Co-transplantation of olfactory ensheathing glia and mesenchymal stromal cells does not have synergistic effects after spinal cord injury in the rat

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Abstract
Background aims. Olfactory ensheathing glia (OEG) and mesenchymal stromal cells (MSC) are suitable candidates for transplantation therapy of spinal cord injury (SCI). Both facilitate functional improvement after SCI by producing trophic factors and cytokines. In this study, the co-transplantation of both types of cells was studied to clarify their additive and/or synergistic effects on SCI.

Methods. A balloon-induced compression lesion was used to produce SCI in rats. OEG, MSC or both OEG and MSC (3 × 10^5 cells of each cell type) were implanted by intraspinal injection 1 week after SCI. The effect of transplantation was assessed using behavioral, electrophysiologic and histologic methods.

Results. Hindlimb function was examined with Basso, Beattie and Bresnahan (BBB) and Plantar tests. Improvement was found in all three groups of transplanted rats with different time–courses, but there was no significant difference among the groups at the end of the experiment. Motor-evoked potentials after SCI decreased in amplitude from 7 mV to 10 µV. Linear regression analysis showed a modest recovery in amplitude following transplantation, but no change in the control rats. Histologic findings showed that the white and gray matter were significantly spared by transplantation after SCI. Functional improvement was achieved with transplantation of OEG and/or MSC, but the co-transplantation of OEG and MSC did not show synergistic effects. The poor migration of OEG and MSC might prevent their concerted action. Pre-treatment with a Rho antagonist and a combination of intraspinal and intravenous injection of the cells might be beneficial for SCI therapy.

Key Words: mesenchymal stromal cells, olfactory ensheathing glia, spinal cord injury, transplantation

Introduction
Cell therapy is a promising approach to restoring locomotor function after spinal cord injury (SCI). Both olfactory ensheathing glia (OEG) and mesenchymal stromal cells (MSC) have been investigated extensively as promising candidates for cell therapy.

The implantation of OEG is known to minimize cavity and scar formation by regulating astrocytes and promoting angiogenesis and myelination (1,2). OEG decrease the levels of chondroitin sulfate proteoglycan (CSPG), which has inhibitory effects on growing axons following SCI (3,4). Thus OEG probably create a permissive environment for spared and regenerating axons after injury by releasing growth factors (NGF, BDNF, GDNF and VEGF) and extracellular matrix molecules (collagen, laminin and fibronecin) in addition to adhesion molecules (5–7). OEG display phenotypes of both Schwann cells and astrocytes. They are genetically closer to Schwann cells than astrocytes, and astrocytes are more related to OEG than to oligodendrocytes (8). These characteristic features of OEG promote the ingrowth of axons from the peripheral nervous system (PNS) to the central nervous system (CNS) beyond the laminin-rich boundary established following SCI (9). Au et al. (10) considered that the production of secreted protein acidic rich in cysteine (SPARC) by OEG regulated laminin-1 production and allowed Schwann cells and astrocytes to interact, resulting in the re-entry of regenerating axons into the CNS.

MSC are multipotent progenitor cells that, in the case of severe tissue ischemia or damage, can be
attracted to the lesion site, where they can secrete cytokines (interleukins) and trophic factors that can exert an inhibitory influence on scarring and apoptosis. They facilitate angiogenesis and neural regeneration (11). Mobilized from the bone marrow (BM), and sorted or expanded in culture, MSC can be delivered to the damaged site by direct or systemic application. Functional improvement resulting from MSC transplantation could be principally because of their neuroprotective and reconstructive influences at the lesion site, resulting from their secretion of various trophic factors such as cytokines and growth factors (12–14), by their down-regulation of caspase-3, which mediates apoptosis (15), and through their immunomodulatory and anti-apoptotic activity resulting from their production of galectin-1, angiopoietin-1, osteopontin and thrombospondin-1 and -2 (16,17), thus fostering functional recovery after SCI (18–20). Their easy accessibility and rapid expansion in culture are attractive characteristics from a therapeutic point of view.

