Experimental Neurology 229 (2011) 238–250

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

GDNF modifies reactive astrogliosis allowing robust axonal regeneration through Schwann cell-seeded guidance channels after spinal cord injury

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ARTICLE INFO

Article history:
Received 13 October 2010
Revised 28 January 2011
Accepted 3 February 2011
Available online 21 February 2011

Keywords:
Axonal regeneration
GDNF
Reactive astrocytes
Schwann cells
Spinal cord injury
Transplantation

ABSTRACT

Reactive astrogliosis impedes axonal regeneration after injuries to the mammalian central nervous system (CNS). Here we report that glial cell line-derived neurotrophic factor (GDNF), combined with transplanted Schwann cells (SCs), effectively reversed the inhibitory properties of astrocytes at graft–host interfaces allowing robust axonal regeneration, concomitant with vigorous migration of host astrocytes into SC-seeded semi-permeable guidance channels implanted into a right-sided spinal cord hemisection at the 10th thoracic (T10) level. Within the graft, migrated host astrocytes were in close association with regenerated axons. Astrocyte processes extended parallel to the axons, implying that the migrated astrocytes were not inhibitory and might have promoted directional growth of regenerated axons. *In vitro*, GDNF induced migration of SCs and astrocytes toward each other in an astrocyte–SC confrontation assay. GDNF also enhanced migration of astrocytes on a SC monolayer in an inverted coverslip migration assay, suggesting that this effect is mediated by direct cell–cell contact between the two cell types. Morphologically, GDNF administration reduced astrocyte hypertrophy and induced elongated process extension of these cells, similar to what was observed *in vivo*. Notably, GDNF treatment significantly reduced production of glial fibrillary acidic protein (GFAP) and chondroitin sulfate proteoglycans (CSPGs), two hallmarks of astrogliosis, in both the *in vivo* and *in vitro* models. Thus, our study demonstrates a novel role of GDNF in modifying spinal cord injury (SCI)-induced astrogliosis resulting in robust axonal regeneration in adult rats.

Introduction

Glial cell line-derived neurotrophic factor (GDNF) and its receptors are widely expressed in the developing (Oppenheim et al., 1995) and adult central nervous system (CNS) (Arenas et al., 1995; Buj-Bello et al., 1995). Two receptors for GDNF, *i.e.* GFRα1 and/or c-Ret, are expressed not only in neurons, but also in Schwann cells (SCs) and astrocytes (Widenfalk et al., 2001). In addition to its effect on neuron survival (Kordower et al., 2000; Perrelet et al., 2002) and axonal regeneration (Iannotti et al., 2003; Mills et al., 2007), growing evidence suggests that the GDNF effect on axon regeneration may be mediated through affecting the behavior of glial cells (Iwase et al., 2005; Paratcha et al., 2003). Whether GDNF plays a role in modification of astrogliosis and the subsequent promotion of axonal regeneration remains unclear.

Reactive astrogliosis, developed in response to injuries of the CNS, significantly impedes axonal regeneration. Following spinal cord injury (SCI), astrocytes at and near the injury border adopt a reactive hypertrophic phenotype; they express elevated levels of glial fibrillary acidic protein (GFAP), and release inhibitory extracellular matrix molecules chondroitin sulfate proteoglycans (CSPGs) (Chau et al., 2004; Predy and Malhotra, 1989). It is the physical and chemical barrier formed by reactive astrogliosis that inhibits axonal regeneration through and beyond injuries in the CNS (Fitch and Silver, 2008; Reier et al., 1983). However, in the developing and mature CNS, astrocytes play multifaceted roles. Radial glial cells, precursors to

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0014-4886/$ – see front matter © 2011 Published by Elsevier Inc.
doi:10.1016/j.expneurol.2011.02.001

astrocytes, are generated alongside neurons and are important for supporting neuronal migration and axon guidance (Vaccarino et al., 2007). In the mature CNS, astrocytes regulate synaptic activity, modulate the extracellular ionic environment and maintain the blood-brain barrier (Abbott et al., 2006; Tanaka, 2007; Walz, 2000). Even after injury, reactive astrocytes may show adaptive plasticity by secreting many cytokines and neurotrophic factors (Aubert et al., 1995; Levison et al., 1996), restoring the extracellular ionic environment (Sykova et al., 1992), and upregulating various cellular surface molecules and extracellular matrix molecules such as L1, laminin, and fibronectin (Alonso and Privat, 1993; Frisen et al., 1993; Le Gal La Salle et al., 1992). Indeed, reactive astrocytes were shown to protect tissue and preserve function after SCI (Faulkner et al., 2004). Thus, a repair strategy aimed at minimizing the inhibitory properties of astrocytes and simultaneously maximizing their growth-promoting properties would be extremely attractive.

Previously, we co-administered recombinant human GDNF (rhGDNF or GDNF) and SCs in semi-permeable guidance channels grafted into hemisected spinal cords and found that GDNF alleviated astroglial reaction and modified morphological properties of reactive astrocytes (Iannotti et al., 2003). However, the role and mechanism by which GDNF mediates such an action remains unclear. The goal of this study was to determine whether GDNF, over-expressed by SCs, would intensify this modification which, in turn, would improve graft–host interfaces leading to enhanced axonal regeneration following SCI.

