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Optimal time for subarachnoid transplantation of neural progenitor cells in the treatment of contusive spinal cord injury☆

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Abstract

This study aimed to identify the optimal neural progenitor cell transplantation time for spinal cord injury in rats *via* the subarachnoid space. Cultured neural progenitor cells from 14-day embryonic rats, constitutively expressing enhanced green fluorescence protein, or media alone, were injected into the subarachnoid space of adult rats at 1 hour (acute stage), 7 days (subacute stage) and 28 days (chronic stage) after contusive spinal cord injury. Results showed that grafted neural progenitor cells migrated and aggregated around the blood vessels of the injured region, and infiltrated the spinal cord parenchyma along the tissue spaces in the acute stage transplantation group. However, this was not observed in subacute and chronic stage transplantation groups. O4- and glial fibrillary acidic protein-positive cells, representing oligodendrocytes and astrocytes respectively, were detected in the core of the grafted cluster attached to the cauda equina pia surface in the chronic stage transplantation group 8 weeks after transplantation. Both acute and subacute stage transplantation groups were negative for O4 and glial fibrillary acidic protein cells. Basso, Beattie and Bresnahan scale score comparisons indicated that rat hind limb locomotor activity showed better recovery after acute stage transplantation than after subacute and chronic transplantation. Our experimental findings suggest that the subarachnoid route could be useful for transplantation of neural progenitor cells at the acute stage of spinal cord injury. Although grafted cells survived only for a short time and did not differentiate into astrocytes or neurons, they were able to reach the parenchyma of the injured spinal cord and improve neurological function in rats. Transplantation efficacy was enhanced at the acute stage in comparison with subacute and chronic stages.

Key Words

neural regeneration; spinal cord injury; subarachnoid space; cell transplantation; neural progenitor cells; time window; grants-supported paper; photographs-containing paper; neuroregeneration

Research Highlights

(1) Neural progenitor cells transplanted *via* the subarachnoid space at the acute, subacute and chronic stages following spinal cord injury could survive and improve neurological function in injured rats.
(2) Grafted neural progenitor cells migrated and aggregated around blood vessels of the injured region, and infiltrated the spinal cord parenchyma along the tissue spaces after acute stage transplantation.
(3) Grafted neural progenitor cells survived long term (8 weeks) and differentiated into astrocytes or oligodendrocytes after chronic stage transplantation.
(4) Grafted neural progenitor cells localized to the parenchyma of injured spinal cords and improved neurological function at the acute stage following injury in rats. Efficacy was enhanced in comparison with transplantation at subacute and chronic stages.

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INTRODUCTION

For the past few decades, a variety of experimental strategies, including neural progenitor cell transplantation, have emerged to facilitate regeneration of injured spinal cords[1]. Transplantation of stem cells has been a focus, and is considered a promising approach for the treatment of central nervous system diseases and injuries to facilitate repair of damaged nervous tissues[1]. Neural progenitor cells are favorable candidates because of their high potential for controlled growth into nerve cells[2]. A study has employed parenchymal injection of cells directly into the lesion site[3], which is a strategy designed to efficiently deliver cells directly to the injury site. In a clinical setting, however, this technique is expensive and invasive, as it requires the use of general anesthesia and risks further damage to the already injured spinal cord.

Intrathecal injection has been widely used to administer peptides[4], drugs[4], neurotrophic factors[5] and cells[6-7] into the brain or spinal cord for various purposes. The purpose of this study was to find the optimal time window for neural progenitor cell transplantation into the cerebrospinal fluid for the treatment of spinal cord injury. Furthermore, we monitored the survival and migration of the grafted cells and improvement in the animal's locomotor activity.

