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Organotypic Spinal Cord Slice Culture to Study Neural Stem/Progenitor Cell Microenvironment in the Injured Spinal Cord

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ABSTRACT

The molecular microenvironment of the injured spinal cord does not support survival and differentiation of either grafted or endogenous NSCs, restricting the effectiveness of the NSC-based cell replacement strategy. Studying the biology of NSCs in *in vivo* usually requires a considerable amount of time and cost, and the complexity of the *in vivo* system makes it difficult to identify individual environmental factors. The present study sought to establish the organotypic spinal cord slice culture that closely mimics the *in vivo* environment. The cultured spinal cord slices preserved the cytoarchitecture consisting of neurons in the gray matter and interspersed glial cells. The majority of focally applied exogenous NSCs survived up to 4 weeks. Pre-exposure of the cultured slices to a hypoxic chamber markedly reduced the survival of seeded NSCs on the slices. Differentiation into mature neurons was severely limited in this co-culture system. Endogenous neural progenitor cells were marked by BrdU incorporation, and applying an inflammatory cytokine IL-1β significantly increased the extent of endogenous neural progenitors with the oligodendrocytic lineage. The present study shows that the organotypic spinal cord slice culture can be properly utilized to study molecular factors from the post-injury microenvironment affecting NSCs in the injured spinal cord.

Key words: spinal cord injury, organotypic slice culture, neural stem cells, hypoxia, inflammatory cytokine

INTRODUCTION

Traumatic injuries to the spinal cord frequently leave permanent neurological disabilities to the victims and impose enormous economic burdens on the families and society, yet there is no single effective therapeutic option to improve functional recovery. Recent studies have shown promises that

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cellular replacement either by transplantation of neural stem or progenitor cells (NSC) or mobilization of endogenous NSCs could be an effective therapeutic option (Rossignol et al., 2007; Meletis et al., 2008; Cao et al., 2010; Tetzlaff et al., 2010). However, inhospitable microenvironment of injured spinal cord has been shown to limit survival and differentiation of either grafted or endogenous NSCs (Monje et al., 2002; Snyder and Park, 2002; Ishii et al., 2006; Kim et al., 2007). For example, survival of NSC continually dropped after transplantation (Okada et al., 2005; Lee et al., 2009). Moreover, differentiation of NSCs into neuronal or oligodendroglial lineages in the injured spinal cord is significantly hampered (Cao et al., 2001; Cao et al., 2002). Therefore, modification of the inhospitable microenvironment would greatly improve the efficacy of NSC transplantation approach for spinal cord injury.

Since the complexity of cellular and molecular composition can hardly be modeled in dissociated cell culture system, studying potential factors that regulate the microenvironment would necessitate *in vivo* animal experiments. However, screening potential candidate factors in *in vivo* system would require a considerable amount of time and cost (Pena, 2010). An *in vitro* system that allows facile manipulation of the microenvironment and yet closely mimics *in vivo* complex tissue microenvironment, therefore, would be desirable for this purpose. The organotypic slice culture allows long-term maintenance of tissue architecture in dish and has been an important tool in neurobiology research (Gahwiler et al., 1997; Noraberg et al., 2005). The current study sought to establish and characterize the organotypic spinal cord slice culture in which exogenous NSCs are seeded or endogenous neural progenitor cells are marked. In this culture system, we performed exemplary experiments to study possible effects of environmental manipulation on biological behavior of either exogenous NSCs or endogenous neural progenitor cells in a complex tissue environment similar to that of *in vivo* system.

