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The roles of neuronal and glial precursors in overcoming chondroitin sulfate proteoglycan inhibition

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ABSTRACT

The extension of axons through the major inhibitory component of the glial scar, chondroitin sulfate proteoglycans (CSPGs), remains a key obstacle for regeneration following spinal cord injury (SCI). We have previously shown that transplants composed of neuronal and glial restricted precursors (NRP and GRP respectively) promote regeneration and connectivity in the injured spinal cord (Bonner et al., 2010, 2011), however, little is known about the properties of these precursors at a cellular level. We now report that NRP-derived neurons, in contrast to dorsal root ganglion (DRG) neurons, have the ability to extend axons and cross over from a permissive substratum (laminin) onto inhibitory CSPG in vitro. Growth cones of neurons derived from NRP, compared to DRG, exhibit significantly lower levels of the CSPG receptors protein tyrosine phosphatase sigma (PTPσ) and leukocyte common antigen-related phosphatase (LAR). GRP-conditioned medium prepared from the same cell densities did not affect the response of primary sensory neurons to CSPG confirming that the ability of NRP-derived neurons to cross onto CSPG is determined intrinsically. However, GRP-conditioned medium collected from high density cultures increased the probability of DRG axons to cross from LN onto CSPG and increased the length of DRG axons extending on CSPG. Collectively, these results suggest that (1) neurons derived from NRPs are intrinsically insensitive to CSPGs due to low levels of receptor expression, and (2) high levels of factors secreted by GRP can reduce the inhibitory effects of CSPG and promote axonal growth. These observations provide mechanistic insights into the specific roles of NRPs and GRPs in promoting regeneration and repair following SCI.

Introduction

The limited regenerative capacity of CNS neurons and the formation of an inhibitory environment at the injury site represent major challenges for regeneration and repair of spinal cord injury (SCI) (Fitch and Silver, 2008; Silver and Miller, 2004). SCI induces the formation of a glial scar composed of several growth-inhibitory molecules, including chondroitin sulfate proteoglycans (CSPGs) (Fawcett, 2006; Rhodes and Fawcett, 2004; Sandvig et al., 2004). CSPGs are upregulated at the lesion site of CNS injuries and present a barrier for axon regeneration (Fitch and Silver, 2008; Jones et al., 2003; Properzi et al., 2003, 2005). In vitro model systems designed to mimic the distribution of CSPG at the lesion site have demonstrated that when axons are initially growing on a permissive substratum they stop/turn when they encounter CSPGs (Dou and Levine, 1994; Hynds and Snow, 1999, 2001; Schmalfeldt et al., 2000; Tom et al., 2004; Yu and Bellamkonda, 2001).

The use of embryonic neurons for cell transplantation is a promising therapeutic strategy for SCI with the potential of repairing damaged circuitry by providing replacement neurons and promoting connectivity. Previous studies have been successful in generating a neuronal relay across the injured spinal cord using a combination of neuronal (NRP) and glial restricted precursor (GRP) cell transplants and neurotrophin support (Bonner et al., 2010, 2011). In these studies, the axons of sensory neurons extended into the injury site and formed synaptic connections with graft-derived neurons. In addition, axons of graft-derived neurons were guided out of the injury site and into the dorsal column nucleus to form a functional relay. Further elucidation of the respective roles of NRP and GRP in reconnecting the injured spinal cord will yield insights into how these cells may be used in a therapeutic context.

We have previously shown that NRP-derived neurons will extend axons on uniform CSPG-coated substrata (See et al., 2010). The goal of the present study was to determine if the axons extending from these neurons could cross over from a permissive substratum into a territory enriched with CSPGs. We report that the axons of NRP-derived neurons

Abbreviations: NRP, neuronal restricted precursor; GRP, glial restricted precursor; DRG, dorsal root ganglia; NGF, nerve growth factor; bFGF, basic fibroblast growth factor; CNS, central nervous system; SCI, spinal cord injury; CSPG, chondroitin sulfate proteoglycan; LN, laminin; PL, poly-L-lysine; PTPσ, protein tyrosine phosphatase sigma; LAR, leukocyte common antigen-related phosphatase; HDCM, high density conditioned medium; SEM, standard error mean.

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readily cross into CSPG enriched substrata in vitro. The ability of these axons to cross onto CSPG correlates with greatly decreased levels of CSPG receptors in the growth cones of these neurons, relative to primary sensory neurons that do not cross onto CSPG. Furthermore, conditioned medium from GRP cultures, the same density as in co-cultures of NRP/GRP, does not affect the crossing of DRG axons onto CSPG. However GRP-conditioned medium from high density cell cultures reduced the inhibitory effects of CSPG on DRG neurons. These results indicate that (1) NRP-derived neurons are intrinsically insensitive to CSPG and (2) GRPs secrete factors that promote axon extension of sensory neurons into inhibitory regions of CSPG. Collectively, these results identify properties of NRPs and GRPs that are beneficial for cell transplantation-based strategies designed to promote regeneration and connectivity in SCI.

Materials and methods

NRP/GRP cultures for CSPG border crossing assays

Cultures of NRP and GRP were obtained from spinal cords of embryonic day 13.5 rat from three separate litters following published protocols (Lepore et al., 2004). Mixed cells of NRP/GRP were plated at a concentration of 10,000 cells per coverslip (18 × 18 mm) containing prepared CSPG borders. Control basal medium contained DMEM-F12 (Gibco), B27 supplement (20 μL/mL; Gibco), Pen-Strep (100 IU/mL; Gibco), BSA (1 mg/mL; Sigma), N2 supplement (10 μL/mL; Gibco), bFGF (10 μg/mL). Control basal medium was supplemented with NT-3 (10 μg/mL; Peprotech) and retinoic acid (10 μM; Sigma) to promote neuronal differentiation. Cultures were fixed 24 h after plating for analysis. Substratum preparation is described below.

