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Transplantation dose alters the dynamics of human neural stem cell engraftment, proliferation and migration after spinal cord injury

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ABSTRACT

The effect of transplantation dose on the spatiotemporal dynamics of human neural stem cell (hNSC) engraftment has not been quantitatively evaluated in the central nervous system. We investigated changes over time in engraftment/survival, proliferation, and migration of multipotent human central nervous system-derived neural stem cells (hCNS-SCns) transplanted at doses ranging from 10,000 to 500,000 cells in spinal cord injured immunodeficient mice. Transplant dose was inversely correlated with measures of donor cell proliferation at 2 weeks post-transplant (WPT) and dose-normalized engraftment at 16 WPT. Critically, mice receiving the highest cell dose exhibited an engraftment plateau, in which the total number of engrafted human cells never exceeded the initial dose. These data suggest that donor cell expansion was inversely regulated by target niche parameters and/or transplantation density. Investigation of the response of donor cells to the host microenvironment should be a key variable in defining target cell dose in pre-clinical models of CNS disease and injury.

1. Introduction

We have previously shown that human central nervous system-derived stem cells propagated as neurospheres (hCNS-SCns) (Uchida et al., 2000) exhibit robust engraftment in the injured spinal cord niche after transplantation at acute, sub-acute or chronic stages of injury in immunodeficient rodent models (Cummings et al., 2005; Hooshmand et al., 2009; Salazar et al., 2010; Piltti et al., 2013a,b). Further, when transplanted into an immunodeficient model in which rejection of the xenograft is minimized, human cells were capable of both proliferation and extensive migration (Anderson et al., 2011), both of which are normal processes for CNS development (Lui et al., 2011; Hatten, 1999; Wilcock et al., 2007). In these paradigms, recovery of function was directly dependent on, and linearly correlated with, hCNS-SCns survival and engraftment, suggesting that integration with the host microenvironment is critical.

Stem cell responses in the host microenvironment depend on both intrinsic properties and extrinsic signals (Watt and Hogan, 2000; Jones and Wagers, 2008; Discher et al., 2009; Johnston, 2009; Voog and Jones, 2010; Wagers, 2012; Faigle and Song, 2013). Understanding the dynamics of donor cell engraftment, proliferation and migration is important for optimizing cell therapies for the injured or diseased CNS. Our recent data indicates that the spinal cord injury (SCI) microenvironment alters the dynamics of transplanted hCNS-SCns when compared to that in uninjured SCI models, suggesting that injury-induced cues and target niche availability play a role in donor cell maturation and migration (Sontag et al., 2014). Additionally, donor cells may also secrete factors that serve as extrinsic cues in a dose or density dependent manner. While it is has generally been assumed that 'more is better' in the context of pre-clinical stem cell transplantation, the capacity of the CNS niche to accommodate or provide cues for donor cell integration is unclear. Few studies have investigated the effect of unmodified human neural stem cell (hNSC) dose in CNS injury or disease models (Ostenfeld et al., 2000; Keirstead et al., 2005; Rossi et al., 2010; Darsalia et al., 2011). Further, none of these studies quantitatively investigated the effect of transplantation dose on the spatiotemporal dynamics of engraftment, or employed a model in which human cell rejection was sufficiently suppressed to permit donor cell expansion (Anderson et al., 2011). Accordingly, in this

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study we investigated the relationship between transplantation dose and early and long-term cell survival, proliferation, and migration as determined by unbiased stereological assessment of donor cells in the injured spinal cord of immunodeficient NOD-*scid* mice at 2 days post-transplantation (2 DPT), 2 weeks post-transplantation (WPT) and 16 WPT.

2. Materials and methods

2.1. Ethics statement

This study was carried out in accordance with Institutional Animal Care and Use Committee regulations at the University of California, Irvine.

2.2. hNSC isolation and culture

hCNS-SCns derivation, culture and characterization were as described (Uchida et al., 2000). Methods and lines used in this study are identical to those described in Cummings et al. (2005), Salazar et al. (2010), and Piltti et al. (2013a,b). Briefly, hCNS-SCns were propagated as neurospheres in X-Vivo 15 medium without phenol red (Lonza, Basel, Switzerland) supplemented with N2, bFGF, EGF, heparin, NAC, and LIF as described previously (Uchida et al., 2000; Cummings et al., 2005). Prior to transplantation, cells (passage ≤12) were dissociated into single cells and adjusted to the following densities: 10,000 (low dose), 100,000 (medium dose), 250,000 (high dose) or 500,000 (very high dose) cells per 5 μl in X-Vivo 15. The highest employed cell dose was selected based on the maximum injection volume at which neither tissue damage or behavioral deficits were observed in uninjured mice as empirically determined in pilot studies (1.25 μl/site); maximum cell packaging density was calculated based on hCNS-SCns size (100,000 cells/μl). Viability of hCNS-SCns after pre-transplantation cell prep and at the end of transplantation day (8–10 h post-cell prep) was >90% (Table S1B).

