Experimental Neurology 233 (2012) 717–732

Contents lists available at SciVerse ScienceDirect

Experimental Neurology

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

Phenotypic analysis of astrocytes derived from glial restricted precursors and their impact on axon regeneration

Christopher Haas [a,1], Birgit Neuhuber [a,1], Takaya Yamagami [a], Mahendra Rao [b,2], Itzhak Fischer [a,*]

[a] Department of Neurobiology & Anatomy, Drexel University College of Medicine, 2900 Queen Lane, Philadelphia, PA, USA
[b] Life Technologies, Frederick, MD, USA

ARTICLE INFO

Article history:
Received 30 August 2011
Revised 12 October 2011
Accepted 1 November 2011
Available online 10 November 2011

Keywords:
Bone morphogenetic protein (BMP)
Ciliary neurotrophic factor (CNTF)
Astrocyte differentiation
Astrocyte transplantation
Spinal cord injury
Axon regeneration

ABSTRACT

Although astrocytes are involved in the production of an inhibitory glial scar following injury, they are also capable of providing neuroprotection and supporting axonal growth. There is growing appreciation for a diverse and dynamic population of astrocytes, specified by a variety of glial precursors, whose function is regulated regionally and temporally. Consequently, the therapeutic application of glial precursors and astrocytes by effective transplantation protocols requires a better understanding of their phenotypic and functional properties and effective protocols for their preparation. We present a systematic analysis of astrocyte differentiation using multiple preparations of glial-restricted precursors (GRP), evaluating their morphological and phenotypic properties following treatment with fetal bovine serum (FBS), bone morphogenetic protein 4 (BMP-4), or ciliary neurotrophic factor (CNTF) in comparison to controls treated with basic fibroblast growth factor (bFGF), which maintains undifferentiated GRP. We found that treatments with FBS or BMP-4 generated similar profiles of highly differentiated astrocytes that were A2B5−/GFAP+. Treatment with FBS generated the most mature astrocytes, with a distinct and near-homogeneous morphology of fibroblast-like flat cells, whereas BMP-4 derived astrocytes had a stellate, but heterogeneous morphology. Treatment with CNTF induced differentiation of GRP to an intermediate state of GFAP+ cells that maintained immature markers and had relatively long processes. Furthermore, astrocytes generated by BMP-4 or CNTF showed considerable experimental plasticity, and their morphology and phenotypes could be reversed with complementary treatments along a wide range of mature–immature states. Importantly, when GRP or GRP treated with BMP-4 or CNTF were transplanted acutely into a dorsal column lesion of the spinal cord, cells from all 3 groups survived and generated permissive astrocytes that supported axon growth and regeneration of host sensory axons into, but not out of the lesion. Our study underscores the dynamic nature of astrocytes prepared from GRP and their permissive properties, and suggest that future therapeutic applications in restoring connectivity following CNS injury are likely to require a combination of treatments.

Introduction

Astrocytes play an important role in the maintenance and function of the nervous system by providing the scaffold for neuronal architecture, modulating the blood–brain barrier, regulating metabolic and trophic activity, and supporting the formation and function of synapses (Freeman, 2010; Nag, 2011; Sofroniew and Vinters, 2010; Zhang and Barres, 2010). Additionally, astrocytes respond to CNS injury in a process referred to as reactive astrogliosis, in which they undergo a spectrum of morphological, biochemical, and functional changes that are triggered by local cues and that depend on the location and time course of the injury process (White and Jakeman, 2008). Astrocytes at the injury site produce an inhibitory glial scar, a hallmark of the pathology that limits axonal regeneration, and secrete chemokines that contribute to the inflammatory process (Fitch and Silver, 2008). However, the process of astrogliosis is dynamic and complex, and there is increasing evidence that astrocytes are capable of providing support for axonal growth and neuroprotection that limits secondary injury (Bush et al., 1999; Faulkner et al., 2004; Fitch and Silver, 2008; Rolls et al., 2009; Sofroniew and Vinters, 2010).