MATERIALS AND METHODS

Culture of human neural stem cells

As an exogenous source of NSCs, we used immortalized human neural stem cell (NSC) line, which has been widely employed in various animal models of CNS diseases (Jeong et al., 2003; Meng et al., 2003; Yasuhara et al., 2006; Lee et al., 2007; Hwang et al., 2009). Preparation and culture of this NSC line has been reported in detail elsewhere (Lee et al., 2009). Briefly, the human NSC line was generated by transducing dissociated cells of fetal human telencephalon tissues (at 14 weeks gestation) by replication incompetent retroviral vector containing v-myc (Flax et al., 1998; Kim, 2004). The permission to use the fetal tissues was granted by the Clinical Research Screening Committee involving Human Subjects of the University of British Columbia, and the fetal tissues were obtained from the Anatomical Pathology Department of Vancouver General Hospital. Cryopreserved NSCs were thawed and cultured in Dulbecco's modified Eagle medium (DMEM; HyClone, Logan, UT, USA) with high glucose supplemented with 5% fetal bovine serum (FBS) and 20 mg/ml gentamicin (Sigma, St Louis, MO, USA) for at least three days before cell seeding. We also generated a NSC line overexpressing green fluorescent protein (GFP) by transducing the human NSC line by retrovirus encoding GFP.

Organotypic spinal cord slice culture and cell seeding procedure

Organotypic spinal cord slice cultures were prepared according to the standard interface method (Stoppini et al., 1991). After decapitation, the brain was removed, and the entire spinal cord block was dissected from P5-7 Sprague Dawley rats through an opening in the ventral side of the spine. Axial slices of the cervical and lumbar cord were dissected and transversely sliced into a 350 um thickness on a McILWAIN tissue chopper (The Mickle Laboratory Engineering Co., Guildford, UK) in sterile Gey's balanced salt solution (Sigma-Aldrich). The slices were then carefully separated with two pairs of fine forceps and transferred to sterile, 30 mm diameter Millipore Milicell-CM (0.4 μm; Millipore, Bedford, MA) culture plate insert, using a glass Pasteur pipette. Five or six randomly selected slices that looked apparently intact and undamaged were transferred and placed on each insert. The inserts were placed in 35 mm diameter

culture wells (six well culture trays; BD Falcon, Franklin Lakes, NJ). Cultures were maintained in 1 ml of the serum-based medium containing 50% Basal Medial Eagle (Sigma-Aldrich), 25% Hank's Balanced Salt Solution (GIBCO), 2.2 g glucose, 1 mM GlutaMAX-I supplement (Invitrogen, Carlsbad, CA), and 20% FBS. Culture plates were incubated at 37°C in a 5% CO_2-95% O_2 humidified incubator. Culture medium was changed 4 hours after harvesting and then twice per week. The level of the medium was adjusted to slightly below the surface of the slices in order to provide a sufficient supply of the culture medium and mixed gases. Cell seeding was performed 7 days after initial slice preparation. The human NSCs were trypsinized just before seeding, and a total of 1,000 cell/1 μl cells for each slice were seeded using a glass micropipette. Special care was taken to avoid touching slices with micropipette. One day after seeding, the culture medium was changed. To induce differentiation of NSCs grown on top of slices, the FBS concentration was lowered to 5%. To identify seeded NSCs, cells were prelabeled with BrdU or DiI. For BrdU prelabeling, cells were treated with 2 μM BrdU dissolved in culture media for 24 hours prior to harvesting for transplantation. The human NSCs were labeled Vybrant™DiI (Molecular Probe) according to the manufacturer's instruction. To mark endogenous proliferating neural progenitor cells, BrdU at a concentration of 1.0 μM was added to the culture media one day before fixation (24 hours incubation). IL-1 β (R&D systems, Minneapolis, MN) was added to the media at a concentration of 20 ng/ml for three days before fixation.

Exposure of cultured slices to a hypoxic chamber

To mimic secondary injury process after spinal cord injury, cultured spinal cord slices were exposed to a hypoxic chamber (Forma Scientific, Marietta, OH). Glucose-free medium DMEM was saturated with nitrogen gas mixture (95% N2, and 5% CO_2) for 40 min to obtain an O_2 gas pressure close to zero, as measured by a dip-type O_2 microelectrode. After saturation, the inserts with spinal cord slices were placed in 1 ml of saturated glucose-free medium DMEM and then maintained at 37°C in a N2 saturated environment. Therefore, the cultured slices were challenged by aglycemic hypoxic stress. After 40 minutes in the chamber, the inserts were moved to the fresh culture medium and atmosphere with 5% CO_2-95% O_2. One day after, NSCs were seeded as described above.

