nat Neurosci 2001;2:369-73.
41. Li Y, Field PM, Raisman G. Regeneration of adult rat corticospinal axons induced by transplanted olfactory ensheathing cells. J Neurosci 1998;18:10514-24.
42. Ramon-Cueto A, Avila J. Olfactory ensheathing glia: properties and function. Brain Res Bull 1998;46:175-87.
43. Ramon-Cueto A , Plant GW, Avila J, Bunge MB. Long-distance axonal regeneration in the transected adult rat spinal cord is promoted by olfactory ensheathing glia transplants. J Neurosci 1998;18:3803-15.
44. Blackemore WF, Franklin RJ. Transplantation of glia cells into the CNS. Trends Neurosci 1991;14:323-7.
45. Huxley AF, Stampfli R. Evidence for salutatory conduction in peripheral myelinated nerve fibers. J Physiol (Lond) 1949;108:315-39.
46. Waxman SG, Brill MH. Conduction through demyelinated plaques in multiple sclerosis: computer simulations of facilitation by short internodes. J Neurol Neuroscurg Psychiatry 1978;41:408-17.
47. Ritchie JM, Rogart RB. Density of sodium channels in mammalian

myelinated nerve fibers and nature of the axonal membrane under the myelin sheath. Proc Natl Acad Sci USA 1997;74:211-5.

48. Waxman SG. Conduction in myelinated, unmyelinated, and demyelinated fibers. Arch Neurol 1977;34:585-9.
49. Moore JW, Joyner RW, Brill MH, Waxman SG, Najar-Joa M. Simulations of conduction in uniform myelinated fibers: relative sensitivity to changes in nodal and internodal parameters. Biophys J 1978;21:147-61.
50. Hines M, Shrager P. Acomputational test of the requirements for conduction in demyelinated axons. Rest Neurol neurosci 1991;3:81-93.
51. Raff Mc, Miller RH, Noble M. A glial progenitor cell that develops in vcitro into an astrocyte or an oligodendrocyte depending on the culture medium. Nature 1983;303:390-6.
52. Groves AK, Barnett SC, Franklin RJ, Crang AJ, Mayer M, Blakemore WF, Noble M. Repair of demyelinated lesions by transplantation of purified O-2A progenitor cells. Nature 1993;362:453-5.
53. Espinosa de los Monteros A, Zhang M, De Vellis J. O-2A progenitor cells transplanted into the neonatal rat brain develop into oligodendrocytes but not astrocytes. Proc Natl Acad Sci USA 1993;90:50-4.
54. Franklin RJ, Bayley SA, Milner R, French-Constant C, Blakemore WF. Differentiation of the O-2A progenitor cell line CG-4 into oligodendrocytes and astrocytes following transplantation into glia deficient areas of CNS white matter. Glia 1995;13:39-44.

55. Sawamura S, Sawada M, Ito M, Nagatsu T, Nagatus I, Suzumura A, Shibuya M, Sugita K, Marunouchi T. The bipotential glial progenitor cell line can develop into both oligodendrocytes and astrocytes in the mouse forebrain. Neurosci Lett 1995;188:1-4.
56. Dubois-Dalcq M, Armstrong RC. In:Martenson RE, editor. The oligodendrocyte lineage during myelination and remyelination. Myelin: biology and chemistry, Boca, Raton: CRC Press; 1992. p.81-122.
57. Yim SH, Farrer RG, Hammer JA, Yavin E, Quarles RH. Differentiation of oligodendrocytes cultured from developing rat brain is enhanced by exogenous GM3 ganglioside. J Neurosci Res 1994;38:268-81.
58. Yim SH, Farrer RG, Hammer JA, Quarles RH. Expression of glycolipids and myelin-associated glycoprotein during the differentiation of oligodendrocytes: comparison of the CG-4 glial cell line to primary cultures. Dev Neurosci 1995;17:171-80.
59. Power J, Mayer-Proschel M, Smith J, Noble M. Oligodendrocyte precursor cell from different brain regions express divergent properties consistent with the differing time courses of myelination in these regions. Dev Biology 2002;245:362-75.
60. Warrington AE, Barbarese E, Pfeiffer SE. Differential myelinogenic capacity of specific developmental stages of the oligodendrocyte lineage upon transplantation into hypomyelinating hosts. J Neurosci Res 1993;34:1-13.

