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障害者対策総合研究事業

移植治療後の慢性期完全脊髄損傷患者のリハビリテーションと脳機能再構成および脊髄再生との関連性についての評価法の開発

平成 25 年度　総括研究報告書

研究代表者　岩月　幸一

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研究代表者　岩月 幸一　大阪大学大学院　医学系研究科　講師

［研究要旨］

慢性期脊髄損傷に対する有効な神経再生療法は未だ無く、残存機能の強化リハビリテーションが現在唯一の治療法とされている。当グループは損傷後半年以上経過した慢性期完全脊髄損傷患者に対して自家嗅粘膜移植を行い、一定の機能回復を見ている。慢性期では下肢筋肉の萎縮による神経栄養因子の枯渇から脊髄前角細胞の変性･下位運動神経の不全が起こり、上位の脊髄神経軸索の再生のみでは十分な機能回復は得られないことが考えられる。よって自家嗅粘膜移植後の効果的なリハビリテーションプログラムを開発する必要性があり、そのために脊髄における軸索再生や脳の神経活動の機能的回復または変化を術前、術後にわたり、経時的に評価する必要性がある。

本研究では、①術前にリハビリテーションを行い、筋肉由来神経栄養因子の産生と下位運動神経の維持を図る、②自家嗅粘膜移植後の脊髄神経軸索の再生、③術後のバイオフィードバックを用いた随意的筋放電の誘発、④長下肢装具およびロボットスーツ HAL 装着による積極的歩行訓練、の一連のプログラムにより、効率的機能再建を目標とする。さらに DTI(Diffusion Tensor Imaging)による損傷脊髄移植部位の軸索再生の可視化、および脳 fMRI による脳神経活動の再構築により機能回復プロセスの客観的指標の開発を目指す。

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A.研究目的

慢性期脊髄損傷に対する有効な神経再生療法は未だ無く、完全脊髄損傷患者においては残存機能の強化リハビリテーションが唯一の治療法となっている。当グループは損傷後１年以上経過した慢性期完全脊髄損傷患者に対して自家嗅粘膜移植を行い、一定の機能回復を見ているが、慢性期では下肢筋肉の萎縮による神経栄養因子の枯渇から脊髄前角細胞の変性･下位運動神経の不全がおこり、上位の脊髄軸索再生のみでは十分な機能回復は得られないことが示唆される。また効果的なリハビリテーションプログラム開発には、脊髄の軸索再生や脳の神経活動の機能的回復を経時的に評価する必要がある。

本申請では慢性期完全脊髄損傷患者に自家嗅粘膜移植を行い、その術前術後に積極的リハビリテーションを導入し、より効率的な下肢機能回復を目指すことを目的とする。

B.研究方法

本研究では機能保存的リハビリテーション･脊髄神経再生･脳神経機能の変化の観点から、下記６つの工程を設ける。

①術前に廃用下肢筋のリハビリテーションにより、筋肉由来神経栄養因子の産生と下位運動神経の維持を図る。②自家嗅粘膜移植による脊髄神経軸索の再生。③術後のバイオフィードバックを用いた随意的筋放電の誘発。④長下肢装具装着による積極的歩行訓練。さらに、これら機能回復のプロセスの客観的指標として、下肢運動指標に加えて、新たに⑤DTI(diffusion tensor imaging)で損傷脊髄移植部位の軸索再生を可視化する。⑥脳 fMRI で脳神経活動の再構築を解明する。

（倫理面への配慮）

本研究は【ヘルシンキ宣言】【臨床研究に関する倫理指針】ならびに本臨床研究実施計画書および同意説明文書を尊守して実施している。

①同意説明と同意取得

研究責任医師等は治療に先立ち、未来医療臨床研究審査･評価委員会の承認を得た同意説明文書を用いて文書による同意を得る。同意取得のため研究責任医師等は、治療への参加に関し、被験者に強制するなど不当な影響を及ぼすことのないよう留意する。

本臨床研究への参加は被験者本人の自由意志による同意を、同意書に署名または記名･捺印し、日付を自ら記入することにより取得する。同意取得後、同意書の写し及び同意説明文書を同意者本人に交付する。

②同意の撤回

一旦書面による同意を行った被験者であっても、嗅粘膜移植術実施前であればいつでも撤回できる。

③臨床研究内容の開示

同意説明を行った患者、または被験者に本臨床研究実施計画書の開示を要求されれば、それに応じるものとする。

④同意書及び同意説明文書の改訂

研究責任医師等は、研究に継続して参加するか否かについて被験者の意思に影響を与える可能性のある情報や、被験者の同意に関連しうる新たな情報を入手した場合には、当該情報を直ちに口頭で被験者に伝える。また、情報提供した旨を診療録に記録し、被験者が研究に継続して参加するか否かを確認する。被験者が未成年の場合は、同時に法定代理人に対してもこれを行う。

C.研究結果

2014年4月30日までに6例の自家嗅粘膜移植術を実施した。うち3例に術後リハビリテーション6ヶ月後から、下肢筋群に随意性の筋電図の発現を認め、運動機能の回復を認めている。うち1名は杖及び短下肢装具使用ながら、500m以上の歩行が可能となっている。また本症例においては、MEP(motor evoked potential)の発現を下肢筋に認めており、これは神経経路の接続を電気生理学的に証明し得たものである。慢性期完全脊髄損傷において、これを確認し得たことは、国際的にも脊髄損傷の医学史上初めてのことである。また本症例においては、脳の運動領野の下肢相当領域の拡大が認められた。他の2例においては未だ実用的ではない。その他の症例においては、体幹の支持性向上によるADLの改善が見られている。全例において、感覚および膀胱直腸障害の回復は認めていない。

回復が得られなかった1例目は術後半年でリハビリを休止、また2例目は随意性筋電図を確認できたものの、規定されていた術後1年でリハビリを中止している。3例目は損傷部位が腰膨大にあり、他症例で認められた腱反射の亢進や不随意運動は認められなかった。4症例目はリハビリテーションの継続とともに回復が得られている。5、6症例目はまだ術後半年であるが、6症例目からは随意性の筋電図が得られている。

D.考察

バイオフィードバックを利用したリハビリテーションの継続により、良好な結果が得られる症例が出ている。しかし腰膨大に存在する運動神経の健常性を示すとされる不随意運動が見られないもしくは弱い症例では、回復が思わしくない。回復が認められた症例においては、脳機能の変化も認められた。

E.結論

自家嗅粘膜移植法による慢性期脊髄完全損傷患者に、一定の回復が得られつつある。同時に

本移植療法の適応症が徐々に明らかになっており、リハビリテーションの継続の重要性も明らかとなっている。

F. 健康危険情報

実施した 6 例において、これまで当研究と関連があると判断される感染症、悪性新生物の発生を認めていない。有害事象として嗅覚低下や、頭痛および脊損領域の痛みが出現した症例もあるが、いずれも術後短期間に解消している。

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Involuntary muscle spasm expressed as motor evoked potential after olfactory mucosa autograft in patients with chronic spinal cord injury and complete paraplegia