The co-transplantation of OEG and MSC may be beneficial for SCI treatment through a combinatory approach: OEG can facilitate regeneration following SCI by providing guiding strands and molecules of the extracellular matrix for axonal ingrowth, while other cell types such as MSC can improve functional outcome by producing trophic factors and cytokotks. In addition, OEG as well as MSC can be used in autologous transplantation protocols, and therefore no additional immunosuppression would be necessary. In this study, the effect of the transplantation of OEG, MSC and the co-transplantation of both OEG and MSC on behavioral, electrophysiologic and histologic outcomes after SCI was studied.

**Methods**

Adult male Wistar rats weighing 270–300 g were used in our experiments. The lesioned animals were divided into four groups. The first group received both OEG and MSC (n = 21). The second and third groups were transplanted with only OEG (n = 22) or MSC (n = 23), respectively. The fourth group was injected with saline as a control (n = 23). All experiments were performed in accordance with the European Communities Council Directive of 24th of November 1986 (86/609/EEC) regarding the use of animals in research, and were approved by the Ethics Committee of the Institute of Experimental Medicine ASCR, Prague, Czech Republic.

**SCI**

Balloon compression was used to create an SCI. A small opening of the thoracic vertebra 10 (T10) was performed. A 2-french Fogarty catheter was inserted below T8, and the balloon was inflated with 15 µL saline for 5 min at T8 (19,21). During the surgical procedure, the body temperature of the animal was maintained at 37°C with a heating pad, and 3% isoflurane in air was administered at a flow rate of 0.3 L/min to prevent edema development as a result of low levels of anesthesia (22). Following the lesioning procedure, the rats were assisted in feeding and urination until they had recovered sufficiently to perform these functions on their own. The animals received gentamicin sulfate (5 mg/kg) for 3 days to prevent post-operative infections.

**OEG preparation and identification**

OEG were obtained from 4-week-old Wistar rats. The olfactory mucosa was scraped from both sides of the nasal septum. After incubation in Dispase II (Roche, Mannheim, Germany), the lamina propria was separated mechanically from the epithelium and dissociated with collagenase type I (Sigma, St. Louis, MO, USA) (23). After a 24-h incubation in Dulbecco’s modified Eagle medium (DMEM)/F12 with 10% fetal bovine serum, the cells were treated with cytosine-β-D-arabinofuranoside (5 × 10–5 M, Sigma) to eliminate rapidly dividing cells, such as fibroblasts, and expanded with bovine pituitary extract (100 µg/mL; Sigma) (24). To assess the purity of OEG cultures, cultured cells on glass slides were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100, blocked in serum, and incubated with primary antibodies overnight at 4°C. Two antibodies were used to identify OEG: mouse anti-p75, low-affinity nerve growth factor receptor (1:100; Chemicon, Temecula, CA, USA), and rabbit anti-S100 (1:200; Sigma). Alexa Fluor 488-conjugated goat anti-mouse IgG and Alexa Fluor 594-conjugated goat anti-rabbit IgG secondary antibodies (Sigma) were used to distinguish the two primary antibodies. Labeled cells were examined in a fluorescent microscope (Zeiss Axioskop, Göttingen, Germany). More than 80% of cultured cells were p75/S100-positive.

**MSC preparation**

BM was extracted from the tibia and femur of 4-week-old Wistar rats by flushing with DMEM. BM cells were dissociated mechanically and plated in DMEM medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 0.1 mg/mL streptomycin (Gibco). MSC were isolated through their adherence to plastic. After a 24-h incubation, non-adherent cells, including hematopoietic stem cells, were removed by replacing the medium (19,25). MSC were used for transplantation after 6–10 passages.
Transplantation