Slice processing and immunohistochemistry

Slices were washed in PBS and fixed in 4% paraformaldehyde for 5 min. Slice was excised from the culture insert together with the attached membrane, and each slice is transferred to a 24 well plate. The slices were permeabilized and blocked by 0.5% triton with 10% goat serum for 2 hours. Then, slices were incubated overnight with primary antibodies at 4°C or 2 hours at room temperature. We used polyclonal NG2 antibody (1 : 1,000; Millipore, Bedford, MA) as a marker for oligodendrocyte progenitors, polyclonal GFAP antibody (1 : 500; Dako, Carpinteria, CA) for astrocytes, Tuj1 (1 : 500; Millipore, Bedford, MA) for immature neurons, CD11b (1 : 300: Abcam, Cambridge, UK) for resident microglia, and BrdU (1 : 500; Serotec, Oxford, UK) for a marker of proliferating NSCs. For BrdU staining, DNA denaturation was achieved by treatment with 2 M HCl at room temperature for 60 min followed by incubation for 30 min with 0.1 M Borate solution (Sigma-Aldrich). After thorough rinsing, slices were incubated by rat IgG secondary antibody tagged with Alexa Fluor 594 or 488 (Molecular Probes, Eugene, OR) for 1 hour at room temperature to visualize antigen-antibody complex.

RESULTS

We first characterized the histological architecture of cultured spinal cord slices. When the cultured slice was view with the transmitted light, the gray matter was apparently distinguished from the surrounding white matter (Fig. 1A). Neurofilament staining revealed clear margin of the dorsal horn where profuse axons and scattered neuronal cell bodies were observed (Fig. 1B). In contrast, axonal fibers in the white matter were sparsely observed, suggesting that axons in the white matter tract underwent some degree of degeneration after disconnected from the cell bodies. In the ventral horn, large neurons suggestive of spinal motor neurons were observed (Fig. 1C, D). They grew long neu-

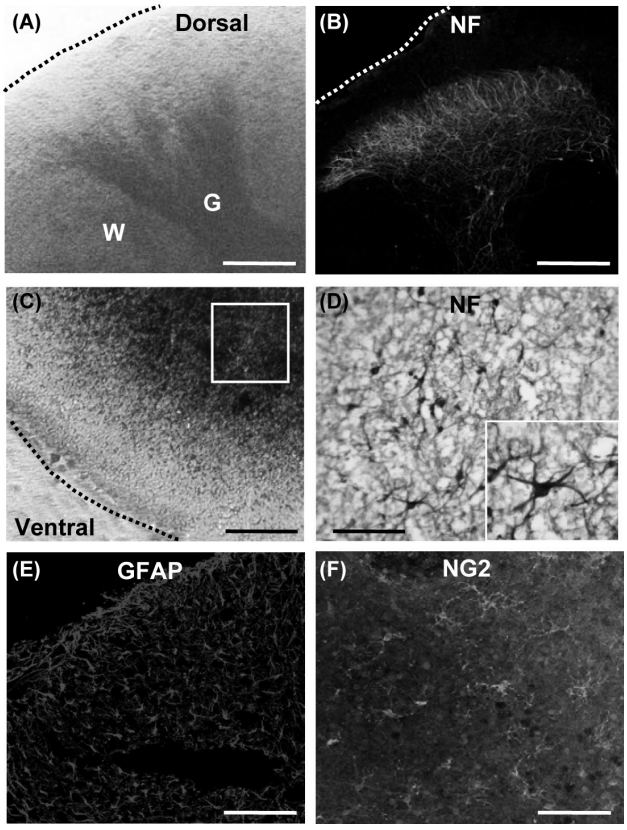

Fig. 1. Characterization of cultured spinal cord slices. (A) A dorsal part of spinal cord slice under the transmitted light showed a clear distinction between the white (W) and gray (G) matter. A dotted line indicates the outer margin of the slice. (B) Immunofluorescent staining with neurofilament (NF) antibody showed profuse axonal branching and scattered neurons in the dorsal horn. (C) A transmitted image of the ventral part of the slice. A dotted line indicates the outer margin of the slice. (D) Neurofilament immunostaining of the region boxed in (C) showed large multipolar neuron in the ventral region. Inset, a magnified image of a neuron. (E, F) Glial cells in the spinal cord slice culture (E) GFAP positive astrocytes, (F) NG2 positive oligodendrocyte progenitors. All scale bars represent 200 μm.