RESULTS

Quantitative analysis of experimental animals

After thoracic spinal cord injury, 136 Sprague-Dawley rats were used in this study. Ten rats were used in the control group (non-treated) and the remaining 126 were randomly assigned to six experimental groups: group A (acute stage, neural progenitor cells treated 1 hour after injury; *n* = 22), group S (subacute stage, neural progenitor cells treated 1 week after injury; *n* = 22), group C (chronic stage, neural progenitor cells treated 4 weeks after injury, *n* = 19), or corresponding media-treated control groups (MA, MS, *n* = 22; MC, *n* = 19). Three rats were chosen randomly from each group for harvesting of spinal cord specimens at 1, 2, 4 and 8 weeks after cell transplantation. All remaining rats were sacrificed 12 weeks after spinal cord injury. All 136 rats were used in the final analysis.

In vitro characterization of neural progenitor cells

During culture in non-differentiation media, neural progenitor cells showed characteristic morphology of small, phase-bright progenitors. Neural progenitor cell viability was greater than 90% before grafting (Figure 1A). At the time of grafting, neural progenitor cell cultures expressed early neural markers, A2B5 (Figure 1B) and nestin (Figure 1C), but did not express mature markers of neurons (NeuN), astrocytes (glial fibrillary acidic protein) or oligodendrocytes (O4) (supplementary Figure 1 online). This evidence confirmed that the cells remained as undifferentiated neural progenitor cells.

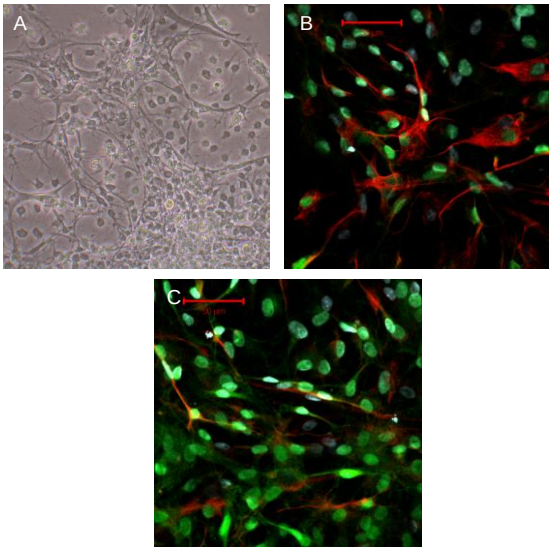

Figure 1 Identification of neural progenitor cells (passage 10).

(A) Characteristic morphology of small, phase-bright progenitor cells (contrast phase microscope, × 200). Cells in cultures expressed early neural markers, A2B5 (B, Alexa 546-labeled, red) and nestin (C, Alexa 546-labeled, red) *in vitro* (immunofluorescence, confocal microscopy, scale bars: 50 μm). All cells in transgenic rats expressed enhanced green fluorescent protein, cell nuclei were stained by 4',6-diamidino-2-phenylindole.

Localization of grafted neural progenitor cells

After transplantation *via* lumbar puncture at the L_{4-5} intervertebral space, group A grafted cells were observed to infiltrate to deeper spinal cord parenchyma by 4 weeks at the T_{9-10} injury region, especially the fiber spaces (Figure 2A). Labeled neural progenitor cells were observed near blood vessels of the injury site (Figure 2B) and also on the pia mater and dura of rostral or caudal injured region, especially the cauda equina region (Figure 3A).

Grafted cell morphologies were irregular. Furthermore, there were no viable enhanced green fluorescent protein-positive cells observed in the central nervous system of group A at 8 weeks after cell transplantation.

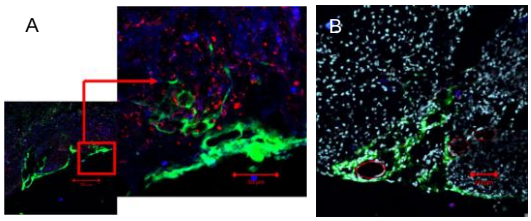

Figure 2 Localization of grafted neural progenitor cells (NPCs) in the acute stage transplantation group at 4 weeks post-transplantation (immunofluorescence, confocal microscopy, scale bars: 50 μm).