The animals were transplanted 1 week after SCI. Just before transplantation, OEG were labeled with the fluorescent dye 10 µM carboxy-fluorescein diacetate succinimidyl ester (CFDA-SE; green fluorescence; Molecular Probes, Eugene, OR, USA), while MSC were labeled with 20 µM carboxy SNARF-1 (red fluorescence; Molecular Probes) or CFDA-SE, to enable subsequent identification of transplanted cells. Labeling was performed according to the manufacturer’s instructions, and the concentrations of the tracers were based on the results of Imazumi et al. (26) and Li et al. (27). The animals were secured in a stereotaxic apparatus by holding the spinal process just anterior to the injured site of the spinal cord with a rat-specific vertebra holder (Cunningham spinal adaptor; Stoelting Co., Wood Dale, IL, USA). The spinal cord was exposed at T8. A total of 3 × 10^5 OEG and/or MSC was injected through a glass pipette at a concentration of 1 × 10^5 cells/µL, into the proximal, central and distal parts of the lesioned spinal cord (each part received 1 µL cell suspension), at a depth of 1 mm below the dorsal surface and a rate of 1 µL/min using a Nano-Injector (Stoelting Co.); OEG/MSC-transplanted animals received six injections (3 × 10^5 OEG and 3 × 10^5 MSC) instead of the three injections received by the other animals. The glass pipette was kept in place after each injection for a further minute to prevent leakage of the cell suspension. The control group received three injections of saline (1 µL/injection), also into the proximal, central and distal parts of the lesioned spinal cord. Cyclosporine (10 mg/kg) and ampicillin (50–100 mg/kg) were administered intraperitoneally 1 day before transplantation and throughout the experiment (for 2 months).

OEG and MSC from green fluorescent protein transgenic rats

To enable more easily distinguishable and stable tracking of transplanted cells, OEG and MSC expressing enhanced green fluorescent protein (GFP) isolated from GFP+ rats were used in 2-month survival experiments. Transgenic Sprague–Dawley (SD-Tg(CAG-EGFP)CZ-004Osb) rats were kindly provided by Dr Masaru Okabe (Osaka University, Osaka, Japan) (28), bred at the laboratory of Dr Martin Marsala (University of California, San Diego, CA, USA) and subsequently sent to our institute and bred in our animal facility.

Functional analysis

Hindlimb locomotor activity after SCI was assessed by the Basso, Beattie and Bresnahan (BBB) test (29) (OEG/MSC, n = 14; OEG, n = 15; MSC, n = 16; control, n = 12). The rats were placed on a floor within a circular enclosure as described by Basso et al. (29). Their hindlimb joint movement, paw placement, weight support, forelimb–hindlimb co-ordination, etc., were evaluated using a 0–21-point scale.

Hindpaw withdrawal latency to a noxious thermal stimuli was assessed with a Plantar test apparatus (Ugo Basile, Comerio, Italy) (OEG/MSC, n = 10; OEG, n = 10; MSC, n = 10; control, n = 10). The animals were placed in a clear plastic chamber and acclimated for 10 min until becoming quiescent. The hindpaw received a heat stimulus through a glass plate. The withdrawal latencies were measured three times for each hindpaw at 5-min intervals. The average of three values was used for statistical evaluation.

Motor-evoked potentials

Rats were anesthetized with ketamine (100 mg/kg). One pair of needle electrodes was placed subcutaneously over the skull for cortical stimulation and another pair in the gastrocnemius muscle for recording. The signals were filtered (1–5000 Hz) and recorded by a Medelec Synergy T-EP EMG/EP Monitoring System (Oxford Instruments Medical Inc., Surrey, UK). Motor-evoked potentials (MEP) were elicited once a week by transcranial stimulation (a single square-pulse electrical stimulus of 0.1 ms in duration) to measure the amplitude, latency to onset and duration of the response (OEG/MSC, n = 9; OEG, n = 10; MSC, n = 11; control, n = 7).

Histologic analysis

To count the number of transplanted GFP cells and examine the possible neural differentiation of the transplanted cells, longitudinal cryostat sections (40-µm thickness) of the spinal cord were made after 4% paraformaldehyde fixation. Every sixth section was chosen for imaging using a fluorescence microscope. The surviving cells were counted in each section, and the total number of cells was estimated according to the volume of the section in which the cells were found. The percentage of surviving transplanted cells was calculated by dividing the estimated total number of surviving cells by the total number of transplanted cells (3 × 10^5 cells/rat). For immunohistochemistry, the sections were blocked with 1% bovine serum albumin, then incubated with antibodies against neural markers, anti-nestin (1:2500; Chemicon) or anti-βIII-tubulin (1:400; Sigma) overnight at 4°C, followed by incubation with the Alexa Fluor 594-conjugated secondary antibody. To assess spared descending axon tracts after SCI, the
corticospinal tract, one of the major descending tracts in the spinal cord, was anterogradely labeled. Two weeks before termination of the experiment, 1 μL of a 10% biotinylated dextran amine (BDA; 10 000 MW; Molecular Probes) was injected bilaterally into the sensorimotor cortex at a depth of 1.5 mm from the surface with a glass micropipette. BDA-injected animals were deeply anaesthetized with ketamine (100 mg/kg) and xylazine (20 mg/kg), their chests opened and transcardial perfusion performed with phosphate buffer followed by 4% paraformaldehyde solution in phosphate buffer. The spinal column was removed and left in paraformaldehyde solution overnight. The spinal cord was then carefully removed from the spinal cord channel and post-fixed in the same solution for at least 1 week. An approximately 5-cm long segment from the same area of each spinal cord was dissected, and 14-μm thick sagittal frozen sections were cut. To visualize the BDA-injected corticospinal tract axons, cryostat sections were incubated with avidin horseradish peroxidase and diaminobenzidine (DAB), following the instructions in the BDA neuronal tracer kit (Molecular Probes). Some sections were stained with hematoxylin-eosin.

