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Research Paper

Human iPS cell-derived astrocyte transplants preserve respiratory function after spinal cord injury

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ABSTRACT

Transplantation-based replacement of lost and/or dysfunctional astrocytes is a promising therapy for spinal cord injury (SCI) that has not been extensively explored, despite the integral roles played by astrocytes in the central nervous system (CNS). Induced pluripotent stem (iPS) cells are a clinically-relevant source of pluripotent cells that both avoid ethical issues of embryonic stem cells and allow for homogeneous derivation of mature cell types in large quantities, potentially in an autologous fashion. Despite their promise, the iPS cell field is in its infancy with respect to evaluating *in vivo* graft integration and therapeutic efficacy in SCI models. Astrocytes express the major glutamate transporter, GLT1, which is responsible for the vast majority of glutamate uptake in spinal cord. Following SCI, compromised GLT1 expression/function can increase susceptibility to excitotoxicity. We therefore evaluated intraspinal transplantation of human iPS cell-derived astrocytes (hIPSAs) following cervical contusion SCI as a novel strategy for reconstituting GLT1 expression and for protecting diaphragmatic respiratory neural circuitry. Transplant-derived cells showed robust long-term survival post-injection and efficiently differentiated into astrocytes in injured spinal cord of both immunesuppressed mice and rats. However, the majority of transplant-derived astrocytes did not express high levels of GLT1, particularly at early times post-injection. To enhance their ability to modulate extracellular glutamate levels, we engineered hIPSAs with lentivirus to constitutively express GLT1. Overexpression significantly increased GLT1 protein and functional GLT1-mediated glutamate uptake levels in hIPSAs both *in vitro* and *in vivo* post-transplantation. Compared to human fibroblast control and unmodified hIPSA transplantation, GLT1-overexpressing hIPSAs reduced (1) lesion size within the injured cervical spinal cord, (2) morphological denervation by respiratory phrenic motor neurons at the diaphragm neuromuscular junction, and (3) functional diaphragm denervation as measured by recording of spontaneous EMGs and evoked compound muscle action potentials. Our findings demonstrate that hiPSA transplantation is a therapeutically-powerful approach for SCI.

1. Introduction

Transplantation of neural stem cells (NSCs) and neural progenitor cells (NPCs) is a promising therapeutic strategy for both neurodegenerative diseases of the central nervous system (CNS) and traumatic CNS injury, including spinal cord injury (SCI), because of the ability to replace lost and/or dysfunctional nervous system cell types, promote neuroprotection, deliver gene factors of interest and provide other benefits (Gage, 2000).

Initial trauma following SCI results in immediate cell death and axotomy of passing fibers. Contusion- and compression-type injuries, the predominant forms of traumatic SCI observed in the clinical population, are followed by an extended period of secondary cell death and consequent exacerbation of functional deficits (McDonald and Becker,

Abbreviations: SCI, spinal cord injury; iPS cells, induced pluripotent stem cells; hIPSAs, human induced pluripotent stem cell-derived astrocytes; GLT1, glutamate transporter 1; PhMN, phrenic motor neuron; C3 (4 5, *etc.*), cervical spinal cord level 3 (4, 5, *etc.*); GRP, glial-restricted precursor; CMAP, compound muscle action potential; NMJ, neuromuscular junction; GFP–hIPSA, lentivirus-GFP transduced hIPSA; GLT1–hIPSA, lentivirus-GLT1 transduced hIPSA; GFP–hFibro, lentivirus-GFP transduced human fibroblast; LV-GFP, lentivirus-GFP; LV-GLT1, lentivirus-GLT1.

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2003). One of the major causes of secondary degeneration following SCI is excitotoxic cell death due to dysregulation of extracellular glutamate homeostasis (Park et al., 2004; Stys, 2004). Exogenous parenchymal administration of glutamate to uninjured spinal cord results in tissue and function loss similar to SCI (Xu et al., 2005). While large increases in glutamate can occur shortly after SCI, elevation can also persist depending on injury severity (Liu et al., 1991; Panter et al., 1990; Xu et al., 2004). In addition to focal increases, levels can also rise in regions removed from the lesion site, possibly *via* a spreading mechanism involving activated glia (Hulsebosch, 2008). Early gray matter loss is likely mediated by NMDA receptors, while delayed loss of neurons and oligodendrocytes, as well as axonal and myelin injury, is thought to be predominantly mediated *via* AMPA over-activation (Stys, 2004). A valuable opportunity therefore exists after SCI for preventing cell injury and functional loss that occur during secondary degeneration. Importantly, secondary degeneration is a relevant therapeutic target given its relatively prolonged time window.

Glutamate is efficiently cleared from the synapse and other sites by transporters located on the plasma membrane (Maragakis and Rothstein, 2004). Astrocytes are supportive glial cells that play a host of crucial roles in CNS function (Pekny and Nilsson, 2005). Astrocytes express the major CNS glutamate transporter, GLT1, which is responsible for the vast majority of functional glutamate uptake and plays a central role in regulation of extracellular glutamate homeostasis in the spinal cord (Maragakis and Rothstein, 2006). Following SCI, astrocyte loss and/or altered GLT1 expression, function and localization can result in further susceptibility to excitotoxicity. For example, we previously found that in rodent models of unilateral mid-cervical (C4) contusion SCI, numbers of GLT1-expressing astrocytes, total intraspinal GLT1 protein expression and GLT1-mediated functional glutamate uptake in ventral horn are reduced soon after injury and this reduction persists chronically (Li et al., 2015). Astrocytes have traditionally been viewed in a negative light following CNS trauma because of their association with disease mechanisms such as glial scarring and pro-inflammatory cytokine release. However, their crucial neuroprotective/homeostatic roles, including GLT1-mediated glutamate uptake, have not been extensively targeted in SCI models using approaches such as NSC and NPC transplantation, despite obvious therapeutic implications (Maragakis and Rothstein, 2006).

