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Research Paper

Serial *in vivo* imaging of transplanted allogeneic neural stem cell survival in a mouse model of amyotrophic lateral sclerosis

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ABSTRACT

Neural stem cells (NSCs) are being investigated as a possible treatment for amyotrophic lateral sclerosis (ALS) through intraspinal transplantation, but no longitudinal imaging studies exist that describe the survival of engrafted cells over time. Allogeneic firefly luciferase-expressing murine NSCs (Luc^+-NSCs) were transplanted bilaterally (100,000 cells/2 μl) into the cervical spinal cord (C5) parenchyma of pre-symptomatic (63 day-old) $SOD1^{G93A}$ ALS mice (n = 14) and wild-type age-matched littermates (n = 14). Six control $SOD1^{G93A}$ ALS mice were injected with saline. Mice were immunosuppressed using a combination of tacrolimus + sirolimus (1 mg/kg each, i.p.) daily. Compared to saline-injected $SOD1^{G93A}$ ALS control mice, a transient improvement ($p < 0.05$) in motor performance (rotarod test) was observed after NSC transplantation only at the early disease stage (weeks 2 and 3 post-transplantation). Compared to day one post-transplantation, there was a significant decline in bioluminescent imaging (BLI) signal in $SOD1^{G93A}$ ALS mice at the time of disease onset (71.7 ± 17.9% at 4 weeks post-transplantation, $p < 0.05$), with a complete loss of BLI signal at endpoint (120 day-old mice). In contrast, BLI signal intensity was observed in wild-type littermates throughout the entire study period, with only a 41.4 ± 8.7% decline at the endpoint. In $SOD1^{G93A}$ ALS mice, poor cell survival was accompanied by accumulation of mature macrophages and the presence of astrogliosis and microgliosis. We conclude that the disease progression adversely affects the survival of engrafted murine Luc^+-NSCs in $SOD1^{G93A}$ ALS mice as a result of the hostile ALS spinal cord microenvironment, further emphasizing the challenges that face successful cell therapy of ALS.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder that is characterized by the progressive loss of motor neurons. The motor neuron degeneration leads to paralysis, respiratory failure, and death within three to five years from disease onset (Chio et al., 2009; Chio et al., 2013). The pathomechanisms of motor neuron degeneration are poorly understood (Boillee et al., 2006), and there is only one FDA-approved drug that marginally extends the life expectancy of ALS patients by three to four months (Bensimon et al., 1994). A long duration between the onset of pathological changes and the manifestation of clinical symptoms in ALS complicates the management of this disease. Considering the complexity of the disease, the development of a therapy that can maintain or restore motor neuron function would provide the most comprehensive approach to treating ALS (Chen et al., 2016).

In the last decade, several preclinical studies showed promising outcomes using stem cells in ALS transgenic animal models (Teng et al., 2012; Xu et al., 2011). In 2009, the FDA approved the first clinical safety trial of direct intraspinal transplantation of human NSCs into patients with ALS (Glass et al., 2012; Riley et al., 2012). One of the major obstacles in successful stem cell therapy is the short-term survival of engrafted cells due to immune rejection (Barker and Widner, 2004). In ALS, several factors can lead to the rejection of transplanted stem cells. For example, disease progression in ALS is accompanied by a multi-phased immune response, and this plays an active role in shaping ALS pathology (Alexianu et al., 2001; Chiu et al., 2009; Yamanaka et al., 2008). An inevitable inflammation due to invasive cell transplantation

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surgery, and the resultant local trauma, could also influence the survival of engrafted therapeutic cells. In addition to the ability to determine long-term survival and the functionality of transplanted cells, real-time information on the accuracy of cell transplantation and adverse outcomes, such as teratoma formation and migration of cells to inappropriate locations, could also greatly affect the outcome of therapy. The current histological methods to analyze transplanted cells are invasive, require euthanasia of the animal, and limit our ability to monitor engrafted cells in real-time over an elongated period of time. In a phase 1/2 open label clinical safety trial, iron oxide-labeled MSCs were injected and tracked in the central nervous system (CNS) by MRI in multiple sclerosis (MS) and ALS patients (Karussis et al., 2010). However, this approach is only suitable for short-term monitoring of immediate cell engraftment, and not for imaging cell survival (Srivastava and Bulte, 2014). Teng et al. (2012) showed that the most potent therapeutic effect for a multi-level NSC transplantation approach is to escalate cell dose, indicating the importance of administrating sufficient cell numbers into critically functioning spinal cord segments to combat ALS pathology. It is therefore pivotal to develop longitudinal imaging strategies to monitor donor cell fate for devising cell therapies for ALS.

In this study, we used two different imaging modalities, computed tomography (CT) and bioluminescence imaging (BLI), to non-invasively visualize the accuracy of cell transplantation and to longitudinally monitor the survival of intraspinally transplanted murine Luc$^+$-NSCs in a transgenic SOD1^{G93A} mouse model of ALS during disease progression.