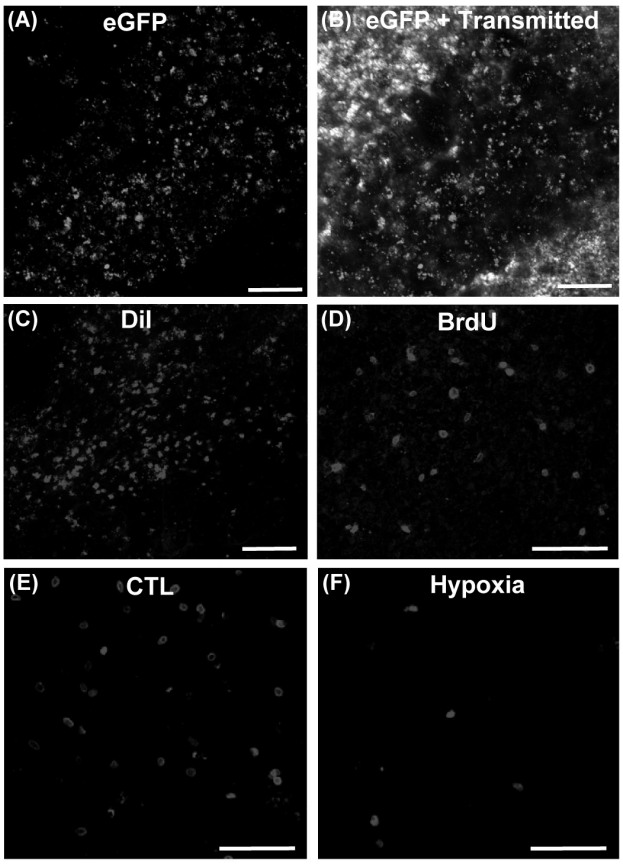

Fig. 2. Survival of neural stem cells co-cultured on spinal cord slices. (A～D) Human neural stem cells (NSCs) were seeded onto the surface of the spinal cord slices and co-cultured up to 4 weeks. The NSCs were prelabeled by eGFP (A, B), DiI (C) or BrdU (D). In a transmitted image (B), NSCs were found to grow on top of the slice. The slices were fixed at 4 weeks after seeding, and the surviving NSCs were readily observed. (E, F) Mimicking post-injury environment by exposure to hypoxic chamber. The survival of NSCs seeded on the slice preexposed to a hypoxic chamber for 40 minutes was markedly reduced compared to that on the control (CTL) slice. All scale bars represent 50 μm.

rites within the slice indicating that the neurons were healthy and made connections with different neurons in the slice. We also characterized glial cells in the spinal cord slices. GFAP staining showed a large number of astrocytes located in both the white and gray matter (Fig. 1E). Numerous oligodendrocyte progenitors expressing NG2 proteoglycan (Dawson et al., 2000) were also observed. However, CD11b (OX42) staining did not show apparent microglial cells in the slice (data not shown), suggesting that no macrophages migrate to the spinal cord to become resident microglial cells before postnatal day 5. Together, these results showed that the spinal cord slices maintain characteristics of the cellular and tissue architecture of the *in vivo* spinal cord tissue.