(A) Grafted NPCs (green) infiltrated into the parenchyma of the central injured region along the tissue spaces, especially the nerve fiber spaces in the white matter (neurofilament, Alexa 546-labeled, red; O4, Alexa 488, blue). Because O4 cannot co-stain with green fluorescent cells, O4 expression was not observed in the grafted cells.

(B) Labeled NPCs are observed adjacent to blood vessels (anti-smooth muscle actin staining, Cy5-labeled, red; nuclei were stained with 4',6-diamidino-2-phenylindole, blue).

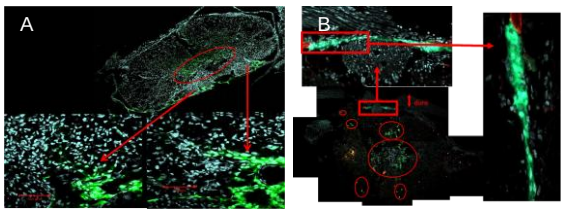

Figure 3 Migration of grafted neural progenitor cells (NPCs) at 4 weeks post-transplantation (4',6-diamidino-2-phenylindole staining cell nuclei blue; confocal microscopy; scale bars: 50 μm).

Green autofluorescence is observed in the core of the injury (circles).

(A) In acute stage transplantation group, transplanted NPCs infiltrate the spinal cord parenchyma and cohere on the surface of the spinal cord at injury region $T_{9–10}$.

(B) In the chronic stage transplantation group, grafted NPCs were observed residing in the scar perimeter around the dura of the injured region (rectangle).

Group S grafted cells were visible, 4 weeks after transplantation, in the spinal pia mater and dura at the T_{9-10} injury region and the cauda equina region. However, no viable enhanced green fluorescence protein cells were observed in the central nervous system at 8 weeks after cell transplantation. In group C, some grafted cells were attached to the cauda equina and remained in the injury space scar from the 1st to the 8th week after cell transplantation (Figure 3B). However, the grafted cells appeared to be resting, with few transplanted cells observed at the intact rostral spinal cord tissue far from the injured segment in both group S and C rats. In group A, a few grafted neural progenitor cells were found on the pia surface of the cerebrum and the cerebellum 2 weeks after cell transplantation, however grafted cells were absent from other parts of the brain, including the hippocampus, striatum and ventricular system. Furthermore, grafted cells did not penetrate into the parenchyma of the brain. No grafted cells were detected to have infiltrated the spinal cord parenchyma in groups S, C, MA, MS and MC from the 1st to the 8th week after cell transplantation.

Differentiation of the grafted neural progenitor cells

Using immunofluorescence staining, some O4- and glial fibrillary acidic protein-positive cells were detected in the core of the grafted cluster attached to the cauda equina pia surface in group C (Figure 4), 8 weeks after cell transplantation. O4- and glial fibrillary acidic protein-positive grafted cells were not detected in groups A, S and C 4 weeks after cell transplantation.

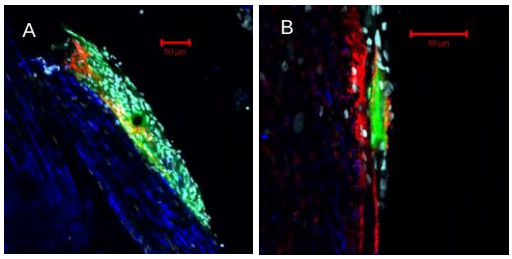

Figure 4 Differentiation of grafted neural progenitor cells (NPCs) at 8 weeks post-transplantation in the chronic stage transplantation group (immunofluorescence, confocal microscopy, scale bars: 50 μm).

The grafted NPCs (green) attached on the pia surface of the cauda equine.

(A) Astrocytes were identified using glial fibrillary acidic protein (Cy5-labeled, red), and axons were identified using neurofilament (Alexa 488-labeled, blue).

