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Transplantation of Schwann cells to subarachnoid space induces repair in contused rat spinal cord

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Abstract

Schwann cell transplantation is well known to induce repair in the injured spinal cord which disables millions of injured patients throughout the world. An ideal route of delivering the grafted Schwann cells to the spinal cord should neither cause more injury nor reinitiate inflammatory events and also provide a favorable milieu to the grafted cells. In this study, we have utilized subarachnoid route to transplant Schwann cells and evaluated their effects in a contusive model of spinal cord injury. Adult rats weighing 100–140 g were experimentally injured by crushing the spinal cord with a titanium clip and then divided into four groups (Tracing, Control, Medium-treated and Schwann cell-treated). Cultured Schwann cells (5×10^4 cells in 5 μl) or medium were injected to the animals of corresponding groups via subarachnoid space at the injured site 7 days after injury. In tracing group, Schwann cells (labeled with Hoechst) demonstrated their presence within spinal cord 7 days after transplantation. Evaluation of locomotor performance of animals for 60 days after injury showed that animals treated with Schwann cells had significant improvement ($P < 0.01$). Similarly, the axon density at the site of injury was significantly higher. The results indicate the efficacy of subarachnoid route for the transplantation of Schwann cells in inducing repair of the contused spinal cord. We conclude that this route can be useful for the transplantation of Schwann cells and offers a hope for the patients suffering from spinal cord injury.
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Schwann cell transplantation to injured spinal cord is a promising therapeutic approach for the patients with spinal cord injury [4]. These cells secrete various neurotrophic factors (such as nerve growth factor (NGF); brain-derived neurotrophic factor (BDNF); glial cell line-derived neurotrophic factor (GDNF); and ciliary neurotrophic factor (CNTF)); produce or secrete extracellular matrix molecules (such as laminin; to which injured axons can attach and extend); and guide regenerating axons into central tracts and remyelinate them [17,20].

Schwann cells are generally transplanted by direct injection into the contused spinal cord [5]. Needle insertion into CNS tissue has been shown to cause inflammation [7] and thus, this route of cell delivery may be invasive for the already injured spinal cord as it reinitiates inflammatory cascades that negatively influence the recovery-inducing role of the grafted cells [1,19]. Moreover, it may not be suitable for widespread and multifocal lesions. Thus, alternate routes of delivery of Schwann cells must be explored. In this study we report the effects of Schwann cell transplantation into subarachnoid space.

Subarachnoid space has been previously used for spinal cord injury/demyelination models to deliver neurotrophins [14], tissue fragments [3] and neurosphere cells [21]. Schwann cells derived from subarachnoid-transplanted sciatic nerve were reported to induce limited remyelination of axons in an ethidium bromide induced model of CNS demyelination [3]. However, no study has been conducted so far to evaluate the effect of Schwann cell transplantation through subarachnoid space for the purpose of treatment of contusive spinal cord injury.

Thirty-three female Wistar rats weighting 100–140 g. were divided into four groups: Tracing group (injured animals

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received Hoechst 33342-labeled Schwann cells; $n = 5$), Control group (injured animals that received no treatments; $n = 12$), Medium group (injured animals treated with subarachnoid injection of Dulbecco's Modified Eagle's Medium (DMEM); $n = 8$) and Schwann group (animals treated with subarachnoid injection of Schwann cells in DMEM as vehicle; $n = 8$). Laminectomy was performed at the level of T_9–T_{10} vertebras after anesthesia by Ketamine (90 mg/kg) and Xylazine (5 mg/kg). Spinal cord at T_{10} level was impinged laterally between blades of a calibrated titanium clip (FT710T, Aesculap, Germany; 1.16 N closing force) for 10 s. No dural rupture was observed in operated animals. Rats were treated with prophylactic antibiotic (Cefazolin; 150 mg/kg, twice a day) and manual bladder evacuation.

To prepare pure neonatal Schwann cell culture, a modified procedure from what was described previously [8] was adopted. Briefly, 1.5-cm of sciatic nerves of rat neonates were harvested and cut into 0.5–1 mm segments after being rinsed in Hank's buffered saline solution (HBSS), and stripped of epineurium and connective tissue. Nerve segments from each donor were placed in DMEM (Sigma, USA) for 6 days during which half of their medium was replaced every 3 days (fasting period to eliminate fibroblasts). The culture medium was then replaced by DMEM plus 10% Fetal Calf Serum (FCS). The culture was maintained for 25 days and half-amount of the medium was replaced every 3 days. Schwann cells were labeled against S100 antigen which is specific for committed Schwann cells [10] and then counterstained with Hoechst 33342. The purity of Schwann cells used for transplantation was 99%.