2.3. Contusion injuries and cell transplantation

Contusion SCI was followed by early chronic hCNS-SCns transplantation into the intact parenchyma, performed as described previously (Cummings et al., 2005; Salazar et al., 2010). Briefly, adult female NOD-*scid* mice (Jackson Laboratory, Sacramento, CA) were anesthetized with isoflurane (VetEquip Inc., Pleasanton, CA), received a T9 laminectomy using a surgical microscope, and a bilateral 50-kDa contusion injury using the Infinite Horizon Impactor (Precision Systems and Instrumentation, Lexington, KY). 30 d post-SCI, mice were re-anesthetized and 1.25 μl of freshly triturated hCNS-SCns suspension injected at four bilateral sites (for a total of 5 μl) 0.75 mm from midline. Two injection sites were at the posterior aspect of T8 (rostral to the site of injury), and two at the anterior aspect of T10 (caudal to the site of injury). Injections were conducted using a Nanoinjector with a micro controller (World Precision Instruments, Waltham, MA) at speed of 417 nl/min, followed by a 2 m delay before withdrawal of the needle, using pulled silicon-treated glass injection tips with a 70 μm ID and 110 μm OD (Sutter Instruments, Novato, CA). For assessing hCNS-SCns proliferation at 2 DPT or 2 WPT, the mice were injected i.p. with 50 mg/kg of BrdU (AbD Serotec, Raleigh, NC) every 12 h starting from the time of transplantation until 2 DPT or 2 WPT.

2.4. Randomization, exclusion criteria, and group numbers

Randomization for group allocation, exclusion criteria, and blinding for histological analysis were conducted as described previously (Cummings et al., 2005; Hooshmand et al., 2009; Salazar et al., 2010). Animals with unilateral bruising or abnormal force/displacement curves after contusion injury, or with a surgical note of poor initial injection due to imperfect needle penetration or back-flow during injection (by blinded surgeon) were excluded from stereological assessments (for exclusions see Fig. 1A). All dose groups in each time cohorts were conducted in parallel, that is, animals received spinal cord injuries at the same age and during the same week of surgery. All animal care, and histological processing/analysis were performed by observers blinded to experimental cohorts or groups. Final group numbers used in histological analysis are listed in Fig. 1A.

2.5. Perfusion, tissue collection, tissue sectioning and immunohistochemistry

Endpoints for assessing the dynamics of hCNS-SCns survival, proliferation and engraftment were selected based on our previous study describing the effect of SCI niche on hCNS-SCns engraftment between 1 DPT and 14 WPT (Sontag et al., 2014). Accordingly, the selected times represent the phases of early engraftment, proliferative expansion in the SCI niche, and the time at which the majority of donor cells have exited the cell cycle and differentiated. Mice were anesthetized and transcardially perfused 2 DPT, 2 WPT, and 16 WPT with 4% paraformaldehyde. The spinal cord T6–T12 segments were dissected based on dorsal spinal root counts, postfixed in 20% sucrose/4% paraformaldehyde overnight and frozen in isopentane at −65 °C. For brightfield stereology, T6–T12 segments were sectioned at 30 μm coronally using a CryoJane tape transfer system (Leica Microsystems Inc., Buffalo Grove, IL). Sections were processed for antigen retrieval using Buffer A (pH = 6) in the Retriever 2100 system (PickCell Laboratories, Amsterdam, Netherlands). For fluorescence conjugated immunohistochemistry, T6–T12 segments were sectioned at 30 μm coronally using a HM 450 microtome (ThermoScientific, Fairlawn, NJ). 2 DPT and 2 WPT cohort T6–T12 spinal segments were trimmed rostrally and caudally 4 mm from the injury epicenter before sectioning. Antibodies and dilutions used are listed in Fig. 1B.

2.6. Stereological analysis

Unbiased stereology was conducted using systematic random sampling with the optical fractionator probe, and StereoInvestigator version 9 (Micro BrightField Inc., Williston, VT). Parameters for the analysis are listed in Table S2. Fluorescence immunostained sections were analyzed from Z-stacks of 15 μm thick optical slices captured in 1 μm intervals using an ApoTome microscope (Zeiss, Maple Grove, MN) and 63× objective.

2.7. Cell migration analysis

For rostral–caudal proportional migration analysis, the number of stereologically quantified human cells located in the rostral versus caudal regions of spinal cord was compared after the sections in each spinal cord were aligned by injury epicenter, designated by the most damaged tissue section with the largest cross sectional lesion area. Maximal distance of cell migration (cell spread rostral and caudal relative to the injury epicenter) was determined by the distance between first and last spinal cord section with $SC121^+$ cells visualized using an inverted microscope (IX71, Olympus, Center Valley, PA).

2.8. Statistical analysis

All data are shown as mean ± standard error of mean (SEM). Statistical analysis was performed using Prism4 software, version 5.0 (GraphPad Software, San Diego, CA). Correlation between transplant dose and total number engrafted cells, survival/expansion, and migration/localization were assessed using Pearson correlation coefficient and linear regression analyses. Comparison of estimated total cell numbers, survival and expansion, maximal rostral–caudal migration distance, mitotic index, and proportion of apoptotic $CC3^+$, $BrdU^+$ or $Ki67^+$ human cells between the groups were analyzed either with one-way analysis of variance (1-way ANOVA) combined with Tukey's post hoc t-test, or Student's 2-tailed t-test. A p value ≤0.05 was considered to be statistically significant.