61. Lachapelle F, Duhamel-Clerin E, Gansmuller A, Baron-Van Evercooren A, Villaroya H, Gumpel M. Transplanted transgenically marked oligodendrocytes survive, migrate and myelinate in the normal mouse brain as they do in the shiverer mouse brain. Eur J Neurosci 1994;6:814-24.
62. Duncan ID, Paino C, Archer DR, Wood PM. Functional capacities of transplanted cell-sorted adult oligodendrocytes. Dev Neurosci 1992;14:114-22.
63. Juurlink BH, Thorburne SK, Devon RM. Protocols for Neural cell culture: Cultures of oligodendroblasts from primary cultures of rat neopallium. 3rd ed. New Jersey: Humana press; 2001, p.149-60.
64. Louis JC, Magal E, Muir D, Manthorphe M, Varon S. CG-4, a new bipotential glial cell line from rat brain, is capable of differentiating in vitro into either mature oligodendrocyte or type-2 astrocyte. J Neurosci Res 1993;31:193-204.
65. Basso DM, Beattie MS, Bresnahan JC. A sensitive and reliable locomotor rating scale for open field testing in rats. J Neurotrauma 1995;12:1-21.
66. McComas AJ, Wilson P. An investigation of pyramidal tract cells in the somatosensory cortex of the rat. J Physiol 1968;194:271-88.
67. Paxinos G. The rat nervous system. San Diego: Academic Press; 1985. p.375-415.
68. Bandtlow CE, Heumann R, Schwab ME, Thoenen H. Cellular localization

of nerve growth factor synthesis by in situ hybridization. EMBO J 1987;6:891-99.

69. Acheson A, Barker PA, Alderson RF, Miller FD, Murphy RA. Detection of brain-derived neurotrophic factor-like activity in fibroblasts and Schwann cells: inhibition by antibodies to NGF. Neuron 1991;7:265-75.
70. Meyer M, Matsuoka I, Wetmore C, Olson L, Thoenen H. Enhanced synthesis of brain-derived neurotrophic factor in the lesioned peripheral nerve: different mechanisms are responsible for the regulation of BDNF and NGF. J Cell Biol 1992;119:45-54.
71. Friedman B, Scherer SS, Rudge JS, Helgren M, Morrisey D, McClain J, Wang DY, Wiegand SJ, Furth ME, Lindsay RM. Regulation of ciliary neurotrophic factor expression in myelin-related Schwann cells in vivo. Neuron 1992;9:295-305.
72. Rende M, Muir D, Ruoslahti E, Hagg T, Varon S, Manthorpe M. Immunolocalization of ciliary neuronotrophic factor in adult rat sciatic nerve. Glia 1992;5:25-32.
73. Widenfalk J, Lundstromer K, Jubran M, Brene S, Olson L. Neurotrophic factors and receptors in the immature and adult spinal cord after mechanical injury or kainic acid. J Neurosci 2001;21:3457-75.
74. Barbin G, Manthorpe M, Varson S. Purification of the chick eye neurotrophic factor. J Neurochem 1984;43:1468-78.
75. Hughes SM, Lillien IE, Raff MC, Rohrer H, Sendtner M. Ciliary

neurotrophic factor injuces type 2 astrocyte differentiation in culture. Nature 1988;335:70-3.

76. Lillien LF, Sendtner M, Rohrer H, Hughes SM, Raff MC. Type 2 astrocyte development in rat brain cultures is initiated by a CNTF-like protein produced by type 1 astrocyte. Neuron 1988;1:485-94.
77. Lillien LF, Sendtner M, Raff MC. Extracellular matrix associated molecules collaborate with ciliary neurotrophic factor to induce type-2 astrocyte development. J Cell Biol 1990;112:635-42.
78. Arakawa Y, Sendtner M, Thoenen M. Survival effect of ciliary neurotrophic factor(CNTF) on chick embryonic motoneurons in culture: comparison with other neurotrophic factors and cytokines. J Neurosci 1990;10:3507-15.
79. Lillien LF, Raff MC. Analysis of the cell-cell interactions that control type 2 astrocye devepopment in vitro. Neuron 1990;4:525-34.
80. Sendtner M, Kreutzberg GW, Thoenen H. Ciliary neurotrophic factor prevents the degeneration of motor neurons after axotomy. Nature 1990;345:440-1.
81. Martinou JC, Martinou I, Kato AC. Cholinergic differentiation factor(CDF/LIF) promotes survival of isolated rat embryonic motoneurons in vitro. Neuron 1992;8:737-44.
82. Barres BA, Schmid R, Raff MC. Multiple extracellular signals are required for long-term oligodendrocyte survival. Development 1993;118:283-95.
83. Kondo T, Raff M. Oligodendrocyte precursor cells reprogrammed to

become multipotential CNS stem cells. Science 2000;289:161-70.

84. Tracey DJ. Ascending and descending pathways in the spinal cord. In: The nervous system, 2 nd ed. San Diego: Academic press; 1995. p.67-80.
85. Saruhashi Y, Young W. Effect of mianserin on locomotory function after thoracic spinal cord hemisection in rats. Exp Neurol 1994;129:207-16.
86. Basso DM, Beattie MS, Bresnahan JC. Graded histological and locomotor outcomes after spinal cord contusion using NYU weight drop device versus transaction. Exp Neurol 1996;139:244-56.
87. Goldberger ME. Partial and complete deaggerentation of cat hindlimb: the contribution of behavioral substitution to recovery of motor function. Exp Brain Res 1988;73:343-53.
88. Goldberger ME. Spared root deafferentation of a cat's hindlimb: hierarchical regulation of pathways mediating recovery of motor behavior. Exp Brain Res 1988;73:329-42.
89. Helgren ME, Goldberger ME. The recovery of postural reflexes and locomotion following low thoracic hemisection in adult cats involves compensation by undamaged primary afferent pathways. Exp Neurol 1993;123:17-34.
90. Ribotta MG, Provencher J, Feraboli-Lohnherr D, Rossignol S, Privat A, Orsal D. Activation of locomotion in adult chronic spinal rats is achieved by transplantation of embryonifc raphe cells reinnervating a precise lumbar level. J Neurosci 2000;20:5144-52.