Spinal compression results in the formation of cavities within the spinal cord tissue and induces degeneration of the spinal cord. The volume of the spared white and gray matter was analyzed at the end of the experiment. After perfusion with 4% paraformaldehyde, a 2-cm long segment of the spinal cord was dissected between 1-cm cranial and 1-cm caudal to the injury epicenter. Serial cross-sections (5 μm thick) were made after paraffin embedding and stained with Luxol-fast blue and Cresyl violet to distinguish the white and gray matter. For volumetric measurements, six sections were selected at 1-mm intervals along the cranio-caudal axis, and whole images of the spinal cord were taken with an Axioskop 2 plus microscope (Zeiss) and analyzed by ImageJ software (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA) (19).

Statistical analysis
The data are expressed as mean ± SEM. A one-way analysis of variance (ANOVA) followed by Tukey’s post-hoc test was used to evaluate differences between groups. Regression analyzes were applied for MEP. A P-value <0.05 was considered statistically significant.

Results

Functional outcome

BBB test. Hindlimb locomotion was examined weekly after SCI using an open-field BBB test. The animals had a BBB score of 0 immediately after the induction of the balloon compression lesion. Some of the three joints in the hindlimb started to regain their ability to move during the first post-surgical week, but the animals never placed their paws on the ground. The control animals achieved BBB scores of 7.08 ± 0.24 at the end of the experiment (9 weeks after SCI; 8 weeks after transplantation) but never supported their body weight on their hind legs. During the first week after transplantation, the transplanted animals already showed a tendency to improve their BBB scores. Among the transplanted groups, the fastest recovery was observed in animals transplanted with OEG. These animals showed significant improvement compared with the controls as early as 4 weeks after SCI (P < 0.05) and their final BBB score was 9.30 ± 0.46. Animals with OEG and MSC co-grafts, even though they received six injections, showed a statistically significant improvement 6 weeks after SCI, with a final BBB score of 9.18 ± 0.44. One week later (i.e. 7 weeks after SCI), similar motor improvement was observed in MSC-transplanted rats (final BBB score of 8.94 ± 0.42) (Figure 1A). All transplanted animals recovered their gait; some of them gained the ability to support their body weight and to perform paw stepping, and two animals from the OEG- and MSC-transplanted groups showed co-ordination of the forelimbs and hindlimbs. However, in conclusion there were no significant differences among any of the transplanted animal groups in terms of overall functional improvement at the end of the experiment (P > 0.05).