To mimic NSC grafting into the spinal cord, we seeded NSCs on top of cultured spinal cord slices using the Hamilton syringe at between 7 to 10 days after initial culture. The seeded hNSCs on spinal cord slice culture were prelabeled by DiI or BrdU. In some experiments, we used GFP expressing hNSCs to identify the seeded cells. Many of the cells survived the seeding procedure and were identified up to 4 weeks after seeding (Fig. 2A～D), the last time point we measured. They were

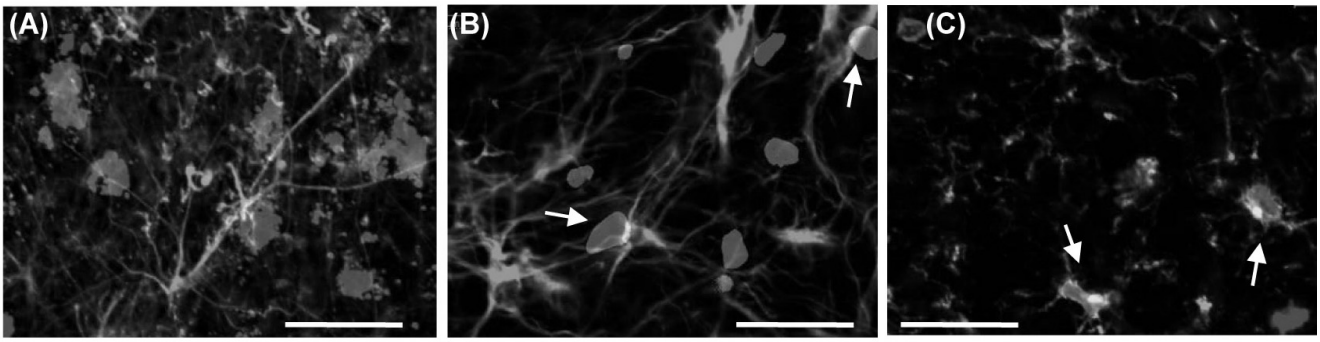

Fig. 3. Differentiation of co-cultured neural stem cells on cultured slices. (A～C) Prelabeled neural stem cells with DiI (A) or BrdU were immunostained with phenotype specific markers such as Tuj-1 (A), GFAP (B), and NG2 (C). There were no NSCs expressing Tuj-1, but some of them differentiated into GFAP positive astrocytes and NG2+ oligodendrocyte lineage cells. Arrows indicate cells with colocalization. All scale bars represent 50 μm.

dispersed throughout the surface of the spinal cord slice, but many of them were found at or near the parts of the slice where they were dislodged from the Hamilton syringe (Fig. 2B). Dispersing cells did not show any preference to either the white or gray matter. These findings indicated that NSCs can be cultured on top of cultured spinal cord slices, providing an opportunity to examine biological behavior of NSCs in an environment which closely mimics *in vivo* spinal cord tissue, yet is still amenable to experimental manipulation. Transplantation of NSCs into injured spinal cord is usually complicated by a poor survival of grafted cells (Okada et al., 2005; Lee et al., 2009). Therefore, increasing survival of grafted NSCs could lead to an improved efficiency of NSC transplantation strategy. To mimic the post-injury microenvironment, cultured spinal cord slices were exposed to hypoxic and aglycemic chamber for 40 minutes. Then NSCs were seeded onto the slice, and the survival of NSCs was compared to NSCs seeded onto control slices without exposure to the hypoxic chamber. We found that pre-exposure of slices to hypoxic and aglycemic chamber markedly reduced the survival of NSCs compared to those grown on control slices (Fig. 2E, F).

The spinal cord has been regarded as non-neurogenic. When NSCs collected from spinal cord were transplanted into spinal cord, they could not differentiate into neurons, whereas the same NSCs differentiated neurons when they were transplanted into the brain (Shihabuddin et al., 2000). We examined differentiation of NSCs co-cultured on spinal cord slices. NSCs did not express neuronal marker Tuj-1 (Fig. 3A). Addition of retinoic acid into the culture medium did not increase Tuj-1 expression (data not shown). Some NSCs (20% of BrdU+ cells) were colocalized with GFAP, indicating differentiation into astrocytic lineage (Fig. 3B). In addition, they were able to differentiate into NG2 positive oligodendrocytic lineage cells (26% of BrdU+ cells; Fig. 3C). Therefore, the microenvironment created by cultured spinal cord tissue does not seem to be conducive to neuronal differentiation of NSCs, lending a support to the notion that spinal cord is non-neurogenic.

