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Transplantation of mesenchymal stem cells enhances axonal outgrowth and cell survival in an organotypic spinal cord slice culture

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ABSTRACT

Mesenchymal stem cells (MSCs) have demonstrated a measurable therapeutic effect following transplantation into animal models of spinal cord injury. However, the mechanism(s) by which transplanted cells promote nerve regeneration and/or functional recovery remains indeterminate. Several studies have suggested that MSCs promote tissue repair via secretion of trophic factors, but delineating the effect of such factors is difficult due to the complexity of the *in vivo* systems. Therefore, we developed an organotypic spinal cord slice culture system that can be sustained for sufficient periods of time *in vitro* to evaluate nerve regeneration as an *ex vivo* model of spinal cord injury. Using this model, we demonstrate that treatment of lumbar slices of spinal cord with lysolecithin induced a significant degree of cell death and demyelination of nerve fibers, but that these effects were ameliorated to a significant extent following co-culture of slices with human MSCs (hMSCs). The results indicate that transplanted hMSCs alter the tissue microenvironment in a way that promotes survival of endogenous cells, including injured neurons, immature oligodendrocytes and oligodendrocyte progenitor cells. This *ex vivo* culture system represents a useful tool to further dissect the mechanism(s) by which MSCs promote regeneration of injured nervous tissue.

Spinal cord injury correlates with the death of various cell types, including neurons, oligodendrocytes, astrocytes and precursor cells. A high percentage of astrocytes and oligodendrocytes in the white matter at the site of injury die within a day after injury. The loss of these cells leads to axonal demyelination. Persistent demyelination leads to further loss of axonal transmission and functional disability [17,18]. Both primary and secondary spinal cord injury have been previously studied using various *in vivo* model systems. Secondary injury has been studied by induced demyelination of the spinal cord *in vivo* with lysolecithin injection into the spinal cord of animals [7,9]. Lysolecithin (lysophosphatidylcholine) is a lipid-containing detergent-like protein with activity similar to that of a membrane-solubilizing agent exhibiting myelinating cell-specific toxicity to cause demyelination.

However, in many cases, the interpretation of the experimental results was difficult due to the complex *in vivo* systems. Therefore, *ex vivo* models, such as organotypic slice cultures, have been used to study spinal cord injury. These *ex vivo* cultures allow for the control of the extracellular environment, easy and repeated access while maintaining intact morphology and local synaptic connections *in vitro* [10,11].

Bone marrow-derived mesenchymal stem cells (MSCs) are multipotent and capable of self-renewal [1]. Many studies have shown that MSCs have the potential to differentiate into various mesodermal tissues, including bone, cartilage, fat and muscle [2,6,12,21]. In addition, several recent studies have reported that MSCs can transdifferentiate into neuron-like cells that expressed various neuronal markers and have functional neuronal activity [14,16,19,20]. Transplantation of MSCs into the spinal cord of animal spinal cord injury models shows significant functional and behavioral recovery [4,8]. Furthermore, due to their autologous character and high plasticity, adult stem cell-based cell therapy using human bone marrow-derived MSCs (hMSCs) is considered as a favorable form of stem cell therapy. Application of hMSCs could be very advantageous for the treatment of neurological disorders, including spinal cord injury [18]. Transplantation of hMSCs into the wounded area can open the possibility of nerve regeneration and also play an important role in the maintenance of endogenous cells by establishing microenvironment more favorable for tissue repair [15].

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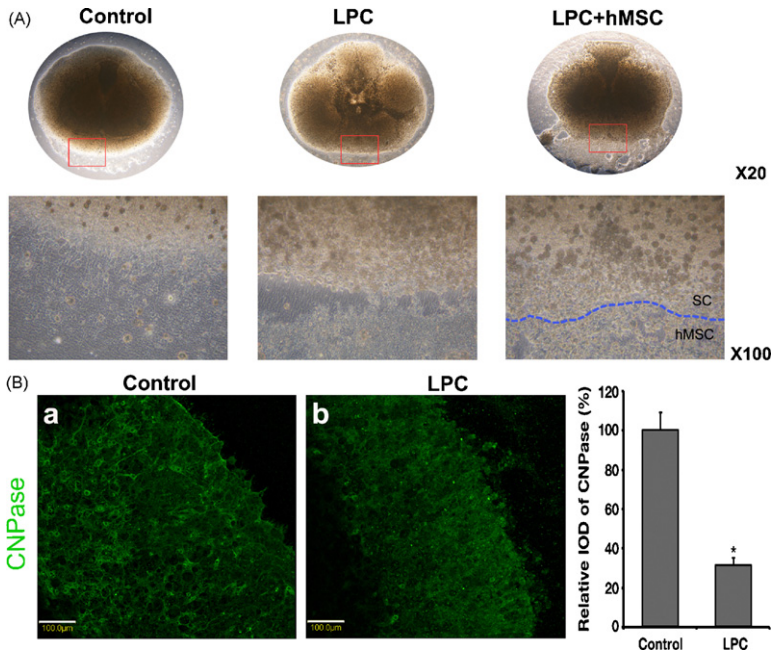