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ABSTRACT

Object: The efficacy of olfactory mucosa autograft (OMA) for chronic spinal cord injury has been reported. New activity in response to voluntary effort has been documented by electromyography (EMG), but the emergence of motor evoked potential (MEP) reflecting electrophysiological conductivity in the central nervous system, including the corticospinal pathway, after OMA, and the best indications for OMA, have not been clarified. Here, we report the emergence of MEPs after OMA and offer recommendations for appropriate indications based on the presence of involuntary muscle spasm (IMS). We used analysis of MEP to examine the efficacy of OMA for patients with complete paraplegia due to chronic spinal cord injury. To clarify the indications for OMA, we investigated the association of IMS and efficacy of OMA. Methods: Four patients, 3 men and 1 woman, were enrolled. The mean age of the cases was 30.3 ± 9.5 years (range, 19 to 40 years). All 4 cases were American Spinal Injury Association (ASISA) grade A. The mean duration from injury to OMA was 95.8 ± 68.2 months (range, 17 to 300 months). Samples of olfactory mucosa were removed, cut into smaller pieces, and grafted into the sites of spinal cord lesions after laminectomy. Postoperative subcutaneous fluid collection, postoperative meningitis, postoperative nosebleed, postoperative infection in the nasal cavity, impaired olfaction, neoplastic tissue overgrowth at the autograft site, new sensory disturbance, and involuntary muscle spasm were investigated as safety issues. Improvements in ASIA grade, variations in ASIA scores, EMG, SSEP, and improved urological function were evaluated as efficacy indicators. Results: There were no serious adverse events in this series. In 2 of the 4 cases, an improvement in motor function below the level of injury was recognized. In one, the motor score was 50 until 16 weeks after surgery, and it increased to 52 from 20 weeks after surgery. In the other, the motor score was 50 until 20 weeks after surgery, and it increased to 52 at 24 weeks after surgery with a further increase to 54 at 48 weeks after surgery. The emergence of MEP was recognized in the latter case at 96 weeks after surgery. The other 2 cases had no improvement in ASIA motor score. Both of these cases who showed improvements in the ASIA motor scores exhibited relative IMS compared with those who had no ASIA motor score recovery. Conclusions: We recognized the emergence of MEPs in a case with complete paraplegia due to chronic spinal cord injury after OMA. IMS might be a candidate of indication of OMA.

Keywords: Olfactory Mucosa Autograft; Spinal Cord Injury; Transplantation; Voluntary Movement; Motor Evoked Potential

1. INTRODUCTION

The olfactory mucosa is an excellent autologous source of adult neuronal precursor cells. The neurons and sustentacular cells of the olfactory mucosa constantly renew themselves throughout life by proliferation of basal global stem cells [1-3]. Furthermore, the mucosa contains olfactory ensheathing cells, which have previously received much attention for their potential application in

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the repair of spinal cord injuries (SCIs) [4-7]. Recent studies of spinal cord axon regeneration have reported good long-term results using various types of tissue scaffolds [8-10]. Olfactory tissue, which allows autologous transplantation, is easily accessible, and can be obtained by a simple biopsy performed through the external nares [11].

We have previously reported that grafts of the olfactory mucosa are effective in restoring functional recovery in rats following spinal cord transection, with histological evidence of neuronal regeneration [12-14]. Lima *et al.* performed a clinical trial of olfactory mucosal autograft (OMA) in humans with chronic traumatic SCI and reported restoration of voluntary electromyography (EMG) responses in 15 of 20 cases (75%) and mean American Spinal Injury Association (ASIA) motor score improvement of 4.95 ± 7.1 over a mean follow-up period of 27.7 months. The range of improvement was various, and some cases demonstrated no response to OMA. Therefore, evaluating the possible factors that could predict the efficacy of OMA will be useful. Inclusion criteria for the study by Lima *et al.* included age, extent of the lesion, and time from the injury, but the authors did not assess the neuronal condition of the severed caudal spinal cord [15]. Furthermore, they did not assess the emergence of motor evoked potential (MEP), which reflects electrophysiological conductivity in the central nervous system, including the corticospinal pathway [16,17], after OMA.

The emergence of involuntary muscle spasm (IMS) after SCI is an indirect measure of the recovery of motor neurons and general motor function [18]. It may indicate plasticity of the spinal cord and the potential for successful regenerative interventions in patients with chronic SCI.

Our pilot study was conducted to examine the emergence of MEP and IMS in OMA patients with chronic SCI and complete paraplegia.

2. MATERIAL & METHODS

2.1. Patient Selection and Inclusion Criteria

This phase I/II nonrandomized, non-controlled, prospective study was approved by the Ethical Committee of the Osaka University Medical School in Osaka, Japan. All procedures were performed after obtaining written informed consent, which included permission to culture and analyze a biopsy from the tissue to be grafted. Patients were fully aware of the experimental nature of the treatment, the uncertain outcomes, and possible side effects including pain, spasticity, autonomic dysreflexia, worsening of motor or sensory function, infection, and unforeseen adverse events.

Patients who had sustained SCI more than 6 months previously with chronic paraplegia (**Table 1**) were included. Our rationale for selecting chronic SCI patients (more than 6 months from injury) was to circumvent the spontaneous recovery bias [19]. The other inclusion criteria of this study were generally consistent with those of Lima *et al.* [15] and comprised ASIA Ggrade A or B; age ≥ 7 and ≤ 40 years; presence of a spinal cord lesion ≤ 3 cm; absence of significant nasal and paranasal sinus pathology; and absence of additional serious medical problems including respiratory disturbance, brain disease, or psychological disturbance.

Four patients were enrolled in the study, 3 males and 1 female. Demographic data, clinical findings, and imaging/radiological characteristics of the patients are presented in **Table 1**. The mean age of the patients was 30.3 ± 9.5 years (range, 19 to 40 years). Injuries were due to traffic accidents in 2 patients, fall in 1 patient, and hemorrhage of unknown origin in 1 patient. The mean maximum lesion size on the vertical axis as measured on both the T1- and T2-weighted MRI was 2.25 ± 0.57 cm (range, 1.55 to 2.94 cm). All 4 patients were ASISA grade A. The mean time from injury to OMA was 95. 8 ± 68.2 months (range, 17 to 300 months).

2.2. Transplantation Protocol and Surgical Procedure

Our procedure essentially followed that reported by Lima *et al.* [15,20]. Samples of olfactory mucosa were removed, cut into smaller pieces, and grafted into the spinal cord lesion site after laminectomy. Microbiological examinations of the nasal cavities were performed routinely before surgery and during the operation just prior to transplantation.

Table 1. Summary of demographic and clinical characteristics of 4 patients with olfactory mucosa autografts (OMA).

Case No. (years)	Age at OMA	Sex	Months Post-SCI	SCI Level	Length of Lesion	AIS Grade
1	40	Male	300	T4-5	2.2	A
2	19	Female	30	T7-9	2.3	A
3	26	Male	17	T12	1.55	A
4	36	Male	36	T7-8	2.94	A

Abbreviations: SCI, spinal cord injury; T, thoracic; AIS, ASIA Impairment Scale.