(B) Oligodendrocytes were identified using O4 (Alexa 546-labeled, red); axons were identified using neurofilament (Alexa 488-labeled, blue).

Neurological function in rats with spinal cord injury

There were no significant differences among MA, MS, MC and control groups ($P > 0.05$), indicating that the animal model was reliable. The Basso, Beattie and Bresnahan scale scores[8] (Table 1) showed that group A had significant hind limb locomotor activity improvements ($P < 0.01$) compared with groups S, C and control from the 2nd to the 12th week after spinal cord injury. There were some differences between groups S and C and between groups S and control at 2–3 weeks ($P < 0.05$), however there were no improvements in groups S and C when compared with the control group at 4–12 weeks after spinal cord injury ($P > 0.05$). There were significant improvements in group A at week 4 after cell transplantation compared with group S at week 5, group C at week 8 and the control group at week 4 ($P < 0.01$). There were no improvements between group S at 5 week, group C at 8 week or the control group at week 4 ($P > 0.05$).

Table 1 Basso, Beattie and Bresnahan scale scores after spinal cord injury

Time after model establishment (week)	Group A	Group MA	Group S	Group MS	Group C	Group MC	Control group
1	2.05±1.43	2.30±1.75	1.95±1.61	1.25±1.65	1.75±1.77	2.20±2.14	2.65±2.08
2	5.75±1.41	4.95±1.32[b]	4.15±1.53[a]	5.55±1.61[b]	3.90±1.68[ab]	4.10±1.33	5.85±1.81[ab]
3	8.20±0.95	7.40±1.27[ab]	6.45±1.05[a]	7.30±1.17[b]	6.50±1.10[a]	7.70±1.59[ab]	7.40±1.35[ab]
4	9.60±1.57	8.20±0.77[a]	7.85±1.23[a]	7.55±1.15[a]	8.05±0.95[a]	7.90±1.29[a]	8.15±0.75[a]
5	9.35±1.42	8.35±1.04[a]	8.15±0.88[a]	8.20±1.28[a]	7.80±1.11[a]	8.00±1.65[a]	8.50±0.95[a]
6	9.45±1.67	8.70±0.87[a]	8.30±0.80[a]	8.10±0.91[a]	8.00±0.80[a]	8.20±1.01[a]	8.55±0.76[a]
7	9.80±1.28	8.75±0.72[a]	8.35±0.94[a]	8.35±0.88[a]	8.20±0.95[a]	8.35±0.75[a]	8.70±0.73[a]
8	10.05±1.43	8.90±0.97[a]	8.50±0.76[a]	8.40±1.31[a]	8.10±0.97[a]	8.55±1.23[a]	8.75±1.02[a]
9	10.10±1.55	8.80±1.44[a]	8.35±1.18[a]	8.05±1.15[a]	8.40±0.94[a]	8.30±0.92[a]	8.70±0.87[a]
10	10.05±1.47	8.85±1.14[a]	8.45±1.19[a]	8.50±0.95[a]	8.40±1.05[a]	8.20±0.77[a]	8.70±1.17[a]
11	10.05±1.76	8.75±0.91[a]	8.60±0.82[a]	8.45±0.51[a]	8.30±0.98[a]	8.75±0.91[a]	8.85±1.23[a]
12	10.10±1.41	8.65±1.04[a]	8.60±1.31[a]	8.75±1.52[a]	8.10±1.12[a]	8.65±1.04[a]	8.75±1.41[a]

Data are expressed as mean ± SD, with 10 rats per group. [a]$P < 0.01$, *vs*. group A; [b]$P < 0.05$, *vs*. group S using analysis of variance and Dunnett's *t*-test. A higher score indicates better neurological function. Group A: Neural progenitor cell transplantation at acute stage; group S: neural progenitor cell transplantation at subacute stage; group C: neural progenitor cell transplantation at chronic stage; group MA, MS or MC: corresponding media-treated control groups of groups A, S, or C.

