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Olfactory ensheathing cell–neurite alignment enhances neurite outgrowth in scar-like cultures

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ABSTRACT

The regenerative capacity of adult CNS neurons after injury is strongly inhibited by the spinal cord lesion site environment that is composed primarily of the reactive astroglial scar and invading meningeal fibroblasts. Olfactory ensheathing cell (OEC) transplantation facilitates neuronal survival and functional recovery after a complete spinal cord transection, yet the mechanisms by which this recovery occurs remain unclear. We used a unique multicellular scar-like culture model to test if OECs promote neurite outgrowth in growth-inhibitory areas. Astrocytes were mechanically injured and challenged by meningeal fibroblasts to produce key inhibitory elements of a spinal cord lesion. Neurite outgrowth of postnatal cerebral cortical neurons was assessed on three substrates: quiescent astrocyte control cultures, reactive astrocyte scar-like cultures, and scar-like cultures with OECs. Initial results showed that OECs enhanced total neurite outgrowth of cortical neurons in a scar-like environment by 60%. We then asked if the neurite growth-promoting properties of OECs depended on direct alignment between neuronal and OEC processes. Neurites that aligned with OECs were nearly three times longer when they grew on inhibitory meningeal fibroblast areas and twice as long on reactive astrocyte zones compared to neurites not associated with OECs. Our results show that OECs can independently enhance neurite elongation and that direct OEC–neurite cell contact can provide a permissive substrate that overcomes the inhibitory nature of the reactive astrocyte scar border and the fibroblast-rich spinal cord lesion core.

Introduction

Olfactory receptor neurons are generated and then project their axons from the peripheral into the central nervous system throughout life (Graziadei and Monti Graziadei, 1985). The regenerative ability of the olfactory receptor neurons is enhanced by olfactory ensheathing cells (OECs), a distinct glia with features of both Schwann cells and astrocytes (Doucette, 1991; Ramón-Cueto and Valverde, 1995). After an olfactory nerve injury, OECs survive and maintain a conduit so that newly generated axons can grow into the damaged inhibitory areas of the adult olfactory system, cross the glia limitans, and contact their olfactory bulb targets (Doucette, 1991; Li et al., 2005). Due to these attributes, OECs are considered a promising treatment following spinal cord injury (SCI; Lu et al., 2002; López-Vales et al., 2006; Kubasak et al., 2008; Ramón-Cueto et al., 1998, 2000; Tabakow et al., 2014; Takeoka et al., 2011; Ziegler et al., 2011).

While adult CNS neurons have a capacity to regenerate, they usually fail to regrow functional axons due to a non-permissive or inhibitory environment. After injury, astrocytic cell bodies hypertrophy, and their processes widen, cluster together, elongate, and display increased glial fibrillary acidic protein (GFAP) immunoreactivity, a response defined as reactive astrogliosis (Barrett et al., 1981; Reier and Houle, 1988; Silver and Miller, 2004; Sofroniew, 2009). A GFAP-positive scar border of reactive astrocytes forms due to both the injury and the invasion of meningeal fibroblasts (Silver and Miller, 2004; Wanner et al., 2013). The processes of newly generated reactive astrocytes become oriented transversely to isolate the intact spinal cord from the lesion core, a response that limits axon regeneration (Barnabé-Heider et al., 2010; Li et al., 2012; Wanner et al., 2013). Additionally, increased chondroitin sulfate proteoglycan (CSPG) and class 3 semaphorin (Sema3) expression contribute to the inhibitory environment formed at the lesion site (Buss et al., 2009; Fitch and Silver, 2008; Hu et al., 2010; Pasterkamp et al., 2001). A scar-like culture model (Wanner et al., 2008) replicates the widespread reduction of neurite outgrowth as a result of reactive astrogliosis and elevation of inhibitory CSPGs: phosphacan, neurocan, and tenascin. The scar-like environment in this model is generated by the addition of two injury-inducing factors to quiescent astrocytes: 1) confrontation with meningeal fibroblasts and 2) mechanical stretch (Wanner et al., 2008).