2.3. Pre- and Postoperative Rehabilitation

All patients underwent preoperative rehabilitation (15 h/week for 4 weeks) and postoperative rehabilitation (15 h/week for 48 weeks). The preoperative rehabilitation was carried out until immediately prior to the operation and baseline parameters were determined after the preoperative rehabilitation in order to confirm stabilized neurological status. Rehabilitation included standard physical therapy strategies encouraging motor function at and below the lesion to facilitate walking training as soon as possible.

2.4. Outcome Measures

Safety and efficacy measures are presented in **Table 2**. Any improvement in the ASIA grade scale or/and lower extremity motor scores was considered evidence of true gains since all patients had ASIA motor scores of 0 for both legs after the preoperative rehabilitation. The pre- and postoperative assessment protocol included an ASIA neurological exam, as described in the International Standards for Neurological and Functional Classification of Spinal Cord Injury Patients [21] as well as standard EMG, with recordings taken after patients were asked to move particular muscles, and somatosensory evoked potentials (SSEP), cortically recorded after tibial nerve stimulation; urodynamic studies; full spinal cord MRI scan; otolaryngological evaluation including a general ear, nose, and throat examination, nasal endoscopy, olfactory evaluation, and computed tomography scan of the nose and paranasal sinuses; and psychological assessment. Psychological testing was intended to detect conditions such as active psychosis, major depression, anxiety disorder, severe mood disorder, suicidal behavior, alcohol addiction, drug addiction, low cognitive resources, and unrealistic expectations about treatment results. Pain was assessed via interviews asking the patients to identify painful areas, describe the pain using standard descriptors, and identify temporal aspects of the pain.

Table 2. Outcome measures.

Safety Measures
Postoperative subcutaneous fluid collection
Postoperative meningitis
Postoperative nasal bleeding
Postoperative infection in the nasal cavity
Impaired olfaction
Neoplastic tissue overgrowth in the transplantation
New sensory disturbance
Involuntary muscle spasm (IMS)
Efficacy Measures
Ability to improve ASIA grade
Variation in ASIA scores
EMG
SSEP
Urological improvement

EMG: Electromyograph; SSEP: Somatosensory Evoked Potential.

We evaluated IMS. There is a variety of tests that attempt to quantify spasticity, but there is no uniformly accepted useful measure [22,23]. Our method was simply to note the emergence of IMS on EMG. IMS was evaluated in the bilateral biceps femoris, anterior tibial, flexor digitorum brevis, femoral quadriceps, gluteus maximus, and gastrocnemius muscles. We watched the emergence of IMS while patients rested in the supine position for 3 min (**Figure 1**).

MEP response to bifocal transcranial magnetic stimulation (TMS) was evaluated in the bilateral rectus femoris muscles. TMS was performed with a coil (7 cm diameter) using a MagPro ×100 (MagVenture A/S, Denmark). Navigation-guided TMS (Brainsight Frameless 1.5; Rogue Research Inc., Montreal, Canada) was used to target the optimal position of each stimulation point. The stimulation hot spot was determined starting about 4 cm rostral of Cz (vertex) [24]. Patients who were not able to produce force were asked to exert as much volitional innervation as possible. The duration of the monophasic transcranial single-pulse stimulus was 100 μs. The sample frequency was 2000 Hz, and a band-pass filter was set at 30 Hz to 1 kHz. TMS was delivered every 5 to 6 s. Three [25] to 5 representative MEPs at the desired stimulus intensity were applied if there was a well-defined response, and up to 10 stimuli were delivered if there was a visible but poorly defined muscle response in order to optimize 3 responses to be stored offline for further analysis [26]. The onset of the fastest response from 4 repeated MEP trials was determined to be the onset latency. The MEP amplitude was calculated from baseline to the negative peak for the largest response out of 4 trials. The intensity of the magnetic stimulus was expressed as a percentage of the maximal stimulator output.

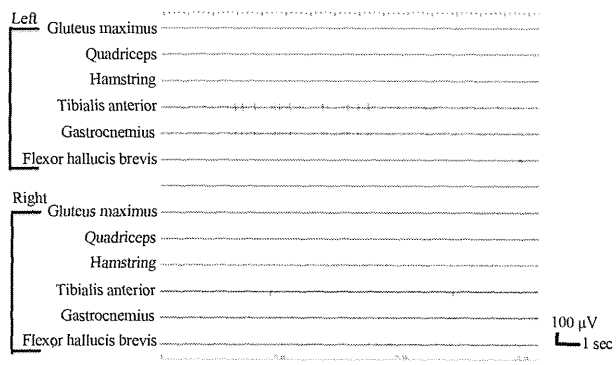

Figure 1. Involuntary muscle spasms were recorded in the left tibialis anterior.

The patients' neurological findings were evaluated preoperatively and 4, 12, 24, 36, and 48 weeks after OMA (MEP was evaluated additionally at 96 weeks).

3. RESULTS

3.1. Safety

No serious adverse event occurred in our series. There was no formation of subcutaneous collection of cerebrospinal fluid along the incision in any case. Two cases had postoperative nosebleed treated with tampon gauze and controlled within 7 days. All cases reported impaired sense of smell. Two regained smell within 12 weeks, and the others regained smell within 48 weeks without any further treatments. No case developed meningitis, nasal infection, or neoplasm after surgery. One case reported transient sensory disturbance consisting of pain around the level of injury (Th4 level) at evaluations both 4 and 12 weeks post-surgery. This disturbance resolved spontaneously without any treatment.

3.2. Efficacy

3.2.1. ASIA Scoring Assessments (Motor)

The data obtained for ASIA motor scores are summarized in **Figure 2**. No change in ASIA motor score was observed in cases 1 or 3, but cases 2 and 4 both demonstrated improved motor function below the level of injury. In case 2, the motor score remained at 50 until 16 weeks after surgery and then increased to 52 from 20 weeks until 48 weeks. In case 4, the motor score was 50 until 20 weeks after surgery and then it increased to 52 at 24 weeks after surgery and further increased to 54 at 48 weeks after surgery.

3.2.2. ASIA Scoring Assessments (Sensory: Pinprick and Light Touch Scores)

No remarkable changes were observed except in case 1, in whom the score decreased after surgery, from 17 to 2 in response to pinprick and from 15 to 2 in response to light touch.

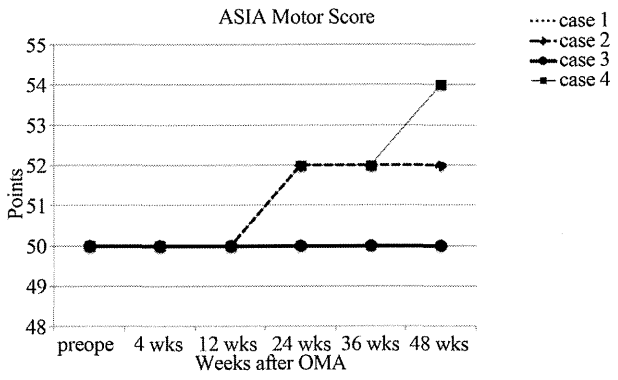

Figure 2. ASIA motor scores. No change in ASIA motor scores was observed in cases 1 and 3, but the score improved from 50 to 52 in case 2 and from 50 to 52 and ultimately 54 in case 4.