For the cells aimed to be transplanted to Tracing group, the cell culture was treated with Hoechst 33342 (Sigma; 5 μg/ml in DMEM) for 45 min at 37 °C before cell harvest and then was washed for several times. The cells were treated similar to the rest of the cells (vide infra), but in separate dishes. The fluorescence activity of these cells was checked before transplantation. In order to harvest Schwann cells, the cell culture was trypsinized (0.25%; Sigma) for 1 min at 37 °C. Thereafter, trypsin was discarded and the dishes were incubated for 5 min at 37 °C. DMEM plus 10% FCS was then added to neutralize trypsin. The cells were detached by pipetting the suspension and the resulting cell suspension was then centrifuged to form a cell pellet. The cell pellet was resuspended in DMEM resulting in a concentration of 1×10^4-viable cells/μl that was determined by hemocytometer counts and trypan blue exclusion assay. The cells were maintained on ice during the surgical procedure.

As 7 days delay between injury and Schwann cell transplantation has been shown to be repair-promoting [9,16], the spinal cords of animals in Tracing, Medium and Schwann groups were exposed 7 days after contusive injury (vide supra). Scar tissues were gently removed until the translucent dura appeared. Dural rupture or cord injury was cautiously avoided during this procedure. A Hamilton syringe (Scientific Glass Engineering, Australia) connected to a 30-Gauge caliber needle was utilized for Schwann cell injection. The needle was inserted through dura into subarachnoid space so as to cause minimal injury to the pial surface of spinal cord. A thin film of CSF beneath the covering meninges represented the subarachnoid space. The tip of the needle was inserted into subarachnoid space 4 mm caudal to the site of injury and was driven rostral for a farther 4 mm. Five microlitres of labeled cell suspension (in Tracing group), DMEM (in Medium group), or intact cell suspension with above concentration, leading to the total number of 50,000 cells (in Schwann group) was injected. The needle was then gently pulled back after few seconds to prevent the transplanted cells from floating out of the subarachnoid space. The wound was then closed.

Animals in Tracing group were then transcardially perfused on seventh day after cell transplantation, using PBS (0.1 M, pH 7.4) followed by 4% paraformaldehyde in PBS. Sections of spinal cords, including the site of injury and 1 cm rostral and caudal to the lesion epicenter, were cryo-sectioned longitudinally in 10 μm thickness. The sections were then examined using Axioskop 2 Plus microscope (Zeiss) fitted with fluorescence optics for Hoechst + nuclei.

Hindlimb locomotor function of animals in Control, Medium and Schwann groups were assessed by two blind examiner, using Basso, Beattie and Bresnahan (BBB) scoring system [2] twice a week for 60 days after injury. The assessments were conducted after bladder evacuation, and were recorded.

After survival period of 60 days, animals in Control, Medium and Schwann groups were transcardially perfused and spinal cord segments including the site of injury were dissected. The injured segments were then placed into paraffin blocks, sectioned longitudinally (10 μm) and stained by Marsland method [6], to evaluate the axon morphology and density at the site of injury. In brief, the slides were cleaned, rehydrated, placed in 20% silver nitrate for 1 h and washed in 10% formalin for 10–15 s. Without washing, sections were flooded with ammoniacal silver solution for 30 s and then flooded with formalin 10% for 1–2 min. After washing in distilled water (SANYO Gallenkamp PLC, UK), sections were fixed in 5% sodium thiosulphate for 5 min, washed, dehydrated, cleaned and mounted.

The number of intact axons was counted at the lesion center, the site considered as the least tissue spared zone, in white matter area stained by Marsland method. All identified axons with a darkly stained extended cylindrical shape were counted from all Marsland-stained tissue slides obtained from each animal and averaged. The quantity was depicted as the axon density per mm^2 of the transverse plane of white matter passing through the lesion center.

Statistical comparisons between experimental groups were performed using repeated measures analysis of variances (ANOVA) followed with the Dunnett T3 analysis as post hoc for BBB scores, and Kruskal–Wallis test followed by appropriate post hoc test for k independent samples [18] for the axon density values. SPSS version 9 was utilized for statistical analysis. Data was presented as mean ± S.E.M.

Seven days after transplantation, Hoechst-labeled Schwann cells were found in subarachnoid space attached to pia matter or migrated into the spinal cord (Fig. 1A). Some cells have migrated into the middle of spinal cord (Fig. 1B). The cells migrated in groups were found both at the site of injury (Fig. 1C) or 1 cm far from injured cord (Fig. 1D).