DISCUSSION

Feasibility of subarachnoid transplantation of neural progenitor cells

Theoretically, mature neurons can play a role in rebuilding neural circuits, whilst mature oligodendrocytes can facilitate remyelination and mature astrocytes can reduce cell death and injury severity. Therefore, neural progenitor cell transplantation could be used for the treatment of central nervous system injury and neural degenerative diseases. Previous studies have indicated that after subarachnoid injection of neural progenitor cells, the grafted cells migrate to spinal cord injury sites[9-10] and multiple sclerosis lesions[11], resulting in improved bladder function and locomotor activity in animal models[12]. Subarachnoid transplantation of neural progenitor cells was found to be feasible for the treatment of spinal cord injury in the acute and subacute stages[13-14]. In this study, we clearly observed that in the acute stage, grafted cells migrated into the spinal cord parenchyma along the tissue spaces, especially the fibrous spaces. This migratory effect may be due to cytokines and growth factors released from dead cells and blood vessels. We also found many neural progenitor cells aggregated at the periphery of blood vessels, which is likely to be due to trophic factors released from the blood[15]. However, evidence is still required to certify that the neural progenitor cells infiltrate the spinal cord parenchyma along the perivascular space. In addition, subarachnoid transplantation of neural progenitor cells was minimally invasive, easy to perform, low in cost and reproducible[16]. Furthermore, transplantation of cells *via* lumbar puncture was also feasible[13]. In response to central nervous system injury, both endogenous and exogenous neural progenitor cells[14] can migrate to diseased sites of the central nervous system. Studies *in vitro* demonstrated that neural progenitor cells migrate to sites of various signaling factors generated at the spinal cord injury site, including cell chemotactic factors[17] and inflammatory cytokines, such as tumor necrosis factor α, interferon γ[18] and platelet-derived growth factor[19], and to injured cells[20] including microglia[21].

Time window of subarachnoid transplantation of neural progenitor cells

It is well known that the relative integrated blood-brain barrier and cerebrospinal fluid-brain barrier are involved in central nervous system functions. It is difficult for non-fat soluble substances and the majority of macromolecular substances to pass through the blood-brain barrier and cerebrospinal fluid-brain barrier to enter the spinal parenchyma. It is more difficult for allogeneic cells to pass through an intact spinal cord. However, grafted cells could play a major role in repair of the spinal cord after entering the spinal parenchyma through interaction with other cells. Therefore, the issue regarding the appropriate time window for cell transplantation may be important for subarachnoid grafted cells. We therefore designed this study to address the issue of the time window. Rauch *et al* [22] found that neural progenitor cells could induce neovascularization after spinal cord injury and could play a role in promoting repair after injury. Our study indicated that grafted neural progenitor cells in the acute stage of injury could migrate to the spinal parenchyma. Although no evidence has indicated that neural progenitor cells can induce neovascularization, grafted neural progenitor cells still

showed the tendency to migrate into the parenchyma of the damage zone and aggregate in the injured regions of blood vessels; however, the mechanism of this occurrence is yet to be verified through further experiments. The aforementioned phenomenon was not observed following cell transplantation during subacute and chronic stages, which differs from a previous study[12] investigating transplantation at the acute stage. First, it is possible that chemotaxis of the grafted neural progenitor cells in the blood vessels and necrotic tissue was evidently reduced, while the injured spinal cord was almost completely repaired with glial scar. Second, the use of an outbred rat strain might be one reason for cell destruction, following rapid rejection during an inflammatory response[23-24]. Finally, cell suspension culture media across cell transplantation groups were different. Fluid collagen, used by Mitsui *et al* [12], may be beneficial for the adhesion of grafted cells. Basso, Beattie and Bresnahan scale score comparisons showed that the hind limb locomotor activity of animals in group A recovered well, whereas animals in groups S, C and the control showed no significant differences after spinal cord injury from weeks 4–12. This indicated that the issue of graft time window was critical in the subarachnoid transplantation of neural progenitor cells. However, the clinical application of subarachnoid transplantation of neural progenitor cells in acute stage continues to be debated[25-27]. Patient condition in the acute stage is often complicated, so a combination of multiple therapies is often required. Subarachnoid transplantation of neural progenitor cells is an invasive therapy, which carries the risk of aggravating the injured spinal cord. Survival of the grafted neural progenitor cells in the acute and subacute stages was quite short (neural progenitor cells were not observed in groups A and S after cell transplantation at 8 weeks). This may be a result of a strong immune reaction due to high inflammatory cell infiltration in the acute or subacute stages.