3.2.3. Electrophysiological Assessment

1) EMG assessment (voluntary movement)

New voluntary activity in response to voluntary effort was documented by EMG at 48 weeks after surgery in cases 2 and 4. In case 2, the activity was recognized in the bilateral tensor fascia lata muscles. In case 4, the activity was recognized in the bilateral hamstring, anterior tibial, femoral quadriceps, gluteus maximus, and gastrocnemius muscles (**Figure 3**). There was no activity in response to voluntary effort shown in cases 1 or 3.

2) SSEP assessment (somatosensory evoked potential)

There were no changes in SSEPs arising from tibial nerve stimulation recorded at the cortical level in any case.

3.3. Urodynamic Studies

No case experienced urge to urinate before or after OMA, and all cases remained unable to urinate by themselves.

3.4. MRI Findings

MRI 48 weeks after transplantation revealed fairly complete filling of cavities and heterogeneous intensities on T1- and T2-weighted images. Gd-enhanced MRI also showed that the grafts were enhanced heterogeneously. No evidence of neoplastic tissue overgrowth was observed in any case (**Figure 4**).

3.5. Involuntary Muscle Spasm

The evaluation for IMS in each case is documented in **Figure 5**. In case 3, IMS was not recognized preoperatively or throughout the follow-up period to 48 weeks after OMA (**Figure 5**). By contrast, in case 4, IMS was recognized preoperatively and throughout the follow-up period to 48 weeks after OMA (**Figure 5**). Each IMS emergence was calculated as 1 point, as summarized in **Figure 6**. At the beginning of the follow-up period, case 1 had more IMS points than case 2, but this difference reversed in the middle and latter half of the follow-up period. Thus, we concluded that that IMS could be recognized in cases 2 and 4 rather than the cases 1 and or 3.

3.6. Motor Evoked Potentials

MEPs were not observed before transplantation in any case, but they were recorded at 96 weeks after OMA in case 4 (**Figure 7**), although not in any other case.

4. DISCUSSION

The information about OMA derived from the studies of

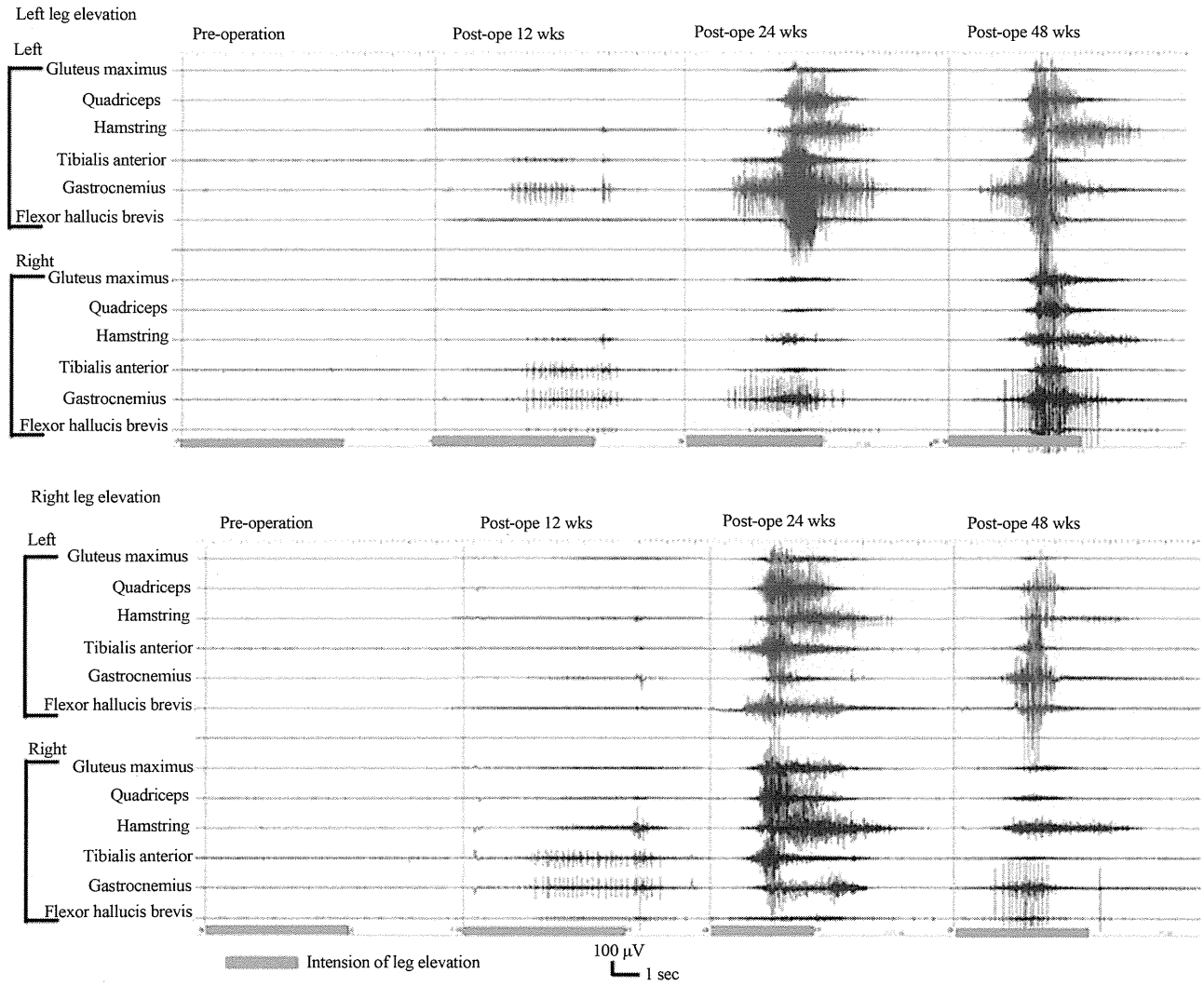

Figure 3. EMG in case 4. New EMG activity in response to voluntary effort was documented at 12, 24, 48 weeks after transplantation. The activity was recognized in the bilateral gluteus maximus, quadriceps, biceps femoris, anterior tibial, gastrocnemius, and abductor hallucis muscles.

Lima *et al.* is invaluable to basic and clinical researchers investigating regeneration in chronic SCI. Their pioneering work in this field revealed that OMA is fairly safe, feasible, and potentially beneficial [15,20]. OMA is advantageous in that it involves transplantation of whole tissue rich in factors that may facilitate neuronal regeneration. We have performed further basic studies of olfactory mucosa transplantation in rats that have supported its feasibility [12,13].