Materials and methods

Generation of purified Schwann cells (SCs) and astrocytes

SCs were purified as described previously (Morrissey et al., 1991; Xu et al., 1995). Briefly, SCs were harvested from the sciatic nerves of adult female Sprague–Dawley (SD) rats (Harlan, Indianapolis, IN) under aseptic conditions, then purified and expanded in culture. Purified SCs (purity >98%) at the third or fourth passage were collected for either *in vitro* experiments or seeding into mini-guidance channels for transplantation. Astrocytes were purified from the cortex of neonatal rat brains (Muir et al., 2002). Cortices from postnatal day (P) 0–1 rats were minced in Hank's Buffered Salt Solution (HBSS) after the removal of meninges, digested in 0.25% trypsin (Sigma, St. Louis, MO), triturated in DMEM with 10% fetal bovine serum (FBS, Sigma), and centrifuged for 5 min at 1000 *g*. The cells were plated in Dulbecco's Modified Eagle's Medium (DMEM) with 10% FBS in tissue culture flasks. Once the cells had reached confluence, they were shaken for 170 rpm for 18 h in an incubator shaker to remove microglia and oligodendrocyte progenitor cells, followed by a 4 day culture in 20 mM anti-mitogen AraC to eliminate the fibroblasts, and then passaged and grown to confluence. Astrocyte cultures were >98% GFAP positive and were maintained in DMEM containing 10% FBS (D10).

Transduction of SC in vitro

SCs were seeded into 6-well plates at a density of 5×10^5 cells/well for *in vitro* enzyme-linked immuno-sorbent assay (ELISA) or into a 25 ml flask at a density of 1×10^6 cells/flask for transplantation. When cells were grown to over 90% confluence, they were pre-treated with 4–6 μg/ml polybrene (Sigma) for 30–60 min, and then infected by lentiviruses expressing either green fluorescence protein (lenti-GFP) or GDNF (lenti-GDNF) for 12 h at a multiplicity of infection (MOI) of 4, resulting in about 50% infection of cells (Abdellatif et al., 2006). Infection media was then replaced with fresh media and, 3 days later, conditioned media in 6 well plates was collected for ELISA. Cells in 25 ml flasks were prepared for transplantation.

ELISA

The GDNF levels secreted by SCs after infection *in vitro* were measured by ELISA (Abdellatif et al., 2006). Three days after infection, the supernatant of SC was collected and centrifuged at 20,000 *g* for 10 min at 4 °C. The procedure for ELISA followed the supplier's recommendations (G1620, Promega, Madison, WI).

Seeding SCs into mini-guidance channels

Semi-permeable 60:40 poly-acrylonitrile/poly-vinylchloride (PAN/PVC) copolymer guidance channels with an outer diameter of 1.25 mm (Provided by Dr. Xuejun Wen, Clemson University, Charleston, SC) were cleaned and sterilized according to the established methods (Bamber et al., 2001; Xu et al., 1999). SCs were suspended in a 60:40 (v:v) of DMEM and Matrigel (MG, Collaborative Research, Bedford, MA) at a final density of 120×10^6 cells/ml and seeded into guidance channels as described previously (Xu et al., 1999). The channel contents include 1) SCs alone (SCs), 2) SCs infected with lenti-GFP (lenti-GFP SCs), 3) SCs co-administered with GDNF protein (GDNF protein + SCs), and 4) SCs infected with lenti-GDNF (lenti-GDNF SCs). In channels when GDNF was co-administered, an amount of DMEM was replaced with an equal volume of concentrated GDNF to achieve a final concentration of GDNF at 5 μg/μl (Iannotti et al., 2003). After seeding, the channel was closed at both ends with PAN/PVC glue and kept in DMEM for 2–3 h at 37 °C to allow polymerization of the MG.

Spinal cord hemisection and transplantation of SC-seeded guidance channels

Adult female SD rats (180–200 g, Harlan) were randomly divided into four groups that received grafts of: 1) SCs alone (n = 10), 2) lenti-GFP SCs (n = 10), 3) GDNF protein + SCs (n = 10), and 4) lenti-GDNF SCs (n = 10). The procedures for spinal cord hemisection and mini-guidance channel implantation, as well as for pre- and post-operative animal care, were described in detail in previous publications (Bamber et al., 2001; Xu et al., 1999). Briefly, a right-sided spinal cord hemisection was performed at the 9th and 10th thoracic (T) levels to create a 2.8 mm gap longitudinally followed by implantation of a 3 mm-long piece of SC-seeded guidance channel into the lesion site. In all groups, rats were sacrificed at 6 weeks post-implantation. All animal handling, surgical procedures, and post-operative care were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and the Guidelines and Policies for Rodent Survival Surgery provided by the Animal Care Committees of Indiana University.

Collection of Schwann cell conditioned medium (SCM)

When cultures of purified SCs in T25 flasks were confluent, they were rinsed twice with DMEM and kept in D10 without or with GDNF (100 ng/ml) for 24 h. Then cultures were replaced with GDNF-free medium and maintained for an additional 4 days before medium collection. The medium was centrifuged and filtrated through a 0.2 μm filter and stored (Millipore, Hertfordshire, UK).

Scratch wound healing migration assay

The scratch migration assay was used to measure two-dimensional cell movement (Boran and Garcia, 2007). After astrocytes were grown to confluence in 24-well plates, a scratch was made on the monolayer using a sterile 200 μl pipette tip. Then the astrocyte cultures were exposed to the following five treatment groups: 1) medium only (D10), 2) GDNF (100 ng/ml in D10), 3) SC conditioned medium (SCM; at 1:1 ration to D10), 4) SCM + GDNF (100 ng/ml), and 5) SCM