DRG cultures

Embryonic day 7 chicken dorsal root ganglia were cultured as explants and supplemented with 20 ng/mL nerve growth factor (R&D Systems, Minneapolis, MN) to promote neuronal outgrowth following published protocols (Lelkes et al., 2006). Dissociated neurons from dorsal root ganglia were obtained from rat embryonic day 19 (E19) and supplemented with 50 ng/mL nerve growth factor. Both sets of neurons (chick and rat) were cultured either in (1) control basal medium or (2) conditioned medium (described below).

NRP/GRP- and GRP-conditioned medium

To address if cells in mixed NRP/GRP cultures are secreting factors that influence NRP-derived axons in crossing onto CSPG, conditioned medium was collected from both mix cultures of NRP/GRP and isolated cultures of GRP. In order to assure that the cell densities (10,000 cells plated on 18 × 18 mm coverslips in 500 μL of medium) in cultures used to generate conditioned medium were equivalent to the density of NRP/GRP cultures used in the NRP crossing border assays, NRP/GRP- and GRP-conditioned medium was collected 24 h after culturing NRP/GRP or GRP at the same density (see above) and on the same substrata (see below) as prepared in the crossing assays. The conditioned medium was then transferred to rat and chick DRG cultures for 24 h.

GRP high density conditioned medium

To determine if GRPs secrete factors that alter the response of primary DRG axons to CSPG borders, GRP-conditioned medium was also tested from high density cultures (GRP-HDCM). Purified rat GRP (P5) was plated onto T25 flasks coated with PL + LN and allowed to recover overnight in basal medium supplemented with bFGF (40 ng/mL; Peprotech). The following day the cells were washed with HBSS and cultured for 3 days in basal medium supplemented with bFGF (30 ng/mL). On the third day media were harvested and utilized for further experimentation. Final concentration of cells was approximately 1 million GRP per mL.

To further investigate the properties of GRP-secreted factors, dilution series were performed with GRP-HDCM. DRG rat neurons were cultured on prepared CSPG borders and cultured with varying dilutions of GRP-HDCM; 100% GRP-HDCM, 50% GRP-HDCM, 10% GRP-HDCM, or 100% control basal medium supplemented with bFGF (30 ng/mL). The concentrations of GRP-HDCM were acquired by diluting GRP-HDCM with control medium.

CSPG border preparation

Coverslips (18 × 18 mm) were coated with 100 μg/mL of poly-D-lysine (PL) (70 KD MW; Sigma) overnight. The following day, coverslips were washed and dried. Using fine forceps, a 1 mm thick strip of filter paper (Fisherbrand P5) saturated with 3 μL of 100 μg/mL chondroitin sulfate proteoglycan (combination of brevican, phosphacan, aggrecan, and versican, CC117; Millipore) was placed on the coverslip. The filter paper was removed once it had completely dried. Following washing, 15 μg/mL laminin (LN) (Invitrogen) was applied to the coverslip for 1 h at 37 °C. Increasing the LN/CSPG ratio directly affects growth cone advance onto the inhibitory CSPG (Snow and Letourneau, 1992), therefore we tested various concentrations of CSPG (10–200 μg/mL) with DRG neurons to determine the appropriate concentration for turning response to CSPG border. For analysis of border assays, we used a concentration of CSPG (100 μg/mL) which showed turning behavior of DRG axons greater than 95%.

Pre-treatment of CSPG borders with conditioned medium

Coverslips containing prepared CSPG borders were pre-treated with 500 μL of either (1) GRP-HDCM or (2) control basal medium for 24 h. Coverlips were washed three times with control basal medium and used for plating rat E19 DRG. Following pre-treatment of substratum, dissociated sensory neurons were cultured with control basal medium supplemented with 50 ng/mL NGF. For positive control, DRG neurons were cultured with GRP-HDCM in parallel.

Immunocytochemistry

Cultures were fixed with 4% PFA (Electron Microscopy Sciences) containing 5% sucrose for 15 min, and incubated in blocking solution containing 10% goat or donkey serum (Gibco) and 0.1% Triton X-100 (Sigma) in phosphate-buffered saline (PBS) for 20 min. To visualize the CSPG border, coverslips were stained using mouse (IgM) anti-CSPG (clone CS-56, 1:500; Sigma). To label neurons, coverslips were stained with either (1) the neuronal specific primary antibody TUJ1 (rabbit monoclonal 1:1000; Covance) for NRP/GRP and rat DRG cultures, or (2) fluorescein-conjugated anti-tubulin monoclonal antibody (DM1A clone, 1:100; Sigma) for chick DRG cultures. For receptor staining, cultures were incubated with anti-beta III tubulin (TUJ1) and either anti-hPTPRσ (goat IgG 1:100; AF3430, R&D Systems) or anti-LAR (H-70) (rabbit IgG 1:200; sc-25434, Santa Cruz). All cultures were stained with primary antibodies overnight at 4 °C, followed by washing and staining with secondary antibodies for 1 h at room temperature. All cultures were stained for DAPI (Vectashield with Dapi) to visualize nuclei of all cells.

Imaging and analysis

For consistency of analysis between cell cultures, images were taken at regions identifying the border between LN and LN + CSPGs (using CS-56 antibody) in which cell density was sparse in order for the number of individual axons and cell bodies to be accurately calculated. All images were obtained and analyzed using an Axiovert