Spinal cord reconstruction using implantation of cells from various sources has gained attention in recent years [27,28]. Neuronal stem cells (NSCs) have the potential to differentiate into both neuronal and glial cells, and are therefore prime candidates for cell replacement therapy following CNS injury. NSCs constitutively secrete significant quantities of several neurotrophic factors that act to support host axonal regeneration after SCI [29]. Partial restoration of function after spinal cord contusion has been achieved by injecting neural/glial precursor NSCs differentiated *in vitro* from mouse embryonic stem cells into the lesion 9 days after injury [30]. However, implantation of NSCs alone did not produce any significant restorative effect because the majority of the NSCs grafted into the spinal cord differentiated into an astrocytic phenotype [29,31]. Although astrocytes can secrete neurotrophic factors and limit the extent of the inflammatory reaction, extensive astroglial scarring within the lesioned area blocks axon growth.

One of the major disadvantages associated with implantation or injection of cells alone is the limited proportion of viable cells surviving at the injury site after the procedure, as cells tend to migrate away from the injury site [32]. To achieve significant functional reconstruction of the spinal cord after SCI, it is necessary to

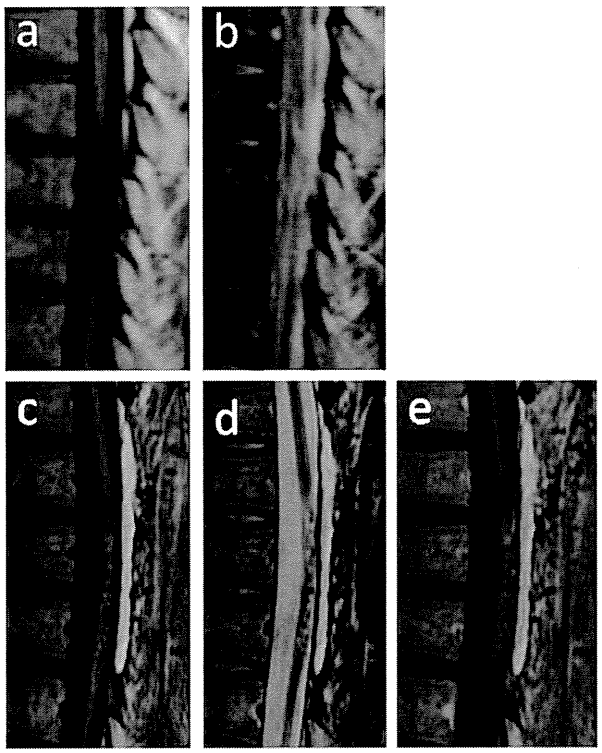

Figure 4. MRI findings in case 2. T1-weighted sagittal image before transplantation showed atrophic change of the thoracic spinal cord (a); T2-weighted sagittal image before transplantation showed an intramedullary high-intensity area (b); MRI scans at 48 weeks after transplantation showed fairly complete filling of cavities and heterogeneous intensity on T1- (c) and T2-weighted images (d); Gd-enhanced MRI showed that the grafts were enhanced heterogeneously (e). No evidence of neoplastic tissue overgrowth was observed.

either populate lesion sites with tissue-specific, regeneration-competent cells or activate endogenous neural progenitor cells to replace or rescue dying cells [33]. The olfactory mucosa seems to be an excellent autologous source of adult neuronal precursor cells. It provides an accessible site for sample biopsy [11] and it contains neurons and sustentacular cells that renew themselves throughout life [1-3] as well as olfactory ensheathing cells that have shown promise in the repair of SCIs [4-7]. These considerations make the nasal mucosa an attractive tissue for potential applications in axonal regeneration. However, while olfactory mucosa may be an ideal tissue for chronic SCI, whether all chronic SCI patients are candidates for OMA remains unclear. Lima *et al.* applied the following inclusion criteria: ASIA impairment grade A or B [34]; age 18 to 40 years; cervical spinal cord lesion below 3 cm or thoracic spinal cord lesion below 4 cm; absence of significant nasal and paranasal sinus pathology; and absence of additional serious medical problems, brain disease, or psychological disturbance [20]. Our present clinical trial generally followed these inclusion criteria and additionally investigated IMS. The emergence of IMS after SCI is an indirect measure of recovery of motor neurons and general motor function [18], and it may function as an indicator for the potential success of regeneration therapy in chronic SCI.

In considering the implications of IMS after SCI in our cases, improved motor function below the level of injury was observed in cases 2 and 4, both of whom exhibited relative IMS before OMA and during rehabilitation after OMA. However, no improvement in motor function was observed in cases 1 and or 3, and neither of them had exhibited relative IMS before OMA or during rehabilitation after OMA (**Figures 5** and **6**). In particular, in case 4, IMS was consistently observed during rehabilitation both before and after OMA (**Figure 6**), and new voluntary muscle activity was recognized in the bilateral hamstring, anterior tibial, femoral quadriceps, gluteus maximus, and gastrocnemius muscles (**Figures 2** and **3**). By contrast, in case 3, no IMS was observed during rehabilitation before or after OMA (**Figures 5** and **6**), and no new voluntary muscle activity was observed (**Figure 2**). The level of injury in this case was Th12. There are motor neurons to generate lower leg movement. These motor neurons were directly injured, and we might not have expected new voluntary muscle activity in such a case. Furthermore, the spinal cord at Th12 is lumbosacral spinal cord, where a central pattern generator (CPG) might exist [4]. The presence of CPG circuitry is thought to be responsible for generation of rhythmic activity within the lumbar cord isolated from brain influence. However, in the absence of descending brain control involved in the initiation of locomotion, CPG activity will be induced by activating afferents from muscles, tendons, and joints by means of peripheral afferent feedback [35]. The CPG in this case might have been injured, and CPG activity might not have been induced by gait training as a peripheral afferent feedback.

We were able to elicit MEPs in case 4. The MEP reflects conductivity in the central nervous system, including the corticospinal pathway [16,17]. MEP induced with transcranial magnetic stimulation allows objective assessment of the integrity of human motor circuitry comprising both the corticospinal tract and peripheral motor nerves [26,36]. The emergence of MEP in case 4 makes this the first report to indicate the recovery of electrophysiological conductivity after complete chronic SCI by any treatment.

In attempts to perform neuronal repair after SCI, strategies to improve the local environment to promote new neuron formation are essential. However, the condition of motor neurons remaining in the spinal cord should also be considered. Patients may not succeed with OMA without functional motor neurons, and the recov

Case 1

		Preope	4 wks	12 wks	24 wks	36 wks	48 wks
Biceps femoris muscle (L5-S2)	Lt				■		
	Rt	■					
Anterior tibial muscle (L4,5)	Lt	■				■	
	Rt			■	■	■	
Flexor digitorum brevis muscle (L5,S1)	Lt		■		■	■	■
	Rt	■	■	■	■	■	■
Femoral quadriceps muscle (L1-3)	Lt	■	■		■		
	Rt	■			■		
Gluteus maximus muscle (L5-S2)	Lt		■				
	Rt						
Gastrocnemius muscle (S1,2)	Lt	■	■			■	
	Rt	■	■	■	■	■	