Endogenous glial progenitor cells are present in adult spinal cord and increase their number in response to spinal cord injury (Horner et al., 2000; McTigue et al., 2001). It is possible that they can differentiate into mature oligodendrocytes that may participate in spontaneous remyelination process (Yang et al., 2006), although the extent of remyelination is often limited. To recapitulate the glial progenitor cells in a complex environment, we marked proliferating neural progenitor cells by BrdU incorporation (Fig. 4A). As expected from the *in vivo* observation (Horner et al., 2000), BrdU positive progenitor cells were never colocalized with neuronal markers (Data not shown). Instead, they showed expression of glial marker such as GFAP (Fig. 4B) and NG2 (Fig. 4C), suggesting that the proliferating neural progenitors are already committed to glial rather than neural lineage. To examine whether the molecular microenvironment in the injured spinal cord could affect the lineage

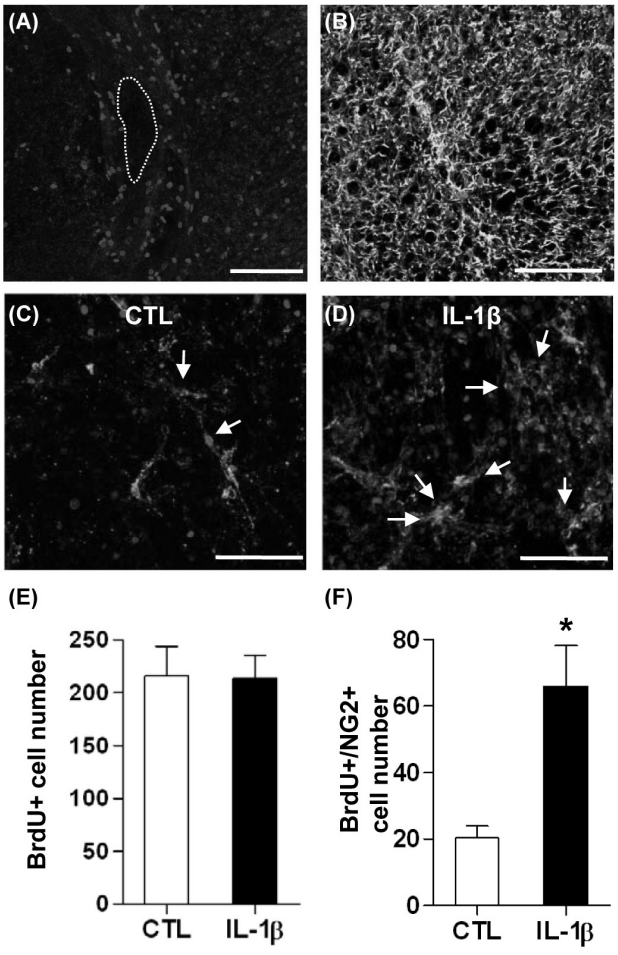

Fig. 4. Influence of inflammatory cytokine on neural progenitor cells in cultured spinal cord. (A) BrdU staining of proliferating neural progenitor cells in a cultured spinal cord slice. A dotted line indicates the margin of the central canal. (B) Visualization of neural progenitor cells differentiated into astrocytes. BrdU staining is shown in red and GFAP in green. (C, D) Influence of inflammatory cytokine IL-1β on differentiation of neural progenitor cells into the oligodendrocytic lineage. IL-1β (D) or PBS (C) as a control (CTL) was added to the culture media for three day. BrdU staining is shown in red and NG2 proteoglycan, a marker of oligodendrocyte progenitor, in green. All scale bars represent 50μm. (E, F) Quantification of BrdU+ cell number (E) or BrdU+/NG2+ cell number (F). Asterisk indicates a statistical difference at *$p<0.05$ by student T-test.

determination of glial progenitor cells, we chose IL-1β which is a well characterized proinflammatory cytokine and know to be unregulated after spinal cord injury (Rice et al., 2007). The number of total proliferating cells was not affected by IL-1β (Fig. 4E). However, addition of IL-1β increased the number of NG2+ oligodendrocyte lineage cells by more than three folds ($p<0.05$, n=3 slices per condition) (Fig. 4C, D, F).