국문요약

신경아교세포의 이식이 척수손상 후 기능회복에 미치는 효과

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사고 등 가장 흔히 일어나는 척수손상 후 손상된 척수 세포의 재생은 어렵기 때문에 살아남은 일부 신경 세포의 기능회복과 재활에 치료의 관점을 두고 있다. 그러나 환자들이 사지마비와 같은 장애가 없이 회복될 수 있는 근본적인 치료를 위해서는 손상된 척수 신경의 재생이 꼭 필요하다. 신경손상 후 수일 혹은 수개월에 걸쳐 발생하는 이차적인 세포 사멸을 방지하는 약제들이 많이 연구되고 있으나, 현재까지는 임상적으로

효용이 증명되었거나 환자에 치료 목적으로 시도된 예는 거의 없으며, 이러한 약제는 특히 만성 척수손상인 경우에는 전혀 도움이 되지 못한다. 따라서 척수의 이차손상을 근본적으로 막을 수 있는 신경재생을 위한 세포의 이식이 치료의 대안이 될 수 있다. 교통사고 등 대부분의 척수손상과 유사한 아급성 척수손상 모델에서 말초신경계가 아닌 중추신경계의 수초형성에 중요한 역할을 하는 희소돌기아교세포를 이식하여 중추신경계 재수초화에 따른 축삭의 기능회복을 규명한 연구는 거의 없는 실정이다. 본 연구에서는 아급성 척수손상 모델에 희소돌기아교 전구세포를 이식함으로써 재수초화에 따른 축삭의 재생을 유도하여 기능적 측면에서의 전도성 회복을 규명하고자 하였다.

실험동물로는 Sprague-Dawley 종 수컷 흰쥐를 사용하여, pentobabital 로 마취하고, 제 9 흉추에서 척추후궁절제술을 실시하였다. 이 부위에 NYU impactor 를 이용하여 아급성 척수손상 모델을 만들고, 손상 1 주일 후 희소돌기아교 전구세포를 이식하였다. 이식 후 2 개월 동안 운동기능의 회복 정도를 행동검사를 통해 확인하고, 재추초화된 축삭의 전도성 회복을

전기생리학적 방법을 통하여 확인하였다. 또한 이식된 세포의 이동 및 축삭의 재생과 재수초화를 조직검사로 관찰하였다.

행동검사 결과 아급성 모델에서 희소돌기 아교세포 이식 6 주후부터 유의미하게 대조군에 비해 BBB 점수가 향상되었다. 전기생리학적 검사에서 체성감각유발전위 (SSEP)는 세포를 이식한 그룹에서 전도성이 빨라지는 경향성을 보였지만 차이는 없었다. 그러나 운동유발전위(MEP)에서는 초기파에서뿐만 아니라 N1-, P1-peak 에서도 대조군에 비해 전도성이 회복되는 경향을 확인하였다. 또한 조직검사에서도 이식한 세포의 증식 및 이동 정도를 알아보기 위해 BrdU 면역염색을 실시하였는데, BrdU 양성인 세포가 손상부위와 손상부위의 상하 주변부로 이동함을 관찰할 수 있었다. Double 면역염색에서 BrdU 양성이며 성숙한 희소돌기아교 세포의 marker 인 CNPase 에 대해 양성인 세포가 대부분 관찰됨으로써, 대부분의 이식된 세포가 희소돌기아교세포로 분화되었음을 확인하였다.

이상에서 살펴본 바와 같이 척수 손상 후 이식한 희소돌기아교 전구세포가 희소돌기 아교세포로 분화하며, 재수초화에 따른 축삭의 재생을 유도하여 기능적 측면에서의 운동성과 전도성 회복을 확인하였다. 따라서 척수손상 후 세포이식을 이용한 신경 재생 연구 즉, 손상된 신경 축삭돌기의 재생, 재신경지배 (reinnervation), 축삭돌기의 재수초화 등을 통한 운동 및 감각 기능의 회복은 일상 장애에 작동될 경우 척수손상 환자의 삶의 질을 크게 향상시킬 수 있을 것이며, 다른 난치성 척수신경 질환에 대한 근본적인 치료 가능성을 제시할 수 있을 것으로 사료된다.

핵심되는 말: 척수손상, O-2A, 세포이식, 행동검사, 전기생리학적 검사, 형태학적 검사