Case 2

		Preope	4 wks	12 wks	24 wks	36 wks	48 wks
Biceps femoris muscle (L5-S2)	Lt				■		
	Rt	■	■		■	■	
Anterior tibial muscle (L4,5)	Lt			■	■	■	■
	Rt	■	■	■	■	■	■
Flexor digitorum brevis muscle (L5,S1)	Lt			■	■	■	■
	Rt	■	■	■	■	■	■
Femoral quadriceps muscle (L1-3)	Lt	■				■	
	Rt				■	■	
Gluteus maximus muscle (L5-S2)	Lt						
	Rt		■		■		
Gastrocnemius muscle (S1,2)	Lt			■	■	■	■
	Rt	■	■	■	■	■	■

Case 3

		Preope	4 wks	12 wks	24 wks	36 wks	48 wks
Biceps femoris muscle (L5-S2)	Lt						
	Rt						
Anterior tibial muscle (L4,5)	Lt						
	Rt						
Flexor digitorum brevis muscle (L5,S1)	Lt						
	Rt						
Femoral quadriceps muscle (L1-3)	Lt						
	Rt						
Gluteus maximus muscle (L5-S2)	Lt						
	Rt						
Gastrocnemius muscle (S1,2)	Lt						
	Rt						

Case 4

		Preope	4 wks	12 wks	24 wks	36 wks	48 wks
Biceps femoris muscle (L5-S2)	Lt	■	■	■	■	■	■
	Rt	■	■	■	■	■	■
Anterior tibial muscle (L4,5)	Lt	■	■	■	■	■	■
	Rt	■	■	■	■	■	■
Flexor digitorum brevis muscle (L5,S1)	Lt	■	■	■	■	■	■
	Rt	■	■	■	■	■	■
Femoral quadriceps muscle (L1-3)	Lt	■	■	■	■	■	■
	Rt	■	■	■	■	■	■
Gluteus maximus muscle (L5-S2)	Lt	■	■	■	■	■	■
	Rt	■	■	■	■	■	■
Gastrocnemius muscle (S1,2)	Lt	■	■	■	■	■	■
	Rt	■	■	■	■	■	■

Figure 5. Evaluation for emergence of involuntary muscle spasm (IMS) in 4 cases. Black boxes represent the emergence of IMS. Blanks indicate no IMS. No emergence of IMS was recognized throughout the follow-up period in case 3. On the contrary, IMS was recognized throughout the follow-up period in case 4. In both cases 1 and 2, moderate IMS was recognized, although more so in case 2 than in case 1.

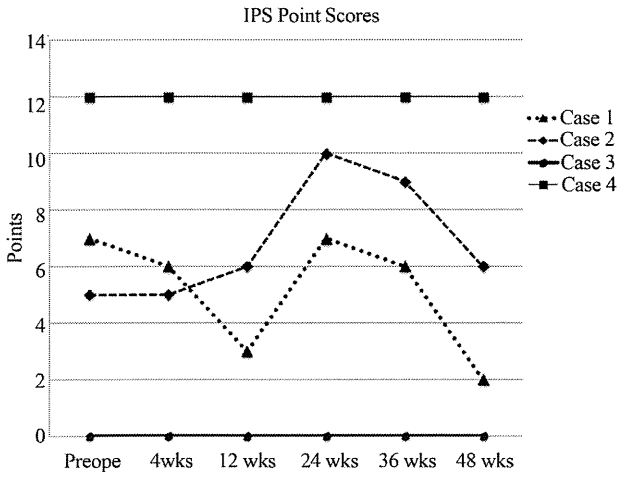

Figure 6. Each emergence of IMS was calculated as 1 point and is summarized in the figure. At the beginning of the follow-up period, case 1 had more points than case 2, but this difference reversed in the middle and latter parts of the follow-up period. Thus, we concluded that relative IMS could be recognized in cases 2 and 4 rather than in cases 1 and 3.

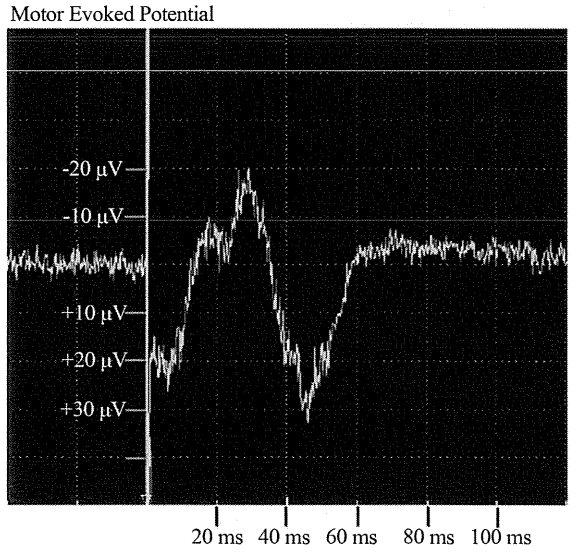

Figure 7. Motor evoked potential (MEP) in response to bifocal transcranial magnetic stimulation (TMS) was evaluated in bilateral rectus femoris muscles. Case 4 exhibited, MEPs in response to TMS.

ery of motor neurons indirectly indicated by IMS may be a predictor of the success of regeneration therapy in chronic SCI. But we have just only four cases in this study. We have to investigate this in our upcoming clinical trial.

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Adult olfactory sphere cells are a source of oligodendrocyte and Schwann cell progenitors

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Abstract The olfactory epithelial layer contains multipotent horizontal basal cells (HBCs) that differentiate into olfactory sensory neurons. Here, we show that rat HBCs express oligodendrocyte progenitor cell (OPC) and astrocyte markers. We generated olfactory sphere (OS) cells in cultures that were derived from adult rat olfactory mucosa. Fluorescence-activated cell sorting and immunofluorescence analyses showed that OS cells also express OPC and astrocyte markers. Interestingly, OS cells underwent oligodendrocyte differentiation in vitro. To study oligodendrocyte differentiation in vivo, OS cells were transplanted into injured rat spinal cords. The transplanted cells integrated into host tissue and differentiated into oligodendrocytes. When transected saphenous nerve ends were encased in collagen-containing silicone tubes with or without OS cells, the transplanted OS cells differentiated into Schwann cells. Our data provide new insights into of the stemness of OS cells.

Introduction

Multipotent horizontal basal cells (HBCs) differentiate into olfactory sensory neurons during normal neuronal turnover as well as during tissue regeneration after injury (Carter et al., 2004; Goldstein et al., 1998; Huard et al., 1998; Iwai et al., 2008; Lindsay et al., 2010). Several extracellular matrix proteins and growth factors control neuronal differentiation of HBCs (Carter et al., 2004; Gokoffski et al., 2011; Newman et al., 2000). Wnt signaling and transcription factor p63 are involved in HBC self-renewal (Wang et al., 2011; Fletcher et al., 2011; Packard et al., 2011). The majority of adult HBCs are derived from neural crest cells (Katoh et al., 2011; Suzuki et al., 2013). Schwann cells, sensory and autonomic neurons of the peripheral nervous system arise from the neural crest as well (Binder et al., 2011).