Plantar test
The withdrawal latency of the hindpaws in response to a thermal stimulus was measured once a week. During the first post-operative week (before transplantation), the average latency of hindpaw withdrawal, determined from three repeated measurements at 5-min intervals, was 7.18 ± 0.30, 6.75 ± 0.24, 7.51 ± 0.27 and 7.18 ± 0.44 s, in OEG/MSC-, OEG- and MSC-transplanted rats and control rats, respectively. The latency of each group slightly decreased during the second week following lesion induction. The control group did not continue to improve further, but the transplanted groups shortened their withdrawal latency during each subsequent week. Significant differences from the control rats (P < 0.05) started to appear in the transplanted rats during the sixth post-surgical week. The final values determined 9 weeks after SCI were 5.83 ± 0.25, 5.69 ± 0.21 and 5.42 ± 0.22 s in OEG/MSC-, OEG- and MSC-transplanted rats, respectively (Figure 1B); these values were significantly different (P < 0.05) from those of the control group (6.72 ± 0.12 s). Hyperalgesia was never observed after OEG and/or MSC transplantation.
and stronger electric stimuli did not increase further their amplitude immediately after the surgery. MEP were elicited weekly from 2 weeks to 6 weeks after SCI. Figure 2A shows representative MEP recorded in individual animals from each group, while Figure 2B shows the maximum amplitudes recorded in all animals, displayed according to the type of transplanted cells: OEG/MSC, OEG, MSC and control. Linear regression was used to analyze the significance of the increase in MEP amplitude over time. There was no significant impact on the slope of the linear regression line in the control rats ($R^2 = 0.004$, $P = 0.278$). The MEP of the control animals did not show any improvement in amplitude and stabilized between 15 and 40 µV 6 weeks after lesioning. In contrast, the transplanted groups showed more improvement than the control group. A significant difference was found 5 and 6 weeks after SCI compared with 2 weeks after SCI ($P < 0.05$) in the OEG/MSC-transplanted group and between 2 weeks and 6 weeks after SCI ($P < 0.01$) in the OEG-transplanted group. Linear regression analysis showed that the weekly increases in amplitude were statistically significant in the OEG/MSC- ($R^2 = 0.122$, $P = 0.002$), OEG- ($R^2 = 0.132$, $P = 0.0002$) and MSC-transplanted groups ($R^2 = 0.037$, $P = 0.038$).

The duration of the response and the latency to onset of the response were 5.55 ± 1.37 ms and 4.81 ± 0.67 ms in the intact animals, respectively. The duration of MEP was extended by 1 ms after SCI. The response duration of MEP quickly decreased in the OEG/MSC- and MSC-transplanted groups, but gradually shortened week by week in the OEG-transplanted and control groups. A difference was observed 1 week after transplantation (2 weeks after SCI) between OEG/MSC- and MSC-transplanted rats and the control rats ($P < 0.05$; data not shown). The latency was not significantly different from normal values throughout the experiment. Linear regression analysis revealed a tendency towards decreased latency in the transplanted groups; in particular, the slope of the linear regression line was statistically significant in the OEG/MSC-transplanted group ($R^2 = 0.095$, $P = 0.044$). In contrast, there was no such tendency observed in the control group (Figure 2C).

Histology

Cell survival. To monitor cell survival in SCI, we examined longitudinal cryosections of the balloon-compressed spinal cord. The green fluorescence expressed by CFDA-SE-labeled OEG was detected with a fluorescent microscope throughout 8 weeks.
of observation after transplantation. However, SNARF-1- or CFDA-SE-labeled MSC were visible for only 4 or 5 weeks after SCI (Figure 3A); subsequently, labeled MSC were not seen. It seemed likely that the concentration of the dye was not adequate for longer observation. Therefore, to enable more reliable tracking of the cells in vivo, OEG and MSC obtained from GFP transgenic rats were used for long-term tracking. Transplants of both types of cells were injected and checked every week in the same way. Surviving MSC (Figure 3B) and OEG (Figure 3C) from GFP rats were easily recognized by their vivid green color in the spinal cord for 8 weeks. None of the studied cell types showed any extensive migration within the spinal cord tissue, as they were almost exclusively found around the injection sites or were slightly diffused by the injection pressure. MSC spread around the injection sites after transplantation, while OEG extended their long processes and remained in close contact with one another. It appeared as if OEG formed conduits, as they do in the lamina propria in situ to ensheath and guide the olfactory axons (Figure 3C). Such dynamic behavior of OEG was also observed in vitro: OEG