DISCUSSION

We found that in organotypic spinal cord slices, regional specificity such as gray and white matter is conserved and cellular diversity and/or complexity is maintained encompassing neurons and glial cells. The fact that the cultured slices retain major characteristics of *in vivo* spinal cord tissue implies that seeded NSCs on spinal cord slices are exposed to the microenvironment very similar to the *in vivo* spinal cord tissue. It has to be mentioned, however, that the cultured spinal cord slices cannot represent all the *in vivo* properties. For example, there were no microglial cells in the slices that play various functions of paramount importance in inflammatory conditions (Ankeny and Popovich, 2009). In addition, the majority of long white matter tracts surrounding gray matter seemed to be degenerated, indicating that contribution of myelin cannot be mimicked in cultured spinal cord slices. We considered, however, that the advantage of *in vitro* system for being easily amenable to experimental manipulation may offset a large part of the differences between the *in vivo* tissue and the organotypic spinal cord slice. Taken together, our findings suggested that organotypic slices can mimic the complex spinal cord tissue environment, and it would be feasible to study environmental factors affecting NSC using in the injured spinal cord.

It has been increasingly clear that adult CNS, especially diseased CNS, is not always favorable to the integration NSCs with host tissue (Bjorklund and Lindvall, 2000; Snyder and Park, 2002; Okano et al., 2003). Injured CNS microenvironment considerably limits the survival of grafted cells (Emgard et al., 2003; Bakshi et al., 2005; Lee et al., 2009), which may pose a significant hurdle to be overcome before NSC transplantation strategy is applied to human patients. Traumatic spinal cord injuries are usually complicated by a breakdown of blood supply leading to tissue ischemia and hypoxia (Chu et al., 2002). As an example of altering the microenvironment in a manner similar to the spinal cord injury, the cultured spinal cord slices were pre-exposed to a hypoxic (aglycemic as well) chamber before the NSCs were seeded on the them. Although hypoxic injury is not supposed to

replicate all the changes related to traumatic injuries, we found that exposure to hypoxic chamber for 40 minutes did make a difference in the survival of seeded NSCs on the slices. It is assumed that hypoxic conditions altered the environment of spinal cord slices to become more inhospitable for NSCs to survive. Therefore, the hypoxic condition used in this experiment can be used to screen potential factors or small molecules that regulate the survival of grafted NSCs in the injured spinal cord.

After spinal cord injury, demyelination of spared white matter significantly hampers spontaneous function recovery (Kim et al., 2007). Therefore, preventing demyelination or promoting remyelination is one of the key strategies to improve function outcomes after spinal cord injury (McDonald and Belegu, 2006). Modifying the microenvironment of the injured spinal cord may improve the extent of oligodendrogenesis and ultimately promote remyelination. We tested whether inflammatory molecules can affect the fate of glial progenitors, especially oligodendrocytic lineage, in the cultured spinal cord slice. The cultured spinal cord slices closely recapitulated *in vivo* glial progenitors since the proliferating cells showed only glial rather than neuronal lineage. Therefore, this culture system allowed a convenient, yet highly relevant assay system to identify a potential environmental factor. It can be envisioned that the organotypic spinal cord culture system is used as a platform of an assay screening a potential candidate from small molecule library to modify inhibitory microenvironment for endogenous neural progenitors (Wang et al., 2006).

To summarize, the current study established the utility of the organotypic spinal cord slices to study neural stem/progenitor cell microenvironment in the injured spinal cord. Co-culture of exogenous NSCs was feasible mimicking post-graft environment. Exposure of the cultured slices to hypoxic chamber mimicked the post-injury environment in that the survival of seeded NSCs was reduced. Cultured spinal cord slices retained the non-neurogenic characteristics of *in vivo* spinal cord tissue since they did not support neuronal differentiation of either exogenous NSCs or endogenous neural progenitors. The cultured spinal cord slices also provided an opportunity to examine the influence of inflammatory environment mimicking post-injury condition on endogenous neural progenitors. Collectively, we conclude that the organotypic spinal cord slice culture can be properly utilized to study molecular factors from the post-injury microenvironment affecting NSCs in the injured spinal cord.

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