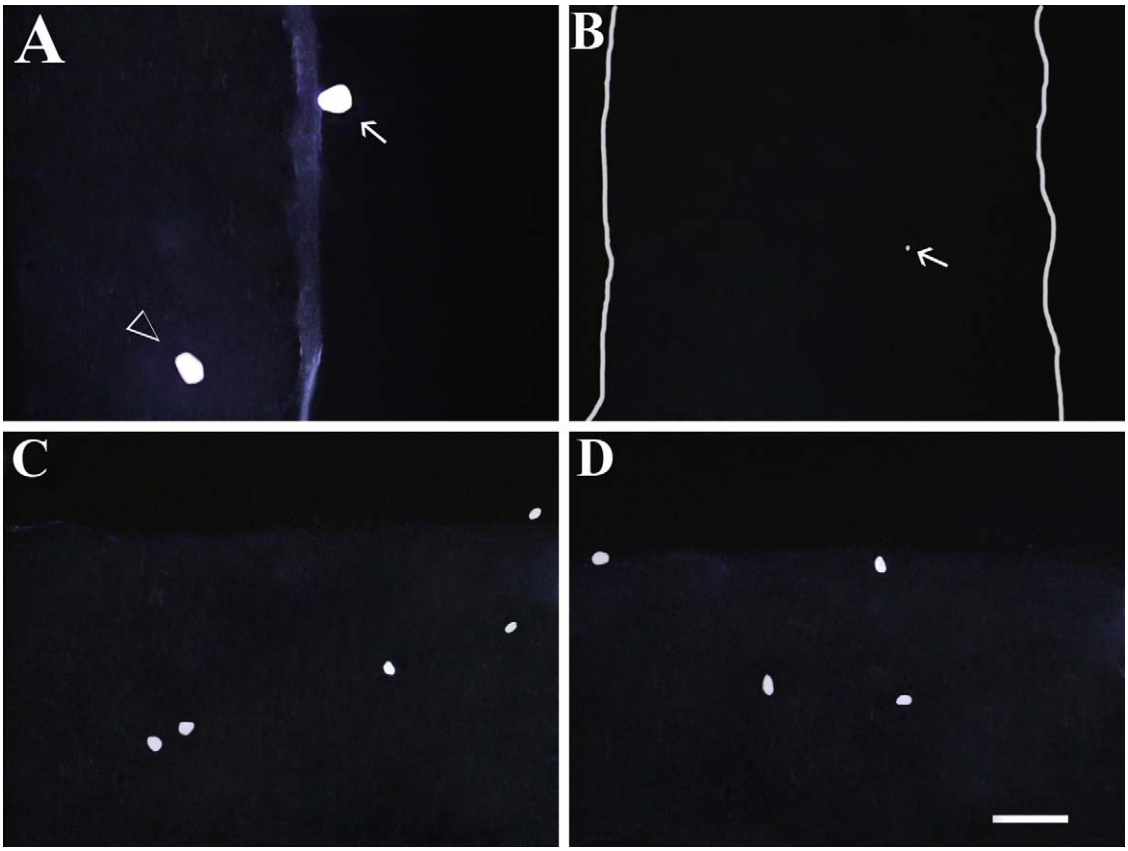

Fig. 1. Tracing Hoechst-labeled Schwann cells 7 days after transplantation to subarachnoid space. (A) A single labeled-Schwann has attached to pia matter (arrow) and the other one has migrated into spinal cord (arrowhead). (B) One Schwann cell (arrow) migrated deep within spinal cord. Groups of migrated Schwann cells were found at the either site of injury (C) or intact area rostral/caudal to injured area (D). Bar = 200 μm (B), 100 μm (C, D), 50 μm (A).

Three groups of animals including Control, Medium and Schwann demonstrated hind limb dysfunction that was maximal during the first few days after the first surgery with the BBB Score of 0 at day 1 post injury (Fig. 2). All groups showed equal BBB Score, 5 days after injury (before transplantation). No drop in the trend of elevating BBB score value of the graph due to the second surgery was observed. The limb dysfunction improved within the following weeks during which the BBB score value of Schwann group outscored that of Medium and Control groups. In Control group, BBB score increased to the score of 9.4 ± 0.3 on day 15 after injury and remained unchanged for the entire period of study. In Medium group the value reached 10.1 ± 0.7 on day 22 and increased to 11.4 ± 0.8 on day 42 after injury and continued more or less as plateau. On the other hand, the score for Schwann group was 13.5 ± 1.1 on day 22 after injury and stayed unchanged for the rest of period. Statistical analysis between groups revealed that cell treatment has made differences in BBB score [F(d.f. 2, 25) = 8.54, $P < 0.001$]. Post hoc analysis proved Schwann group to be significantly recovered compared with both Control and Medium groups ($P < 0.01$). Finally, one animal in Control group, two in Medium and five in Schwann groups were found to have BBB score of 13 (persistent coordinated hindlimb locomotion) or higher at the end of 60 days period.