Transplantation-based targeting of astrocytes provides a number of key benefits. Grafts can be anatomically delivered to precise locations for achieving neuroprotection of specific populations of cells (Lepore et al., 2008b). Alternative strategies such as gene therapy only target one/several specific genes (s), while astrocyte transplantation can participate in the restoration of a host of astrocyte functions. Transplantation also provides for long-term astrocyte integration and therapeutic replacement. For example, the lasting nature of dysregulation of extracellular glutamate homeostasis after SCI (Lepore et al., 2011a,2011c) calls for longer-term maintenance of therapeutic effects, both with respect to early cell loss occurring during secondary degeneration and outcomes of SCI associated with more persistent pathophysiology of glutamate signaling such as chronic neuropathic pain (Gwak et al., 2012; Hulsebosch, 2008).

To achieve translation of NSC/NPC-based interventions, clinically-relevant cell sources that address scientific, practical and ethical considerations must be extensively tested in relevant models of CNS disease. These cell types also need to be evaluated in the context of patient-relevant functional outcomes such as respiratory function. Induced pluripotent stem (iPS) cells are pluripotent cells generated from adult somatic cell types *via* expression of combinations of pluripotency-related factors, avoiding ethical issues of embryonic stem cells (Takahashi et al., 2007b). This technology allows for homogeneous derivation of cell types in large quantities for applications such as transplantation, potentially in an autologous fashion from the eventual recipient or from allogeneic sources (Das and Pal, 2010; Kiskinis and Eggan, 2010). Despite the promise of this approach, the iPS cell transplantation field is still in the early stages of evaluating therapeutic usefulness in relevant SCI models (Salewski et al., 2010).

Respiratory compromise is a major problem following cervical spinal cord trauma. Cervical SCI represents greater than half of all human cases, in addition to often resulting in the most severe physical and psychological debilitation (Lane et al., 2008). Respiratory compromise is the leading cause of morbidity and mortality following SCI. While a growing literature exists on respiratory function in animal models of SCI (Lane et al., 2008, 2009), few studies have examined cellular mechanisms involved in protection of this vital neural circuitry, and little work has been conducted to test therapies for targeting cervical spinal cord-related functional outcome measures such as breathing. Phrenic motor neuron (PhMN) loss plays a central role in respiratory compromise following cervical SCI. The diaphragm, a major inspiratory muscle, is innervated by PhMNs located at cervical levels 3–5 (Lane et al., 2009). PhMN output is driven by descending pre-motor bulbospinal neurons in the medullary rostral ventral respiratory group (rVRG) (Zimmer et al., 2007). Cervical SCI results in diaphragmatic respiratory compromise due to PhMN loss and/or injury to descending bulbospinal respiratory axons. The majority of these injuries affect mid-cervical levels (Shanmuganathan et al., 2008) (the location of the PhMN pool), and respiratory function following mid-cervical SCI is significantly determined by PhMN loss/sparing (Strakowski et al., 2007). Although use of thoracic models has predominated, cervical SCI animal models have recently been developed (Aguilar and Steward, 2010; Awad et al., 2013; Gensel et al., 2006; Lane et al., 2012; Lee et al., 2010; Sandrow-Feinberg et al., 2009, 2010; Sandrow et al., 2008; Stamegna et al., 2011), including our own (Nicaise et al., 2012). Because of the relevance of astrocyte and GLT1 dysfunction to PhMN loss/injury following cervical trauma, we targeted transplantation in the present study to cervical spinal cord ventral horn in a cervical contusion SCI model.

We previously investigated the therapeutic efficacy of transplanting rodent-derived glial-restricted precursors (GRP), a class of lineage-restricted astrocyte progenitor cell (Li et al., 2014). We transplanted either undifferentiated GRPs or GRP-derived astrocytes (pre-differentiated *in vitro* prior to injection) into our model of cervical contusion SCI, and found that both cell types survived, localized to the ventral horn and efficiently differentiated into mature astrocytes. However, animals injected with GRP-derived astrocytes had higher levels of intraspinal GLT1 expression than those injected with undifferentiated GRPs, suggesting that pre-differentiation enhanced the *in vivo* maturation of these cells. We also observed that modifying GRP-derived astrocytes to constitutively express GLT1 was more effective in achieving *in vivo* GLT1 expression and for protecting PhMNs.

Given the importance of astrocytes in SCI pathogenesis, the observations of GLT1 dysfunction following SCI, and our previous success targeting astrocyte GLT1 using rodent-derived glial progenitor cells, in the present study we evaluated intraspinal transplantation of hiPS cell-derived astrocytes (hIPSAs) into ventral horn following cervical contusion SCI as a novel therapeutic strategy for reconstituting GLT1 function. Specifically, we examined the *in vivo* fate of hIPSAs transplants in the injured spinal cord of both mouse and rat models of cervical contusion SCI, including long-term survival and integration, astrocyte differentiation, maturation into GLT1-expressing cells and safety. We also tested the therapeutic efficacy of hIPSA transplantation for protection of PhMNs and preservation of diaphragm function.

Derivation of cell types from iPS cells represents a relevant approach for clinical translation; therefore, it is critical to test both the safety and efficacy of these transplants in a patient-relevant SCI model. Importantly, previous work has shown that human- and rodent-derived versions of a given stem/progenitor type do not necessarily show similar *in vivo* fate or therapeutic properties in the disease nervous system. For example, we previously demonstrated that, following transplantation into the $SOD1^{G93A}$ rodent model of ALS, human glial progenitors cells show more persistent proliferation, greater migratory capacity, reduced efficiency of astrocyte differentiation, and decreased GLT1 expression