Survival and differentiation of grafted neural progenitor cells and promotion of functional recovery of rat hind limbs

In this study, we found that grafted neural progenitor cell clusters adhered to the cerebral pia mater of the cauda equina region and to the region of spinal cord injury after cell transplantation for 8 weeks. However, no grafted neural progenitor cells were observed in groups A or S at 8 weeks. We considered that subarachnoid transplantation of neural progenitor cells during the chronic stage would avoid the peak of inflammation after spinal cord injury and that the immune reaction would be comparatively less. Grafted cells may have been limited by fiber scar, so few transplanted cells were found at the intact rostral spinal cord tissue far from the injured segment in groups S and C. After cell transplantation, O4- and glial fibrillary acidic protein-positive cells were observed in the regions of cluster centers that were affiliated with the cauda equina region at 8 weeks in group C. Conversely, immunohistochemical staining revealed that O4- and glial fibrillary acidic protein- positive neural progenitor cells were absent in groups A, S and C at 4 weeks. This may be because: (1) the time frame was too short for neural progenitor cells to differentiate after cell transplantation, (2) increased inflammatory factors were harmful and inhibited differentiation of neural progenitor cells, (3) neural progenitor cell numbers had reduced drastically during the acute and subacute stages because of the inflammatory response, thus grafted cell numbers were too few to be observed for expression of enhanced green fluorescence protein, O4 and neuronal nuclei. Conditions in the subarachnoid space to promote grafted neural progenitor cells to differentiate into gliocytes or neurons need further investigation. However, previous studies have indicated that neural progenitor cells do have the potential for differentiation *in vivo* [28-29].

Recovery of hind limb locomotor function in rats after spinal cord injury requires a time frame of about 3 to 4 weeks. Our study indicated that the Basso, Beattie and Bresnahan scale scores in rats, 3 weeks after spinal cord injury in group A, showed significant improvement when compared with other groups. Conversely, Basso, Beattie and Bresnahan scale scores at each time point in groups S, C and control showed no significant difference after 4 weeks, indicating that neural progenitor cell transplantation was beneficial for recovery of the hind limb in rats. Ideally, cellular grafts should: (1) provide a conducive substrate for axonal growth, (2) remyelinate axons, (3) replace damaged neurons or introduce neurons that can serve as “relays” to re-establish axonal connections, and (4) support axon regrowth/ remyelination *via* soluble factors secreted by grafted cells[30]. Our study found that none of the grafted cells migrated to the parenchyma of the injured region, which was observed after subarachnoid transplantation of neural progenitor cells in the subacute and chronic stages. However, although the affiliated grafted cell clusters were observed in the scar tissue space in the injured region, there was no evidence that the cells migrated from the scar tissue into the spinal parenchyma. It would therefore be difficult for the neural progenitor cells to repair the injured neural circuits and to facilitate remyelination. Basso, Beattie and Bresnahan scale scores indicated that subarachnoid transplantation in the subacute stage played a small role in the repair of neural function of the hind limbs in rats. But whether this cell transplantation technique is

suitable for treatment of chronic spinal cord injury patients needs to be discussed. The following issues must therefore be considered for the treatment of spinal cord injury using subarachnoid transplantation of neural progenitor cells: (1) limitations of scar formation (including glial scar and fiber scar), (2) central nervous system immunological rejection of transplanted cells, and (3) survival time and differentiation conditions of grafted neural progenitor cells in the subarachnoid space. In light of the aforementioned conditions, clinical cell transplantation into the subarachnoid space is progressing from bench to bedside.