2. Materials and methods

2.1. Isolation of NSCs

All animal procedures were approved and conducted in accordance with the guidelines of our Institutional Animal Care and Use Committee (IACUC). Animals were maintained in a normal day-night cycle (12/12 hr) with access to food and water *ad libitum*. Allogeneic Luc$^+$-NSCs were isolated from the brain of a homozygous firefly luciferase transgenic mouse (FVB background, Jackson Laboratory, Bar Harbor, ME, stock # 008450) at the E13.5 stage as previously described (Ferrari et al., 2010). Briefly, cells were maintained in serum-free DMEM/F12 medium (Invitrogen, Carlsbad, CA) supplemented with 10 ng/ml epidermal growth factor (EGF; Invitrogen) and 10 ng/ml basic fibroblast growth factor (bFGF; Invitrogen). After seven days of culture, cells had grown to free-floating neurospheres. For passaging, spheres were dissociated using 1 ml Accutase (Life Technologies) for 10 min at room temperature. Single cells were resuspended in the same medium.

2.2. BLI of Luc$^+$-NSCs in vitro

Luc$^+$-NSCs were plated into poly L-lysine/laminin-coated 96-well plates at 10,000, 1000, and 100 cells per well. For bioluminescence (BL) signal measurements, the medium was removed and 30 mg/ml D-luciferin (Sigma-Aldrich, St. Louis, MO) in 10 mM phosphate buffered saline (PBS), pH = 7.4 was added. The BL signal was measured using an IVIS SpectrumCT In Vivo Imaging System (Perkin Elmer, Waltham, MA). Images were acquired at one-minute intervals for 10 min until peak signal was observed. BL signal was quantified by the creation of regions of interests (ROIs) over the wells, with the data expressed as photon flux (p/s).

2.3. Cell preparation for transplantation

On the day of transplantation, neurospheres were collected, washed with PBS, and then treated with 1 ml Accutase (Life Technologies, Carlsbad, CA) for 10 min at room temperature to make a single cell suspension. The cells were then centrifuged at 200x*g* for five minutes and the supernatant was removed. The cell pellet was resuspended in PBS and the cell number was determined using an automated cell counter (Cellometer Auto 2000, Nexcelom Bioscience, Lawrence, MA).

2.4. Immunosuppression

Mice were immunosuppressed by intraperitoneal (i.p.) daily administration of a cocktail of tacrolimus (FK-506) + sirolimus (Rapamycin) (1 mg/kg each; LC Laboratories; Woburn, MA), beginning five days before cell transplantation, and then, continuously until sacrifice.

2.5. Cell transplantation

Twenty male SOD1^{G93A} ALS mice (B6SJL-Tg(SOD1*G93A)1Gur/J: Stock # 002726) (Jackson Laboratory) and 14 male wild-type littermates were used in this study. The animals were divided into three groups: (i) SOD1^{G93A} ALS mice (n = 14) transplanted with Luc$^+$-NSCs; (ii) wild-type littermates (n = 14) transplanted with Luc$^+$-NSCs; and (iii) control SOD1^{G93A} ALS mice (n = 6) injected with PBS.

Cells were transplanted in the cervical spinal cord region of 63-day old mice as previously described (Lepore et al., 2011). In brief, animals were anesthetized with 350 mg/kg chloral hydrate i.p. (Sigma). Each mouse received two grafts (bilaterally at the C5 cervical spinal cord region) of 100,000 NSCs/site (in 2 μL saline) into the ventral horn. Control SOD1^{G93A} ALS mice were injected with only 2 μL saline/site into the ventral horn. Cells were delivered using a 10 μL Hamilton Gastight syringe with an attached 30-gauge 45° beveled needle (Hamilton; Reno, NV). The injection pipette was secured to a manual micromanipulator (World Precision Instruments; Sarasota, FL) attached to an 80° tilting base. The tip was lowered to a depth of 0.75 mm below the surface of the cord and was held in place for two minutes before and after cell injection to minimize backflow. Cells were delivered under the control of a micro-syringe pump controller (World Precision Instruments) at a rate of 0.5 μl/min.

2.6. Computed tomography

CT images were acquired with an IVIS SpectrumCT In Vivo Imaging System (Perkin Elmer) using 50 kVp X-rays at 1 mA of current, 50 ms exposure time, and with an aluminum filter. A total of 720 projections, spaced 0.5° apart, were acquired and the CT volume was reconstructed using Living Image 4.3 software (Perkin Elmer), which provided a field of view (FOV) of 12.0 × 12.0 × 3.0 cm and an isotropic resolution of 0.15 mm.

2.7. BLI of intraspinally engrafted Luc$^+$-NSCs

Bioluminescent images of the animals were acquired using the cooled CCD camera of the same IVIS SpectrumCT instrument. For each animal, anesthesia was induced using 2% isoflurane gas in oxygen, and 150 mg/kg body weight of D-luciferin (Sigma-Aldrich) was injected i.p.. Images were acquired 10 min after injection to maximize the BL signal. To generate two-dimensional (2D) bioluminescent images, no emission filter was used during imaging. Images were quantified by drawing ROIs over the cervical spinal cord region and the data were expressed as photon flux (p/s). To generate three-dimensional (3D) bioluminescent images, four spectrally resolved images were acquired using emission filters at 600, 620, 640, and 660 nm, with a bandwidth of 20 nm each. Imaging parameters were an exposure time of 180 s, an aperture of f/1, a FOV = 13 cm, and 2048 × 2048 pixel resolution. Pixel binning was set to an 8 × 8 bin width for an effective image resolution of 256 × 256 pixels. Imaging parameters were identical for both 2D and 3D imaging.