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Glial restricted precursors maintain their permissive properties after long-term expansion but not following exposure to pro-inflammatory factors

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ABSTRACT

Glial restricted precursors (GRP) are a promising cellular source for transplantation therapy of spinal cord injury (SCI), capable of creating a permissive environment for axonal growth and regeneration. However, there are several issues regarding the nature of their permissive properties that remain unexplored. For example, cellular transplantation strategies for spinal cord repair require the preparation of a large number of cells, but it is unknown whether the permissive properties of GRP are maintained following the process of *in vitro* expansion. We used rat GRP isolated from the embryonic day 13.5 spinal cord to compare the properties of early (10–20 days) and late (120–140 days) passage GRP. We found that late passage GRP showed comparable effects on neurite outgrowth of adult rat DRG to early passage GRP in both *in vitro* co-culture and conditioned medium experiments. In addition, to further examine the effects of the inflammatory cascade activated in the aftermath of SCI on the microenvironment, we studied the direct effects of strong inflammatory mediators, Lipopolysaccharide and interferon gamma (LPS and IFNγ, respectively), on the properties of GRP. We showed that exposure to these pro-inflammatory mediators altered GRP phenotype and attenuated their growth-promoting effects on neurite outgrowth in a dose dependent manner. Taken together, our data suggest that GRP maintain their growth-promoting properties following extensive *in vitro* passaging and underscore the importance of modulating the inflammatory environment at the injured spinal cord.

Abbreviations: GRP, Glial restricted precursors; SCI, Spinal cord injury; LPS, Lipopolysaccharide; IFNγ, Interferon gamma; OPC, Oligodendrocyte precursor cell; NRP, Neuronal restricted precursors; ES cells, Embryonic stem cells; TRL4, Toll-like receptor 4; IFNγRα, Interferon gamma receptor alpha

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1. Introduction

Spinal cord injury (SCI) is a devastating disorder characterized by the disruption of ascending and descending axonal pathways and death of a variety of cells in the central nervous system (CNS) (Simpson et al., 2012; Verma et al., 2008). One of the most significant obstacles for repair following SCI is the inability of injured neurons to regenerate and restore connectivity (Fitch and Silver, 2008). Transplantation of neural progenitors is a promising therapeutic strategy that has the potential to replace lost cells, modulate the inhibitory microenvironment of the CNS, and promote axonal growth (Eftekharpour et al., 2008; Tetzlaff et al., 2011).

Extensive studies using multiple types of cells such as Schwann cells, olfactory ensheathing glial cells, neural stem cells, fate-restricted neural/glial precursor cells, and bone marrow stromal cells have been examined for their therapeutic potential for SCI repair (Falnikar et al., 2014; Lopez-Vales et al., 2006; Medalha et al., 2014; Mendonca et al., 2014). Once appropriate candidates for cell transplantation have been identified, the transition to clinical applications requires culturing and expansion to prepare adequate cell stocks and to determine whether the cells retain their original properties. These are particularly important considerations for primary cells derived from fetal or adult tissue, which unlike embryonic stem (ES)-derived cells, are a limited resource. Several studies have shown that some cells retain their growth-supportive properties following expansion (Akesson et al., 2007; Radtke et al., 2010; Tang et al., 2000; Zhu et al., 2014), but in other cases the transplantation protocols have been restricted to a low cell passage (Huang et al., 2006; Saberi et al., 2008). It is therefore important to define the expansion parameters for specific cell types and use these data to develop appropriate transplantation protocols.