The issue of the optimal time window for subarachnoid transplantation of neural progenitor cells for the treatment of spinal cord injury needs to be considered. Cell transplantation performed in the acute stage indicates that grafted cells are capable of aggregating around the blood vessels of the injured region, from which they then migrate into the spinal parenchyma of the injured region *via* tissue spaces, and promote repair of hind limb locomotor activity in rats.

MATERIALS AND METHODS

Design

A randomized, controlled animal experiment.

Time and setting

Experiments were performed in the First Affiliated Hospital of General Hospital of Chinese PLA from March 2008 to September 2011.

Materials

Embryonic (14 ± 0.5 days old) transgenic donor Sprague-Dawley rats (SOD G93A, RRRC, USA) and 136 adult female Sprague-Dawley rats, weighing approximately 250–300 g (Vital River Laboratory Animal Technology Co., Ltd., Beijing, China; license No. SCXK (Jing) 2007-0001) were used in this study. All experimental protocols were in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[31].

Methods

Neural progenitor cell culture

Neural progenitor cells were isolated from embryonic cerebral vesicles of transgenic donor rats (outbred Sprague-Dawley) expressing green fluorescence protein (RRRC #65). Briefly, embryos were isolated in a dish containing Dulbecco's modified Eagle's medium/F12 (Sigma, St. Louis, MO, USA). After peeling away the meninges, the cerebral vesicles were incubated in collagenase type I (10 mg/mL) / dispase II (20 ng/mL)/ Hank's Buffered Salt for 30 minutes at 37°C . Cells were then plated in neural progenitor cell complete media (Dulbecco's modified Eagle's medium/F12) containing 10% bovine serum albumin (1 mg/mL, Sigma) B27 (5 ng/mL; Invitrogen, Carlsbad, CA, USA), basic fibroblast growth factor (20 ng/mL; PeproTech, Rocky Hill, NJ, USA), N2 (10 ng/mL; Invitrogen), epidermal growth factor (20 ng/mL; PeproTech) and chick embryo extract (10%, SLI Ltd., West Sussex, UK) on laminin- coated (20 ng/mL; Invitrogen) plastic plates. Cells were passed every 3–5 days for a total of 10 passages.

The neural progenitor cell population was dissociated from culture plates using 0.05% trypsin/ ethylenediaminetetraacetic acid, washed and re-suspended at a concentration of 4×10^6 cells (in Dulbecco's modified Eagle's medium/F12) for transplantation. Cells were placed on ice throughout the grafting process. Before completion of the grafting procedure, cell viability was assessed using the trypan blue assay[29]. The phenotype of neural progenitor cells was verified before grafting by staining for nestin and stem cell marker A2B5 (A2B5 for neural progenitor cells), neuronal nuclei (for mature neurons), glial fibrillary acidic protein (for astrocytes) and O4 (for oligodendrocytes)[29].

Establishment of thoracic spinal cord injury model

A laminectomy was performed at the $T_{9\text{-}10}$ region under pentobarbital sodium (40 mg/kg) anesthesia. The rod of the impactor (10 g) was centered above T_{10} and dropped from a height of 25 mm to induce a consistent partial and incomplete spinal cord injury[32]. Muscle and skin were then sutured in layers after injury. Cefazolin (25 mg/kg) was administered for 5 days to prevent urinary tract infections. Urinary bladders were emptied manually twice daily until urinary function was recovered. All surgeries were performed by one blinded operator.