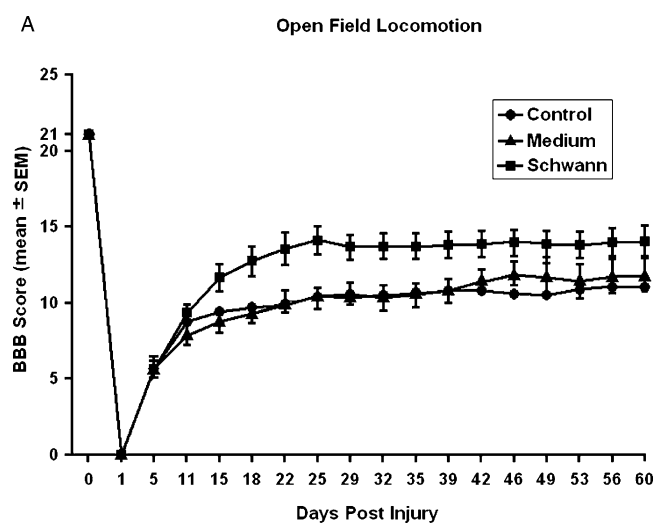

Fig. 2. Effect of subarachnoid Schwann cell transplantation with 7 days delay after injury on BBB score measurements. Subarachnoid Schwann cell transplantation improves hind limb performance. The graph depicts the BBB scores of control (Control), medium-treated (Medium) and Schwann cell-transplanted (Schwann) groups. Schwann cell-treated group was compared against both Control and medium-treated groups and showed significant recovery in locomotion activity ($P < 0.01$).

The effect of subarachnoid injection of Schwann cells on axon density was assessed in Marsland-stained 10-μm thick longitudinal sections obtained from all animals. Control, Medium and Schwann groups represented an axon density of 23916.7 ± 2013.1 per mm^2, 27865.0 ± 2622.5 per mm^2 and 39606.3 ± 4096.7 per mm^2, respectively (Fig. 3A–C). Statistical analysis revealed significant differences of Schwann group with Control ($P < 0.01$) and Medium ($P < 0.05$). However, between Control and Medium groups, there was no significant difference ($P = 0.21$) in axon count values.

Tracing of subarachnoid Schwann cells has provided direct evidence for their presence within injured spinal cord. Subarachnoid-transplanted Schwann cells can directly interact with the host tissue and exert their regenerative effects that have been previously shown for intramedullary transplanted cells. Schwann cells, unlike their precursors, have unique ability to promote their own survival in the injured tissue by the autocrine signals that involve insulin-like growth factor (IGF), platelet-derived growth factor-BB (PDGF), neurotrophin-3 (NT-3) and laminin which supports long term survival of Schwann cells [11,13]. Remyelination of perivascular axons in CNS by Schwann cells in transplanted nerve tissue in subarachnoid space has been previously shown in a model of CNS demyelination [3] and suggested that Schwann cells were able to migrate to the site of injury via perivascular space. In a recent report [21], neurosphere cells were injected through 4th ventricle into CSF in a compression model of spinal cord injury. The grafted cells tended to gather at the injury-affected site, and showed extensive invasion into the spinal cord tissues.

Our results of BBB score in Schwann cell group indicate significant recovery as compared with the other two groups. We evaluated BBB score of animals during the time between injury and transplantation (on 5th day) to ensure an equal basal level of neural deficit among experimental groups. Although our BBB graph is not quantitatively comparable to that of Takami et al. [19], but the results show similar qualitative patterns with a sharp decrease at the time of injury and gradual subsequent rise to an intermediate value then after.

Histological examinations revealed a higher index of axon density and healthier appearance of axons at the site of injury in Schwann cell-treated group, which is compatible with previous reports utilizing Schwann cell transplantation [12]. The possibility of defusing growth factors secreted in subarachnoid space by Schwann cells and their effect on partial recovery cannot be excluded. Growth factors delivery and direct Schwann cell injection have been both shown previously to induce locomotor recovery, and can explain the achieved results in behavioral recovery [4,14].