OECs may overcome the injury site inhibition and promote neurite sprouting and outgrowth by providing both an adhesive cellular

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substrate and permissive soluble factors (Chung et al., 2004; Kafitz and Greer, 1999; Pellitteri et al., 2009; Sonigra et al., 1999). Indeed, OECs express multiple adhesion molecules involved in axon outgrowth, secrete trophic factors, and ensheath growing axons to protect them from inhibitory molecules (Doucette, 1990; Lipson et al., 2003; Ramón-Cueto and Valverde, 1995; Woodhall et al., 2001). Trophic factors, such as brain-derived neurotrophic factor (BDNF), contribute to the ability of OECs to enhance axon regeneration on an inhibitory substrate (Ruitenberg et al., 2003; Runyan and Phelps, 2009), but the contact-mediated OEC–neuron interactions are not well studied.

The growth-promoting characteristics of OECs lead to their use as therapeutic cellular grafts following SCI. Over the past decade, a number of studies reported that OECs support axon regeneration *in vivo*, even after a complete transection (Kubasak et al, 2008; López-Vales et al., 2006; Lu et al., 2002; Ramón-Cueto et al., 1998, 2000; Tabakow et al., 2014; Takeoka et al., 2011; Ziegler et al., 2011). Despite the reported functional improvements, most of these studies could not identify OECs post-implantation and consequently the OEC interactions in a SCI environment remain unclear. In the present study, we used an established model of SCI that recapitulates the inhibitory environment of the astroglial scar and its fibroblast border (Wanner et al., 2008) to test how OEC transplantation facilitates neurite regeneration. We identified OEC–neurite alignment as a critical regulator of neurite outgrowth on the growth-inhibitory substrates in scar-like cultures.

Materials and methods

Astrocyte–meningeal fibroblast co-culture

Methods to prepare co-cultures of astrocytes and meningeal cells (predominantly fibroblasts, but also microglia and blood vessels) were similar to those reported in Wanner et al. (2008) and Wanner (2012). Astrocytes were cultured from neonatal rat cerebral cortices in 5% fetal bovine serum (FBS, Hyclone, Logan, UT) and upon reaching confluency, cultures were switched to a mixture of 1:1 DMEM and Ham's F12 (D/F medium, Gibco) supplemented with 5% horse serum. Approximately 200,000 astrocytes were seeded onto deformable membranes (962 mm^2 Bioflex 6-well plates, Flexcell Int. Corp., Hillsborough, NC) previously coated with collagen (Fig. 1; control culture). Astrocyte cultures were slowly withdrawn from serum and kept serum-free until the addition of meningeal cells.

Meningeal fibroblasts were isolated from newborn rat cortical meninges, dissociated and resuspended with 3% trypsin, collagenase, and DNAse I. They were grown for 5 days on poly-L-lysine-coated dishes (PLL; Sigma, St. Louis, MO). Meningeal fibroblasts were added to the astrocytes on deformable membranes after 4 weeks *in vitro* with 130,000 cells per culture in 10% FBS/DF medium (Fig. 1). Astrocyte–fibroblast co-cultures grown on deformable membranes were given two short pressure pulses (3.5–3.8 psi) with a pressure controller (Ellis et al., 1995) that induced abrupt membrane deformation and mechanical trauma to the cells (Fig. 1; scar-like culture).

OEC primary culture

Olfactory bulbs were collected from 8–10 week old Sprague–Dawley rats, and the leptomeninges were removed to reduce fibroblast contamination. Methods to prepare OEC primary cultures were adopted from Ramón-Cueto et al. (2000). OECs were dissected from the first two layers of the olfactory bulb and washed in Hank's Balanced Salt Solution (HBSS, Gibco, Rockville, MD) prior to tissue centrifugation at 365 *g* for 5 min. The tissue pellet was first resuspended in 0.1% trypsin and HBSS without Ca^{2+}/Mg^{2+} (Gibco), then placed in a 37 °C water bath, and mixed intermittently for 10 min. D/F medium supplemented with 10% FBS and 1% Penicillin/Streptomycin (P/S, Gibco) was used to inactivate trypsin prior to centrifugation. Dissociated cells were rinsed and centrifuged 3 times and then plated into 25 cm^2 culture flasks pre-coated with 0.05 mg/ml PLL. Cells were maintained at 37 °C for 7 days, and D/F medium was changed every 2 days.