Traditionally, astrocytes have been defined by their stellate appearance and their characteristic expression of glial fibrillary protein (GFAP),

Abbreviations: AP, alkaline phosphatase; APC, astrocyte precursor cell; bFGF, basic fibroblast growth factor; BMP, bone morphogenetic protein; CNS, central nervous system; CSPG, chondroitin sulfated proteoglycans; CTB, cholera toxin subunit B; CNTF, ciliary neurotrophic factor; DAPI, 4′6′ diamidino-2-phenyindole; FBS, fetal bovine serum; GFAP, glial fibrillary protein; GRP, glial restricted precursors; LN, laminin; NPC, neural precursor cells; NRP, neuronal restricted precursors; O-2A, oligodendrocyte-type 2 astrocyte precursor cell; OPC, oligodendrocyte precursor cell; PLL, poly-L-lysine; SCI, spinal cord injury; SE, standard error.

* Corresponding author at: Department of Neurobiology and Anatomy, Drexel University College of Medicine, 2900 Queen Lane, Philadelphia, PA 19129, USA. Fax: +1 215 843 9082.
E-mail address: IFischer@drexelmed.edu (I. Fischer).
[1] These authors had equal contribution and are designated as co-first authors.
[2] Present address: NIH-Center for Regenerative Medicine.

0014-4886/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.expneurol.2011.11.002

but there is growing evidence for a heterogeneous population of multiple subtypes of astrocytes capable of performing a diverse array of functions that are temporally, regionally, and contextually specific (Hewett, 2009; Matyash and Kettenmann, 2010; White and Jakeman, 2008; Zhang and Barres, 2010). Given the essential role of astrocytes during neural development, particularly for the stimulation of axonal growth, maintenance of neuronal survival, and promotion of synapse formation (Zhang and Barres, 2010), astrocytes derived from the developing CNS have been suggested for treatment of CNS injury including spinal cord injury (SCI). Indeed, the transplantation of neonatal astrocytes into a model of brain injury or of SCI, resulted in reduced scar formation, decreased host reactive astrogliosis, and the modest support of axonal growth into the lesion site (Joosten et al., 2004; Kliot et al., 1990; Smith et al., 1986).

The complexity of astrocyte populations is reflected not only by multiple pathways that can give rise to such cells – neural stem cells, radial glia, and glial precursors – but also in the diversity of glial precursors themselves (Liu and Rao, 2004). Tripotential GRP derived from the embryonic spinal cord, based on their expression of the surface marker A2B5, are capable of self-renewal as well as differentiation into two phenotypic classes of astrocytes -type 1 and 2- and oligodendrocytes (Rao et al., 1998). Grafting experiments of GRP into the intact CNS demonstrated their ability to survive, migrate, and differentiate along glial lineages, generating both astrocytes and oligodendrocytes (Herrera et al., 2001; Hill et al., 2004). Transplantation of GRP into a contusion model of SCI showed that GRP-derived astrocytes modulate the environment of the lesion and promote neuroprotection, reduction of glial scar formation, and increased axonal growth into the permissive graft environment (Hill et al., 2004) but, even when combined with cAMP, resulted in only modest recovery of autonomic function (Nout et al., 2011). The O-2A cells, originally isolated from the optic nerve (Raff et al., 1983), represent an intermediate population that can be derived from GRP (Gregori et al., 2002). O-2A are bipotential and can generate type 2 astrocytes and oligodendrocytes *in vitro*; *in vivo*, O-2A cells only give rise to oligodendrocytes (Gregori et al., 2002) and have therefore been referred to as O-2A/OPC.

The generation of different types of astrocytes with distinct properties during development or following injury is determined by the interaction of an intrinsic expression program with external signals that depend on the state of the environment (Freeman, 2010; White et al., 2010). Similarly, the differentiation of glial precursors into various astrocyte subtypes depends on the specific population of precursors and the factors present in the culture. Yet, it is important to recognize that subsets of progenitors will spontaneously differentiate in culture, as the intrinsic program of the cells modulates the process of cell division and differentiation together with culture conditions (Noble and Mayer-Proschel, 1997; Wu et al., 2002). Nevertheless, treatment of GRP cultures with FBS resulted in the production of A2B5−/GFAP+ astrocytes with a fibroblast-like flat morphology, whereas exposure to basic fibroblast growth factor (bFGF) together with ciliary neurotrophic factor (CNTF) produced mostly process-bearing A2B5+/GFAP+ astrocytes (Rao et al., 1998). Bone Morphogenetic Proteins (BMP) have also been implicated in promoting astrocyte differentiation over other neuronal or glial lineages not only from GRP, but also from other neural stem cells (D'Alessandro et al., 1994; Gomes et al., 2003; Gross et al., 1996). However, the actions of BMP can be multifaceted because of opposing effects of signaling through different receptors (Sahni et al., 2010), as evidenced by mixed results obtained *in vivo* where inhibition of BMP was reported to expand the lesion, resulting in poor functional recovery (Enzmann et al., 2005) or, in other studies, to enhance axonal growth and recovery of function following SCI (Matsuura et al., 2008). Similarly, some studies have shown that grafting astrocytes predifferentiated with BMP-4 into a hemisection model of SCI preferentially supported axonal growth and promoted recovery using either rat (Davies et al., 2006, 2008) or human cells (Davies et al., 2011). Another study using a contusion injury showed that transplants of human GRP or astrocytes predifferentiated by BMP-4 promoted improvement of the lesion environment with only modest functional recovery (Jin et al., 2011), similar to the effects of undifferentiated rat GRP in a contusion injury (Hill et al., 2004; Nout et al., 2011).