Cell transplantation

Neural progenitor cells were injected into the lumbar subarachnoid space at the corresponding time point. Under sodium pentobarbital (40 mg/kg) anesthesia, a 1 cm midline incision was cut into the skin over the $L_{3\text{–}5}$ spinous process region, and the skin was retracted. A 29-gauge needle (BD Neonatal, 29G1; BD Medical Systems, San Jose, CA, USA) was inserted into the spinal canal at $L_{4\text{–}5}$. Proper needle placement was confirmed by a sudden loss of resistance at the time of entry, a tail flick and cerebral spinal fluid flow into the needle hub. Cells were then

administered over a period of 1 minute. 80 μL of F12 media solution containing neural progenitor cells (4×10^6 cells), or the same volume of F12 media, was injected as a single dose into the subarachnoid space. The muscle and skin were sutured tightly. The control group was considered as an untreated control and received no cell transplantation or media injection. Cell transplantations were performed by one blinded operator.

Immunohistochemical and immunofluorescent staining

Animals were deeply anesthetized with pentobarbital sodium. Rats were sacrificed by transaortic perfusion with 100 mL of 4°C PBS followed by 150 mL of 4% paraformaldehyde. The whole spinal cord and brain were removed from the spinal column and skull after perfusion. One spinal cord specimen from each group was cut sagittally, while the others were cut coronally at 1, 2, 4 and 8 weeks. All brain specimens were cut coronally. All brain and spinal cord tissues were post-fixed in 4% paraformaldehyde overnight and transferred into a 30% sucrose solution, frozen, and cut into 6 μm-thick slices using a cryostat (Leica, Heidelberg, Germany). Sections were mounted on glass slides and stored at –70°C. Tissue sections and cultured cells were washed in PBS, blocked in 10% goat serum (Invitrogen) for 1 hour at room temperature and incubated in primary antibody solution at 4°C overnight. Both monoclonal and polyclonal antibodies were used to identify the grafted cells. A number of primary antibodies were used to assess the phenotype of the cells. Mouse anti-rat nestin (1:300; Chemicon, Santa Cruz, CA, USA) and A2B5 (1:500; Chemicon) monoclonal antibody were used to identify undifferentiated neural progenitor cells. Neurons were identified using mouse anti-rat neuronal nuclei monoclonal antibody (1:100; Chemicon). Astrocytes were identified using mouse anti-rat glial fibrillary acidic protein monoclonal (1:100; Chemicon) and rabbit anti-glial fibrillary acidic protein polyclonal (1:200; Dako, Glostrup, Denmark) antibodies. Oligodendrocytes were identified using rabbit anti-O4 polyclonal antibody (1:250; Chemicon). Rabbit anti-neurofilament polyclonal antibody (1:100; Chemicon) and mouse anti-rat smooth muscle actin monoclonal antibody (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used to label axons and blood vessels respectively. Samples were incubated for 2 hours at room temperature with secondary antibodies: Alexa 488- or 546-labeled goat-anti-mouse/rabbit IgG (1:400; Molecular Probes, Eugene, OR, USA) or Cy5-labeled goat-anti-mouse IgG (1:400, Jackson Immuno Research Labs, Inc., West Grove, PA, USA). Samples were counterstained with 4',6-diamidino-2-phenylindole (1:1 000; Sigma) to identify nuclei and mounted under coverslips with anti-fade mounting media (Fluorosave, CN Biosciences, La Jolla, CA, USA). Images were acquired using confocal microscopy (Zeiss LSM 510 Meta, Oberkochen, Germany).

Basso, Beattie and Bresnahan scale score

Hind limb locomotor activity of the animals in each group (10 rats/group) was assessed by two blinded examiners using the Basso, Beattie and Bresnahan scoring scale[8] once a week for 12 weeks after injury. Assessments were conducted 1 hour after bladder evacuation every Monday morning.

Statistical analysis

SPSS 13.0 software(SPSS, Chicago, IL, USA) was used for statistical analyses. All data are represented as mean ± SD. Statistical analysis was performed using analysis of variance and Dunnett's test. *P* values < 0.05 were regarded as statistically significant.

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