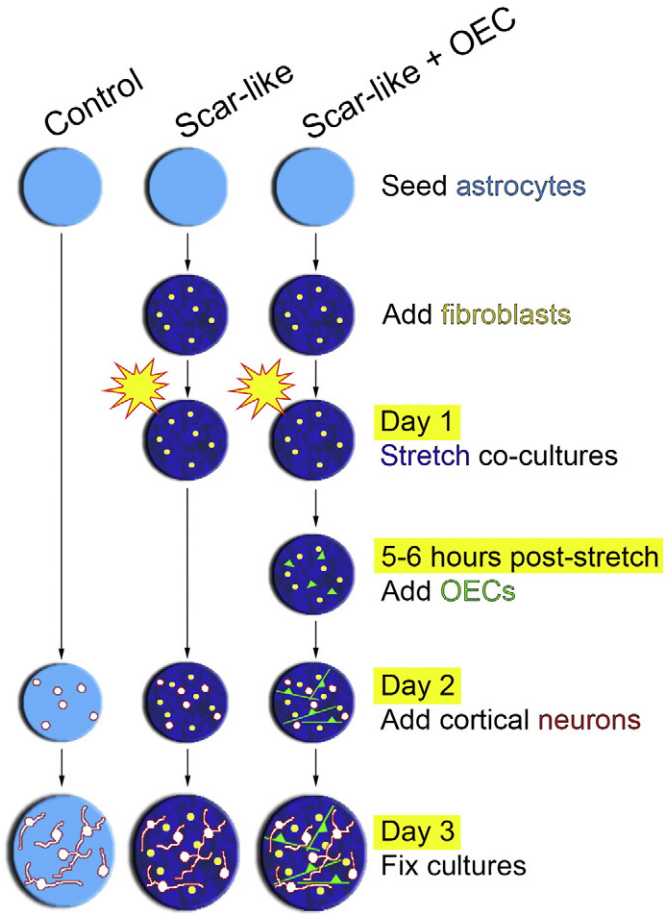

Fig. 1. Schematic of experimental design and timeline. Experiments were conducted on either quiescent astrocytes (control) or co-cultures of stretched astrocytes and meningeal fibroblasts (scar-like). Mature astrocytes (light blue background) were cultured on deformable membranes and first confronted with fibroblasts to induce astrogliosis (dark blue background). Then astrocyte–fibroblast co-cultures were mechanically stretched 5–6 h prior to the addition of OECs labeled with Cell Tracker Green (scar-like + OEC). One day later, cerebral cortical neurons (P6–P8) were added to all experimental conditions. Neurons were grown for 24 h and fixed.

OEC immunopurification

Hydrophobic Petri dishes were coated overnight with Biotin-SP-conjugated AffiniPure goat anti-mouse IgG (1:1000; Jackson ImmunoResearch Laboratories, West Grove, PA) in 50 mM Tris buffer. Dishes were washed 4 times with 25 mM PBS (Gibco) and then incubated overnight with antibody against p75-nerve growth factor receptor (anti-p75-NGFR, 1:5; clone 192, Chandler et al., 1984) at 4 °C. Dishes were rinsed 3 times with 25 mM PBS and treated with a mixture of PBS and 0.5% BSA for 1 h at room temperature. Before immunopanning cells, antibody-treated dishes were washed with PBS and DMEM.

OEC primary cultures were treated with 0.25% trypsin in HBSS without Ca^{2+}/Mg^{2+} for 3 min at 37 °C before trypsin inactivation with D/F medium. Cells were centrifuged at 216 *g* for 10 min and resuspended in D/F medium. Cells were added to pre-treated anti-p75-NGFR dishes and incubated at 37 °C for 10 min. Unbound cells were washed off, and a cell scraper was used to recover bound cells which were then subjected to a second immunopanning. Purified p75-NGFR-positive OECs were resuspended, plated on PLL-coated culture flasks, and incubated at 37 °C for 7 days with medium changed every 2 days. Purified OECs