In this study we systematically evaluated multiple preparations of rat GRP populations prepared by different methods and assessed their morphological and phenotypic properties following differentiation into astrocytes with FBS, BMP-4, or CNTF and compared them to controls that were maintained at a precursor state with bFGF. We found that treatments with FBS or BMP-4 generated differentiated astrocytes with similar expression profiles, A2B5−/GFAP+. However, treatment with FBS generated the most mature astrocytes, with a distinctive, near-homogeneous, uniformly flat morphology, whereas BMP-4 astrocytes were heterogeneous with a stellate appearance. Treatment with CNTF allowed for differentiation of GRP into an intermediate state of GFAP+ cells that maintained immature markers and had relatively long processes. Furthermore, astrocytes generated by BMP-4 or CNTF (but not FBS) showed considerable experimental plasticity; their phenotype could be reversed with complementary treatments along a wide range of mature–immature stages. Importantly, when GRP or GRP treated with BMP-4 or CNTF were transplanted acutely into a dorsal column lesion of the spinal cord, all of them survived, generated permissive astrocytes that supported axon growth, and supported regeneration of host sensory axons into but not out of the lesion.

Material and methods

Harvest and culture of rat neural precursor cells (NPC)

Rat NPC were harvested from embryonic day 13.5 spinal cords of transgenic Fischer-344 rats that express the marker gene human placental alkaline phosphatase (AP) (Mujtaba et al., 2002) and prepared as described previously (Han et al., 2002; Lepore and Fischer, 2005). Cells were plated in GRP basal medium (DMEM/F12 [Invitrogen, Carlsbad, CA], BSA [1 mg/mL; Sigma-Aldrich, St. Louis, MO], B27 [20 μl/mL; Invitrogen], Pen/Strep [50 IU/mL; Invitrogen], N2 (10 μl/mL; Invitrogen]) supplemented with bFGF (20 ng/mL; Peprotech, Rocky Hill, NJ) and NT-3 (10 ng/mL; Peprotech) on culture dishes coated with poly-L-lysine (PLL; 15 μg/mL; Sigma-Aldrich) and laminin (LN; 15 μg/mL; Invitrogen).

Isolation of GRP

Different protocols were used to prepare purified rat GRP in 3 separate preparations.

GRP preparation 1 (GRP1)

This method was based on a culturing protocol that was used previously to purify progenitor cells (Hunter and Bottenstein, 1990; Mabie et al., 1997) and was adapted for GRP. NPC were cultured for 10 days on PLL/LN-coated culture dishes in GRP basal medium supplemented with 20 g/mL bFGF prior to freezing of cultures at 2×10^6 cells/mL in freezing medium (80% GRP basal medium + 10 ng/mL bFGF + 10% Chick Embryo Extract + 10% DMSO) at −80 °C. Following freezing, GRP were thawed and plated on PLL-coated culture dishes in GRP basal medium supplemented with 20 ng/mL bFGF and expanded to passage 3.

GRP preparation 2 (GRP2)

This method utilized fluorescence-activated cell sorting based on A2B5 expression by GRP as described previously (Han et al., 2004; Lepore et al., 2008; Mujtaba et al., 2002; Wu et al., 2002). Sorted cells were plated onto tissue culture flasks coated with PLL/LN and expanded in GRP basal medium supplemented with 20 ng/mL bFGF.