Figure 2. Representative MEP recorded in transplanted and control rats 6 weeks after SCI (A). The maximum amplitudes (µV) (B) and the latency (ms) (C) obtained from individual animals are shown as a scatterplot at 1-week intervals following transplantation or saline injection (Tx). The linear regression lines are shown as solid lines.
were plotted at 1-mm increments from the injury epicenter (Figure 4A, B). The cross-sectional area of the spared white and gray matter was always smaller at any 1-mm distant sectional point in the control group than in the transplanted groups, and some of differences in area reached statistical significance. When the total volume of the white and gray matter (mm$^3$) was calculated from area measurements and summed from 21-mm long segments, a larger volume of spared white matter was observed in rats implanted with any type of cell (36.9 ± 1.9 mm$^3$, 38.8 ± 1.2 mm$^3$ and 38.9 ± 0.8 mm$^3$ in the OEG/MSC-, OEG- and MSC-transplanted rats, respectively) compared with 30.7 ± 0.9 mm$^3$ in the control animals ($P < 0.05$; Figure 5A). The volume of gray matter calculated from 21-mm long segments revealed that OEG/MSC and MSC transplants were more effective in terms of gray matter sparing (Figure 5B; $P < 0.01$). When the volume of the spared white and gray matter was compared between the cranial half and caudal half segments from the injury epicenter, a significant difference in the volume of the white matter was found in the caudal segments between OEG-transplanted rats and control rats ($P < 0.05$). The white matter was mainly spared in the thoracic segments caudal to the injury epicenter in OEG-transplanted rats, while in OEG/MSC- and MSC-transplanted rats the white matter was equally preserved in the cranial and caudal segments. On the other hand, the gray matter was more spared in cranial segments in both OEG/MSC- and MSC-transplanted animals ($P < 0.05$). The volume of the gray matter estimated from the area between 2-mm cranial and 2-mm caudal to the injury epicenter, where the cells were transplanted, showed that the gray matter was prominently spared around the injection sites (Figure 5C, D). The volume of the gray matter in the transplanted animals was significantly different from that in the control animals ($P < 0.01$). Anterograde tracing disclosed that when a larger volume of the white matter was spared, the corticospinal axons were more visible in the spared white matter (Figure 3F). Axons were scarcely seen around the injury epicenter in the control rats (Figure 3E).

**Discussion**

The subacute phase, up to 3–4 weeks after SCI, is the therapeutic window for cell therapy in humans as well as in animal models (30). Cell survival is better when the cells are transplanted in the subacute phase rather than in the acute phase of SCI (31). Cells transplanted immediately after SCI encounter a hostile environment characterized by inflammation, the up-regulation of pro-inflammatory cytokines attempted to grasp each other by tangling their long processes (Figure 3D). At the end of the experiments, the number of surviving cells was counted. There were great differences between individual animals even in the same group. The percentage of surviving MSC and OEG was 0.41–0.96% and 0.34–1.72%, respectively. Similar cell survival rates were observed in the co-transplanted group. Nestin and βIII-tubulin staining did not reveal any positive cells among the transplanted OEG or MSC 8 weeks after transplantation.

**Morphometric evaluation**

The area of the spinal cord was calculated from serial cross-sections 1-cm cranial and 1-cm caudal from the injury epicenter, which was identified as the thinnest part of the thoracic spinal cord. The cross-sectional areas (mm$^2$) of the white and gray matter
Co-transplantation of OEG and MSC

create a supportive environment for neural sprouting by removing cell debris, including inhibitory components for axonal growth such as CSPG and myelin-associated glycoprotein (33). When CSPG...
production is blocked immediately after SCI, spontaneous recovery is decreased. CSPG seems to exert a beneficial effect on the injured spinal cord in the acute phase by activating microglia and macrophages (34). However, the inhibitory regulation of microglial activity is required in the later phase of SCI because neural cell damage proceeds as a result of cytokines and free radicals released from proliferating microglia (35). Thus OEG and MSC were transplanted at 7 days after SCI in our study.

In agreement with previous studies (19), our results show that hindlimb locomotion improved in OEG- and MSC-transplanted rats. However, their co-transplantation did not yield further additive and synergistic functional improvements. OEG and MSC appear to have a propensity to migrate towards an injury site (36–38). The GFP OEG and MSC transplants were predominantly observed around the injection sites. In our experiments, OEG and MSC were injected inside the lesions, so they would not need to migrate any considerable distance. On the other hand, their poor migration might be one of the features of OEG obtained from the lamina propria (39). Su et al. (40) suggested that post-injury up-regulation of Nogo-A on oligodendrocytes enhanced the adhesion of OEG via activation of Rho-A and limited their migration. The increased activation of Rho after SCI (41,42) also induced the formation of MSC focal adhesions (43,44). Poor cell migration might interfere with the concerted actions of OEG and MSC, which were implanted by separate injections in our experiments. Pre-treatment with a Rho antagonist or a Rho kinase inhibitor might be beneficial for the co-transplantation of OEG and MSC to facilitate cell migration as well as promote axonal growth and prevent apoptosis in the injured spinal cord (40,45).