Oligodendrocyte progenitor cells (OPCs) exist in the central nervous system (CNS). OPCs differentiate into oligodendrocytes in response to injury and demyelination. Although OPCs are multipotent, their fate remains controversial (Nishiyama et al., 2009). OPCs in the CNS express NG2

Abbreviations: CNS, central nervous system; EGFP, enhanced green fluorescent protein; FACS, fluorescence-activated cell sorting; HBCs, horizontal basal cells; MACS, magnetic activated cell sorting; OPC, oligodendrocyte progenitor cell; OS, olfactory sphere; SCI, spinal cord injury.

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and platelet-derived growth factor receptor α (PDGFRα) (Nishiyama et al., 1996; Pringle et al., 1992) and generate oligodendrocytes and Schwann cells in induced demyelinated lesions (Zawadzka et al., 2010).

Attempts to expand olfactory stem cells according to the free-floating cell aggregate central nervous system (CNS) neurosphere culture method (Reynolds and Weiss, 1992) have been described (Barraud et al., 2007; Sicard et al., 1998; Carter et al., 2004). Olfactory spheres (OSs), clusters of cells generated by culturing the olfactory mucosa, contain cells expressing markers of neural stem cells, glial cells, and neural cells (Murrell et al., 2005; Othman et al., 2005; Murdoch and Roskams, 2008; Tome et al., 2009; Krolewski et al., 2011), but little is known about the properties of OS cells. For example, rat embryonic olfactory mucosa generates 2 distinct types of spherical cell clusters (Tome et al., 2009). One type contains multipotential cells with mesenchymal stem cell-like characteristics and originates in the lamina propria. The second cell type of spherical cell clusters displays epithelial cell characteristics and originates in the olfactory epithelium. Barraud et al. also used OS culture to expand neonatal olfactory mucosa-derived progenitor cells (Barraud et al., 2007).

In the present study, we found that rat HBCs and OS cells express OPC and astrocyte markers. We used a serum-free culture method to generate OSs from adult rat olfactory mucosal cells. We show that OS cells differentiated into oligodendrocytes and Schwann cells in vitro and in vivo.

Materials and methods

Animals

Eight-week-old male Sprague–Dawley rats (wild-type or transgenic SD-Tg rats) were obtained from Japan SLC, Inc. (Hamamatsu, Japan). The transgenic rats carry the enhanced green fluorescent protein (EGFP) gene driven by the ubiquitously expressed CAG promoter (Ito et al., 2001). All procedures were performed in accordance with the guidelines of the Laboratory Animals Care and Use Committee of the Osaka University, Faculty of Medicine (Osaka, Japan). Every effort was made to minimize the number of animals used and to limit their suffering.

Tissue preparation and olfactory sphere culture

The olfactory mucosa was dissected as reported (Aoki et al., 2010). Briefly, the olfactory mucosa was located in the caudal part of the septum and identified by its yellowish color. The tissue was removed from each side of the septum, avoiding contamination with other tissues such as the olfactory bulb and cribriform plate. The mucosa was dissociated mechanically and treated for 60 min at 37 °C with a mixture of collagenase (Wako, Osaka, Japan), dispase (Sanko Junyaku, Tokyo, Japan), DNase I (Sigma-Aldrich, St Louis, MO), and hyaluronidase (Sigma-Aldrich) in Dulbecco's modified Eagle medium (DMEM)/F12 medium (DF; Life Technologies, Carlsbad, CA). Dissociated cells (1×10^6/ml) were plated onto poly(2-hydroxyethyl methacrylate)-coated dishes in DF medium supplemented with B27 (Life Technologies), 20 ng/ml basic fibroblast growth factor (bFGF; Sigma-Aldrich), 20 ng/ml epidermal growth factor (EGF; Sigma-Aldrich), 5 μg/ml heparin (Sigma-Aldrich), and antibiotic–antimycotic solution (Life Technologies). Cultures were incubated at 37 °C in a 5% CO_2 atmosphere, and the medium was changed every 2 or 3 days.

The midline septum and attached olfactory mucosa were dissected. The olfactory mucosa was embedded in optimal cutting temperature (OCT) compound (Tissue-Tek Cryomold, Sakura Finetek, Tokyo, Japan) and cut into 10-μm-thick serial sections in the axial plane. The sections were treated for 1 h at room temperature in blocking solution (phosphate-buffered saline (PBS) containing 4% Block Ace, DS Pharma Biomedical Co., Ltd., Osaka, Japan, and 0.1% Tween 20), incubated overnight at 4 °C with the primary antibody, washed, and incubated overnight at 4 °C with the secondary antibody.

Differentiation culture

After 7 or 8 days in culture, OSs were plated on polyornithine-coated 4-well chamber slides (Becton Dickinson, Franklin Lakes, NJ) or dishes (Asahi Glass Co., Ltd., Tokyo, Japan). Cells were cultured for 5 days in DF medium supplemented with N2 (Life Technologies), B27, 20 ng/ml bFGF, 20 ng/ml EGF, and antibiotic–antimycotic solution. For dissociation cultures, spheres were treated with trypsin-ethylenediaminetetraacetic acid (EDTA, Life Technologies), plated onto polyornithine-coated 4-well chamber slides at approximately 5×10^3 cells per well, and cultured for 5 days in supplemented DF medium as described above.

Magnetic activated cell sorting (MACS)

The olfactory mucosa was dissociated into single-cell suspensions using the Neural Tissue Dissociation Kit (P) (Miltenyi Biotec Inc., Auburn, CA). Myelin debris was magnetically labeled with the Myelin Removal Beads II (Miltenyi Biotec Inc.) and removed, and the cell suspension was loaded onto a MACS Column, which is placed in the magnetic field of a MACS Separator (Miltenyi Biotec). The magnetically labeled myelin is retained within the column. The unlabeled cell fraction is magnetically labeled with the anti-GLAST (solute carrier family 1 member 3, Slc1a3; Excitatory Amino Acid Transporter 1, Eaat1; astrocyte cell surface antigen-1, ACSA-1) MicroBead (Miltenyi Biotec). The cell suspension is loaded onto a MACS Column placed in the magnetic field of a MACS Separator. The magnetically labeled cell is retained within the column. The unlabeled cell fraction comprises GLAST-negative cells. After removing the column from the magnetic field, the magnetically retained GLAST-positive cells were eluted.

Fluorescence-activated cell sorting analyses (FACS)

Fluorescence-activated cell sorting was performed on a FACS Canto II (BD Biosciences, San Jose, CA). Antibodies were as follows: phycoerythrin (PE)-conjugated mouse anti-rat CD54 (intercellular adhesion molecule 1 (ICAM-1) (BD Biosciences), allophycocyanin (APC)-conjugated mouse anti-rat GLAST antibody (Miltenyi Biotec), PE-conjugated isotype control mouse IgG1 (BD Biosciences), APC-conjugated isotype control mouse IgG2a (Miltenyi Biotec), anti-NG2-Fluorescein (mouse monoclonal antibody; R&D Systems, Inc., Minneapolis, MN),

anti-Mouse IgG1 Isotype Control-Fluorescein (mouse monoclonal antibody; R&D Systems, Inc.), anti-A2B5-Biotin (Miltenyi Biotec), anti-Mouse IgM-Biotin (Miltenyi Biotec), and anti-Biotin-FITC (Miltenyi Biotec).