This study shows efficacy of subarachnoid-transplanted Schwann cells in promoting repair in contused model of injured spinal cord. Administration of the cells through subarachnoid space does not disrupt tissue integrity and thus prevents reinitiation of inflammatory cascades, which have been shown to negatively influence survival or therapeutic potential of the transplanted cells by releasing factors such as IL-1α, IL-6, TNF-α [15], apoptosis-inducing caspase-3, IL-1β, Monocyte Chemoattractant Protein (MCP)-1, Macrophage Inflammatory Protein (MIP)-1β, MIP-1α and Intercellular Adhesion Molecule (ICAM)-1 [1]. We conclude that subarachnoid cell delivery can be considered as an alternate route for delivery of Schwann cells in clinical studies.

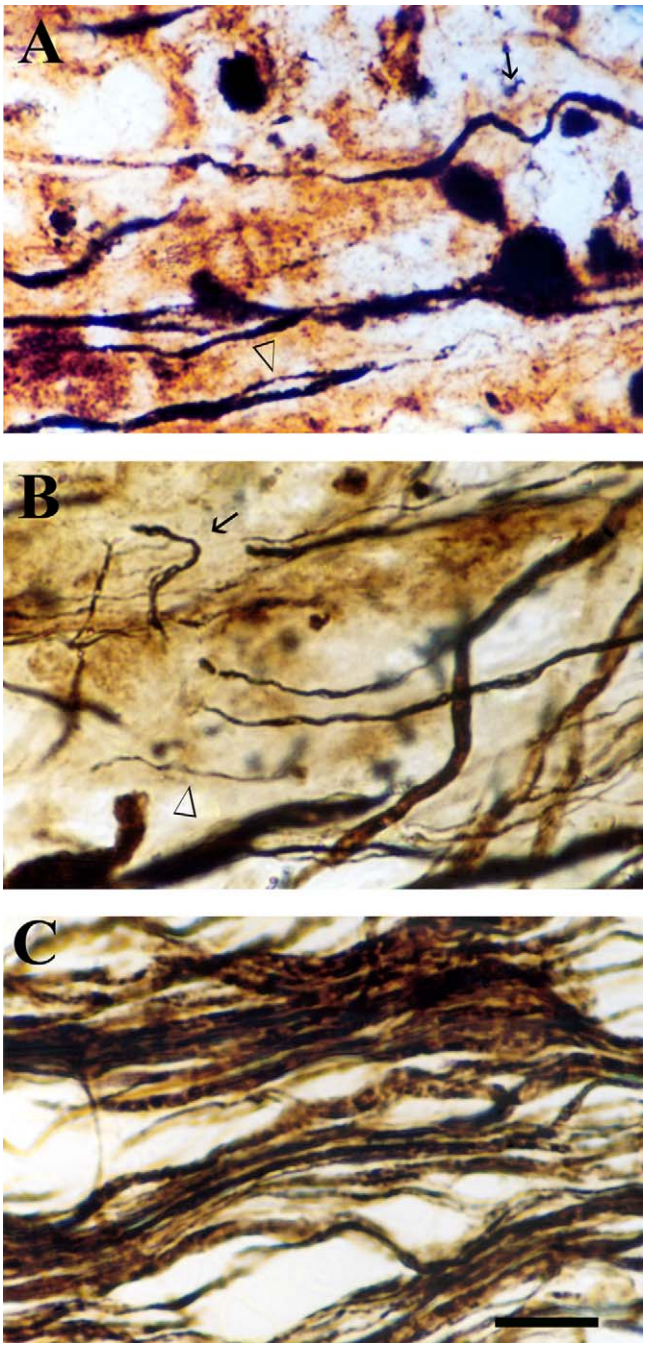

Fig. 3. Axons at the injured zone of spinal cord white matter obtained from Control (A), Medium (B) and Schwann (C) samples cut in longitudinal direction and stained by Marsland method. (A) Axons are thinner (arrowhead) and track in a tortuous manner (arrow) in degenerated white matter at the injured site. (B) Axons appear changed pathologically, regarding axon's diameter and length (arrowhead) and tracking (arrow). (C) Axons are comparatively thicker, well-organized and elongated, tracking in a straight way. Statistical analysis accomplished on the value of axon density in injured white matter area of spinal cord showed significant difference between Schwann cell-treated and both medium-treated ($P < 0.05$) and Control ($P < 0.01$) groups. Bar = 10 μm.

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