To treat SCI, cell therapy is used to ameliorate a hostile niche for neuronal sparing and regeneration, including the prevention of scar formation, inhibitory factors and demyelination, and to establish new neural circuits to bypass the injured sites. After SCI, the severed axons sprout and send collaterals into the gray matter (46–48) and new circuits are mediated by propriospinal neurons in the incompletely damaged spinal cord (49,50). Functional recovery is very much dependent on this newly established route. The MEP recorded once a week showed very variable amplitudes in each group and even in the same animal. The positions of the electrode for recording were not exactly the same for every measurement. Therefore, permanent implantation of electrodes into the muscle (51) or spinal cord (52) might be beneficial for periodic recordings during a long-term experiment if they do not affect the animal’s locomotion and behavior. Even though the MEP had variable amplitudes and remained within microvolt-levels, the trend of their increasing amplitude, analyzed during a 1-month observation period, was statistically significant in the transplanted groups. The control group did not show any improvement in amplitude, but the transplanted groups improved their amplitude week by week; in particular, the OEG-transplanted group showed
Co-transplantation of OEG and MSC

The initial mechanical trauma and subsequent inflammatory processes lead to the apoptosis of oligodendrocytes, which results in demyelination (55,56). OEG remyelinate demyelinated axons after SCI either by themselves (57) or by facilitating the entry of host Schwann cells into the injured spinal cord (58). The tendency towards shorter MEP latencies was significant only in the co-transplanted group. The remyelination process might be partly involved in this functional improvement.

Sensory afferent inputs play an important role in more sophisticated locomotion (59). None of the transplanted rats achieved a BBB score higher than 15, which requires parallel paw position during locomotion. The animals could not correct their paw position after receiving sensory information when the paw was placed on, and lifted from,

Figure 5. The total volumes (mm$^3$) of the white and gray matter calculated from area measurements and summed from 21-mm long segments of the lesioned spinal cord. The volume of the white matter was larger in the transplanted groups than in the control group (A) (**P < 0.05, ***P < 0.01). The gray matter was well-spared in the OEG/MSC- and MSC-transplanted rats compared with the controls (B) (**P < 0.01). The volumes (mm$^3$) of the white and gray matter were estimated from the area between 2-mm cranial and caudal to the injury epicenter, where the cells were implanted. There was no significant difference in the white matter volume (C) but the gray matter was remarkably well preserved around the injection sites (D) (**P < 0.01).
the floor. Our results did not show the same clear improvement in the Plantar test as was observed in a previous study where MSC were implanted intravenously (19). Because intraspinal injection may cause additional damage to the dorsal columns, the results from the Plantar test may not be as reliable in the present study as in our previous MSC study. Although the transplantation of both types of cells showed similar results, their mechanisms of action are different in restoring the damaged spinal cord. It has been shown that intravenous injection of MSC 7 days after SCI improves locomotor activity and significantly increases spared white matter in the lesion area (19). However, no sparing of gray matter was observed. MSC are known to have immunomodulatory effects (60). Therefore, their systemic injection may influence the levels of pro- and anti-inflammatory cytokines, and this might result in a trophic effect on functional recovery. Also, the lesion is not fully developed at 7 days after SCI, so the MSC may act as a neuroprotective agent. The slow improvement observed in the Plantar test might therefore be because of these different routes of cell injection: intraspinal versus intravenous.

Cell counts showed the poor survival of OEG and MSC implanted into the lesioned area. The direct injection of each cell type spared the gray matter in our study, but the injury epicenter is not a habitable environment for transplanted cells (31). Subarachnoid injection is an alternate and promising route for cell administration in order to avoid additional damage to the spinal cord and enable more efficient cell delivery to the spinal cord (61,62), but further study is required to elucidate the environment in the cerebrospinal fluid (CSF) for transplanted cells. MSC injected into the fourth ventricle were subsequently transported to the subarachnoid space in the spinal cord through the CSF and promoted behavioral recovery after SCI, but these MSC had disappeared by 3 weeks after injection, even using an immunosuppressant (63). Repeated subarachnoid administration of cells might be effective in promoting the long-term effects of the transplanted cells on the damaged host tissue.