OSs were dissociated with trypsin-EDTA and enzyme-free Cell Dissociation Buffer (Life Technologies) for 5 min and 10 min, respectively at 37 °C. OS cells (1×10^5) were first reacted with antibodies against ICAM-1, NG2, GLAST, and A2B5 for 60 min at 4 °C and washed. Cells were further incubated with anti-Biotin FITC antibody for 30 min at 4 °C. Cells were washed, resuspended, and then passed through a 40 μm filter immediately before sorting of 2×10^4 cells. Data were analyzed using FLOWJO software v6.2.1 (Tree Star, Ashland OR).

Immunocytochemistry

OS cells were fixed with 4% paraformaldehyde (PFA) and incubated for 1 h at room temperature in blocking solution, incubated overnight at 4 °C with the primary antibody, washed, and incubated overnight at 4 °C in the secondary antibody. The primary antibodies were as follows: anti-microtubule-associated protein 2 (MAP2, 1:200 rabbit polyclonal antibody, Abcam, Cambridge, UK), anti-β3-tubulin (Tuj1, 1:200 mouse monoclonal antibody, Cell Signaling Technology, Beverly, MA), anti-glial fibrillary acidic protein (GFAP, 1:300 mouse monoclonal antibody, Cell Signaling Technology or 1:2 rabbit polyclonal antibody from Dako, Glostrup, Denmark), anti-O4 (1:200 mouse monoclonal antibody; Neuromics, Edina, MN), anti-nestin (1:200 mouse monoclonal antibody, Abcam), anti-oligodendrocyte transcription factor 2 (Olig2, 1:300 sheep polyclonal antibody; Abcam), anti-p75 NGF Receptor (1:50 rabbit monoclonal antibody, Abcam), anti-PDGFRα (1:200 rabbit polyclonal antibody, Abcam), and anti-receptor-interacting protein (RIP, 1:100 rabbit monoclonal antibody Cell Signaling Technology). Monoclonal antibody BMG6H8 directed against BrdU was obtained from Boehringer–Mannheim (1:10 mouse 5-bromo-2-deoxyuridine labeling and detection kit II, Cat. No. 1 299 964; Roche, Indianapolis, IN).

The secondary antibodies used were DyLight 488-conjugated goat anti-mouse (1:200; Kirkegaard and Perry Laboratories, Inc. (KPL), Gaithersburg, MD), DyLight 549-conjugated goat anti-rabbit (1:200; KPL), and Cy5-conjugated donkey anti-sheep (1:200; Jackson ImmunoResearch, West Grove, PA). The slides were then counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, Vector Laboratories, Burlingame, CA). Cell phenotypes and cell counts were assessed from images captured using a confocal laser fluorescence microscope (FV-1000D; Olympus, Tokyo, Japan). The percentage of BrdU-, Olig2-, MAP2-, and GFAP-positive cells are shown as the mean ± standard deviation (SD) of 3 images from 3 independent experiments. Primary and secondary antibodies are listed in Table 1.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

RNA was extracted from spheres harvested from differentiating cultures using commercially available kits (QIAGEN, Hilden, Germany). Total RNA (1 μg) was reverse transcribed using the OneStep reverse transcriptase-polymerase chain reaction (RT-PCR) Kit (QIAGEN). The primers and size in base pairs (bp) of the expected amplicon were as follows: *Glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*), CCTCT GGAAAGCTGTGGCGT and TTGGAGGCCATGTAGGCCAT, 430 bp; *Nestin* (*Nes*), CAGGCTTCTCTTGGCTTTCTGG and TGGTGAGGG TTGAGGTTTGT, 431 bp; *tubulin, beta 3 class III* (*Tubb3*), TGCGTGTGTACAGGTGAATGC and AGGCTGCATAGTCATTTCC AAG, 240 bp; *glial fibrillary acidic protein* (*Gfap*), ACCTCGG CACCCTGAGGCAG and CCAGCGACTCAACCTTCCTC, 141 bp; *S100 protein, beta polypeptide, neural* (*S100β*)), GGATGTCTG AGCTGGAGAAG and ACTCCTGGAAGTCACACTCC, 222 bp; *2′,3′-cyclic nucleotide 3′ phosphodiesterase* (*Cnp*), CCGGAGACATAG TGCCCGCA and AAAGCTGGTCCAGCCGTTCC, 450 bp. Reverse transcription was performed at 50 °C for 30 min. PCR was performed at 94 °C for 30 s, 56 °C for 40 s, and 72 °C for 50 s. The PCR cycles for *Gapdh*, *S100β*, and *Cnp* were 30 and 40 for *Nes*, *Tubb3*, and *Gfap*.

Spinal cord injury (SCI) model and cell transplantation

Rats were anesthetized using sevoflurane and O_2, and laminectomy was performed at the midthoracic vertebrae 7/8. Each spinous process of midthoracic vertebrae 6/9 was fixed using a stabilizing forceps (MK-BPT 400, Muromachi Kikai Co., Tokyo, Japan). Using an operating microscope, the dorsal surface of the dura mater was exposed and SCI was induced using an SCI device (100 kdyn, dwell time, 5 s; Infinite Horizon Impactor, Muromachi Kikai Co., Tokyo, Japan). Dwell time is the time that the impactor remains extended and in contact before retracting. All rats received subcutaneous gentamicin (8 mg/kg) daily for 7 days. Nine days after SCI surgery, rats were transplanted with cells using a Hamilton syringe connected to a micro-glass pipette with a stereotaxic injector (Narishige, Tokyo, Japan). Rats with incomplete injury (Basso, Beattie, and Bresnahan [BBB] score above 1 at 9 days after injury) were excluded. The cells for transplantation were prepared from trypsinized sphere cells and diluted in DF media to 2.5×10^4 cells/μl. The micropipette tip was inserted into the spinal cord at the epicenter of the injury, and 2 μl of cell suspension was injected at 1 μl/min. After transplantation, all rats received daily subcutaneous injections of cyclosporine (10 mg/kg) as an immunosuppressant and gentamicin (8 mg/kg) for 7 days. Rat tissues were examined 4 weeks post-transplantation. The rats were anesthetized, and tissues were fixed by transcardial perfusion with 100 ml PBS followed by 4% PFA. The spinal cord was fixed in 4% PFA overnight, cryoprotected in 30% sucrose, embedded in OCT compound (Tissue Tek, Sakura Finetek, Tokyo, Japan), and frozen. Sections (10-μm-thick) were sagittally cut from the blocks using a cryostat (CM1510S; Leica, Tokyo, Japan).

Transected saphenous nerve model and cell transplantation

Rats were anesthetized using sevoflurane and O_2, and the bilateral saphenous nerves were exposed. A nerve segment was resected to create a 10-mm gap between the proximal and distal stumps. The stumps of the right saphenous nerve were placed in a silicon tube containing type IA collagen gel (Cell Matrix Corporation; Nitta Gelatin Inc., Osaka, Japan).