Fig. 1. Organotypic spinal cord slice culture as an *ex vivo* model of spinal cord injury. (A) Histological examination of slice cultures (DIV8). Control: untreated culture; LPC: lysolecithin-treated demyelinated culture; LPC + hMSC: hMSC-transplanted demyelinated culture. The contact region between the spinal cord slice (SC) and transplanted hMSCs is demarcated. (B) The relative IOD of CNPase immunoreactivity in slice cultures. The IOD is normalized to control and expressed as mean ± S.E.M. ($n = 3$; $^*p < 0.05$ vs. control). The myelin marker CNPase was stained with monoclonal anti-CNPase and goat anti-mouse Alexa 488 antibodies (green). Myelin immunostaining is broken and punctate in lysolecithin-treated slice culture compared to the control. Scale bars: 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Therefore, to examine whether there are any protective and axonal outgrowth-facilitating properties by hMSCs, we transplanted hMSCs into a lysolecithin-demyelinated organotypic spinal cord slice culture. Our study clearly demonstrated that transplanted hMSCs significantly increased both axonal outgrowth of nerves and survival of endogenous cells in an *ex vivo* model of spinal cord injury.

Cryopreserved adult hMSCs (Poietics Normal Human Mesenchymal Stem Cells) were purchased from Cambrex, and cultured in MSC growing medium (Cambrex) at 37 °C with 5% CO_2.

Sixteen-day-old Sprague–Dawley rats were anaesthetized with Avertin, the spinal cord was aseptically extracted and placed in ice-cold HBSS (Invitrogen). Next, the spinal cord was cut into 400 μm thick slices using a tissue chopper (McILWAIN). The slices of lumbar parts of spinal cord were placed onto Millicell-CM inserts (Millipore) in six-well plate with 50% MEM with Earl's salts (Invitrogen), 25% HBSS, and 25% horse serum (Invitrogen) supplemented with 20 mM HEPES (Sigma) and 6 mg/ml of D-glucose. The slices were incubated at 37 °C with 5% CO_2 and media was changed twice a week.

At 7 days *in vitro* (DIV), the slices were treated with 0.5 mg/ml lysolecithin (Sigma) for 17 h at 37 °C as previously described in a cerebellar slice culture system [3]. Then, the media was changed with 1 ml of fresh slice culture medium. hMSCs (5×10^4 cells/2.5 μl of PBS) were transplanted directly into the dorsal column of spinal cord slices using Eppendorf Cell Tram Injector (Eppendorf). All slices were incubated for 1–3 weeks at 37 °C with 5% CO_2.

The slice cultures were fixed with 4% paraformaldehyde overnight at 4 °C, permeabilized with 0.5% Triton X-100 in 1% BSA for 10 min, blocked with 0.1% Triton X-100 in 3% BSA for 1 h at room temperature and incubated overnight at 4 °C with monoclonal anti-CNPase (myelin 2,3′-cyclic nucleotide 3′-phosphodiesterase; Covance), polyclonal anti-NF-M (neurofilament-M; Chemicon) or monoclonal anti-Hu (human nuclei; Chemicon) antibodies. Then, the slices were incubated with secondary antibodies, either goat anti-mouse Alexa 488 (Molecular Probes) or goat anti-rabbit Cy3 (Jackson Laboratories).

Each immunostaining was visualized by confocal z-stack laser scanning microscope (Olympus), digitally captured and quantified by morphometric image analysis (integrated optical density, IOD) with the use of a National Institutes of Health program (Scion Image).

Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) staining was performed using In Situ Cell Death Detection Kit (Roche Diagnostics) according to manufacturer's instructions.

The Statistical Package for Social Sciences (SPSS) program was used to perform the data analysis. The data set was tested for normality with Shapiro–Wilk test and *Q*–*Q* normality plots; equality of variance was also tested with the Levene test. Then, one-way analysis of variance was used to determine the difference among three groups. Multiple comparisons with Dunnett T3 method was used to investigate between group differences under probability $p < 0.05$.

Spinal cord slices from postnatal day (*P*) 16 rats were successfully cultured for up to 3 weeks, and their morphological and structural integrity were well preserved. Axonal outgrowth of spinal nerves was observed in the control, while it was destroyed in the lysolecithin-treated slices. However, this destruction of axonal outgrowth was restored after hMSCs were transplanted into the slices (Fig. 1A). We also observed clear demyelination in our organotypic spinal cord slice culture immediately after treatment with 0.5 mg/ml lysolecithin for 17 h. The control slices were smoothly stained with the myelin marker CNPase in the white matter regions. On the other hand, in addition to a decrease in CNPase immunoreactivity, the CNPase staining in the lysolecithin-treated slices appeared broken and punctate. We quantified the changes in CNPase staining induced by lysolecithin in slice cultures by the integrated optical density (IOD) of the CNPase immunoreactivity (Fig. 1B). The IOD was normalized to controls, and expressed as mean ± S.E.M. As shown in Fig. 1B, the relative IOD of CNPase was decreased up to approximately 31% in lysolecithin-treated cultures compared to controls (31.43 ± 4.00%; $p < 0.05$).