Another issue concerning transplantation following CNS trauma relates to the presence of pro-inflammatory molecules, which are released at the injury site and elicit an immune response leading to astrogliosis and glial scar formation (Donnelly and Popovich, 2008; Fitch and Silver, 2008; Sofroniew, 2009). The inflammatory environment encountered by transplanted cells may produce changes that will affect the phenotypic and functional properties of transplanted cells. These changes can be studied *in vitro* by modeling the direct effects of pro-inflammatory molecules on particular cell types. Lipopolysaccharide (LPS) and interferon gamma (IFNγ) represent powerful, canonical innate inflammatory mediators and have been used extensively to study cellular responses to inflammation (Dafny and Yang, 2005; Fok-Seang et al., 1998; Fu et al., 2014; Lehnardt et al., 2003). Although the responses of a variety of cell subtypes, such as astrocytes, microglia/macrophages, and oligodendrocyte precursor cells (OPCs) to inflammation have been previously documented (Tanner et al., 2011; Walter et al., 2011; Zamanian et al., 2012), the changes to the properties of multipotent glial restricted precursors (GRP) following exposure to inflammatory factors has not been examined.

The present report continues our previous studies, which have shown that a combination of neuronal and glial-restricted precursors (NRP/GRP) promoted host axon regeneration of sensory neurons into the injury site to form synaptic connections with transplant-derived neurons and created a function relay across the injury (Bonner et al., 2011; Lepore and Fischer, 2005). The presence of GRP was shown to be a necessary and sufficient component for regeneration, creating a permissive environment even in the presence of inhibitory chondroitin sulfate proteoglycans (CSPGs), a major inhibitory component of the glial scar (See et al., 2010). Conditioned medium harvested from rat GRP also promoted neurite outgrowth of DRG neurons *in vitro*, suggesting that the secretion of soluble factors was responsible for the growth (Ketschek et al., 2012).

In the present study we wanted to examine the properties of GRP in the context of their potential application for SCI repair, with specific aims designed to test their ability to support axonal growth following *in vitro* expansion and exposure to inflammatory factors. Here, we demonstrate the consistently permissive nature of late passage GRP (P19-21; grown over 120 days) on neurite outgrowth from adult rat DRG utilizing two experimental protocols, direct DRG–GRP co-culture and the use of conditioned medium, indicating the role of secreted factors. In addition, we examined the direct effects of pro-inflammatory mediators, LPS and IFNγ, on the phenotypic and functional properties of GRP with respect to neurite outgrowth using co-culture experiments. This treatment resulted in a reduced capacity to support neurite outgrowth, which correlated with phenotypic changes of GRP. Taken together, these results underscore the therapeutic potential of GRP to serve as permissive cellular transplants in SCI, even after prolonged expansion *in vitro*, but emphasize the importance of the inflammatory microenvironment of the injured spinal cord and its potential influence on the properties of GRP.

2. Results

2.1. *Characterization of late passage GRP*

We have previously shown that serum-free defined basal medium supplemented with basic fibroblast growth factor (bFGF) maintains rat GRP in an undifferentiated state for at least 14 days as compared to media supplemented with FBS, BMP-4 or CNTF, which induce differentiation into astrocytes (Haas et al., 2012). Here, we followed a similar culturing method using basal medium supplemented with bFGF, but continued to grow GRP for over 19 passages (ranging from 120 to 140 days *in vitro*) to compare early and late passage GRP with respect to their proliferative potential, morphology, and lineage specific markers. Early passage GRP showed robust proliferation with a population doubling time (PDT: 22.3±1.1 h), while late passage GRP showed reduced proliferation (PDT: 27.5±1.5 h), as shown in Fig. S1A (see Supplemental information). Immunostaining with Ki67, a marker of cell proliferation, supported these results, showing a large population of cells positive for Ki67 (Fig. S1B), with 60.0±1.3% early passage GRP and 47.2±2.4% of late passage GRP staining for Ki67 ($P=0.03$). Early passage GRP had a typical morphology of a non-differentiated progenitor characterized by small refractive cell bodies and a short stellate or bipolar appearance (Fig. 1A). The vast majority of late passage GRP showed a comparable morphology with only a small number of cells displaying larger cell bodies and multi-branching shapes or cells with a flattened morphology, typical