Vainety et al. (21) showed that balloon compression leads to a complete loss of gray matter, a partial loss of white matter and copious vacuoles. Our morphometric analysis also revealed that the cross-sectional area of the gray matter around the injury epicenter was 0–0.013 mm² in the control animals. However, the gray matter always remained spared in the treated animals. The white matter was equally spared in the lesioned spinal cord with the OEG/MSC and MSC transplants, while OEG transplants preserved the white matter in the caudal segments more than in the cranial segments. The gray matter was spared to a greater extent in the MSC/OEG- and MSC-transplanted rats, especially in the spinal cord cranial to the injury epicenter. In other words, the gray matter sparing improves with the aid of MSC transplantation, while the white matter sparing resulting from OEG transplantation is likely to protect spinal tract axons from degeneration during the early stages of SCI, which in turn might lead to the faster recovery of simple locomotor function. Moreover, analyzing the volume between 2-mm cranial and 2-mm caudal to the injury epicenter, where the OEG and/or MSC were mainly implanted, there was no statistical difference in the volume of the white matter between the transplanted and control groups. In contrast, a significant difference was found in the volume of the gray matter. OEG and MSC had the ability to spare the white and gray matter in the injured spinal cord; in particular, the gray matter was well preserved around the damaged area of the spinal cord where the transplants were directly injected. The gray matter is more susceptible to metabolic damage than white matter. Balloon compression includes ischemic damage to the spinal cord and leads to metabolic failure in the gray matter. We showed that the transplants spared the gray matter from such damage. Without OEG and/or MSC treatment, the gray matter scarcely remained and the white matter was poorly spared. OEG and MSC appear to secure more descending and ascending axons by sparing the white matter. Therefore, they can provide more opportunities to reconstruct lost connections by sending collaterals from the spared axons to the gray matter, which was also spared by the transplants. In the gray matter, interneurons can re-establish or maintain neural connections with the PNS and CNS. If OEG and/or MSC were not implanted, the animals never showed the ability to support their body weight on their hind legs. However, we could not find any unambiguous relationship between the volume of the spared spinal cord tissue and behavioral improvement. Functional recovery must be considered from the viewpoint of synaptic reconstruction, and the white and gray matter sparing induced by transplantation increases the feasibility of this reconstruction.

Although MSC have the multipotency to differentiate into mesodermal and non-mesodermal lineages (64), they are prone to differentiate into the mesodermal lineage. Their potential to differentiate into neuronal cells is limited and remains controversial (65,66). In our experiment, nestin-positive and βIII-tubulin-positive transplanted cells were not found 2 months after transplantation. Although we cannot dismiss the possibility of neural differentiation in the transplanted cells, which might help to bridge the damaged neural circuits, the trophic effects of the transplanted cells on the resident neurons are more likely to affect functional improvement positively after neural damage than the possible differentiation of the transplanted cells (67).
Thus OEG and MSC could foster locomotor function by sparing the spinal cord, but their combined advantages provide no added benefit to SCI therapy. Additional treatment might be required to facilitate their migration, leading to co-operation of the two types of cells, or different combinations of transplants and/or trophic factors might need to be considered for further functional improvement. In a previous study, the intravenous injection of MSC spared the white matter after SCI, but not the gray matter (19). Comparing the spared volume of white and gray matter between the intravenous injection used in our previous study and the intraspinal injection of MSC used in the current study, we found significantly more gray matter sparing following intraspinal injection. If the spared volume in the control animals is considered 100%, the spared volume of the white matter following intravenous injection was 150% and 134% following intraspinal injection, while the spared volume of the gray matter was 137% following intravenous injection and 220% following intraspinal injection. In addition, recovery in the BBB test was fastest during the first few weeks post-grafting in those animals that received OEG transplants. Taken together, we speculate that to maximize the advantageous effects of co-transplantation, direct implantation of OEG into the spinal cord lesion accompanied by intravenous injection of MSC might be another, perhaps more efficacious, approach to the co-transplantation of OEG and MSC.

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