Neuroscience 139 (2006) 513–530

LONG-TERM FATE OF NEURAL PRECURSOR CELLS FOLLOWING TRANSPLANTATION INTO DEVELOPING AND ADULT CNS

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Abstract—Successful strategies for transplantation of neural precursor cells for replacement of lost or dysfunctional CNS cells require long-term survival of grafted cells and integration with the host system, potentially for the life of the recipient. It is also important to demonstrate that transplants do not result in adverse outcomes. Few studies have examined the long-term properties of transplanted neural precursor cells in the CNS, particularly in non-neurogenic regions of the adult. The aim of the present study was to extensively characterize the fate of defined populations of neural precursor cells following transplantation into the developing and adult CNS (brain and spinal cord) for up to 15 months, including integration of graft-derived neurons with the host. Specifically, we employed neuronal-restricted precursors and glial-restricted precursors, which represent neural precursor cells with lineage restrictions for neuronal and glial fate, respectively. Transplanted cells were prepared from embryonic day-13.5 fetal spinal cord of transgenic donor rats that express the marker gene human placental alkaline phosphatase to achieve stable and reliable graft tracking. We found that in both developing and adult CNS grafted cells showed long-term survival, morphological maturation, extensive distribution and differentiation into all mature CNS cell types (neurons, astrocytes and oligodendrocytes). Graft-derived neurons also formed synapses, as identified by electron microscopy, suggesting that transplanted neural precursor cells integrated with adult CNS. Furthermore, grafts did not result in any apparent deleterious outcomes. We did not detect tumor formation, cells did not localize to unwanted locations and no pronounced immune response was present at the graft sites. The long-term stability of neuronal-restricted precursors and glial-restricted precursors and the lack of adverse effects suggest that transplantation of lineage-restricted neural precursor cells can serve as an effective and safe replacement therapy for CNS injury and degeneration. © 2006 Published by Elsevier Ltd on behalf of IBRO.

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Abbreviations: AP, human placental alkaline phosphatase transgene; A/P, anterior/posterior; C, cervical; DMEM, Dulbecco's modified Eagle medium; D/V, dorsal/ventral; E, embryonic day; EDTA, ethylenediaminetetraacetic acid; ES, embryonic stem; GRP, glial-restricted precursor; L, lateral; NPC, neural precursor cell; NRP, neuronal-restricted precursor; NSC, multipotent neural stem cell; PBS, phosphate-buffered saline.

0306-4522/06$30.00+0.00 © 2006 Published by Elsevier Ltd on behalf of IBRO.
doi:10.1016/j.neuroscience.2005.12.043

Key words: neural stem cells, neural progenitors, grafts, neurogenesis, NRP, GRP.

The use of neural precursor cells (NPCs) for therapeutic transplantation is a promising strategy for the treatment of CNS injuries and neurodegenerative disorders because of the potential for replacement of lost or dysfunctional cells (Fischer, 2000). Transplanted cells offer additional benefits, including neuroprotection, the potential for delivery of therapeutic factors such as neurotrophic proteins and mobilization of endogenous NPCs (Rao and Mayer-Proschel, 2000). The importance of studying NPCs has been further highlighted by the observation that while it is possible to obtain and replace glial cells by grafting glial precursors, the generation of neurons in the adult non-neurogenic CNS is more challenging. Thus, pure populations of multipotent neural stem cells (NSCs) show poor survival in most regions of the brain (Lepore et al., 2004), while the differentiation of neuronal precursors is inhibited in the injured CNS (Cao et al., 2002b).

The success of effective transplantation strategies, particularly replacement of neurons, requires that the grafted NPCs survive and integrate potentially for the life of the transplant recipient (Bjorklund et al., 2003). In addition, it is important to demonstrate that transplants do not cause adverse outcomes such as tumor formation, persistence of a pronounced immune response, migration to inappropriate locations or differentiation to unexpected cell types. Unfortunately, few studies have examined the long-term fate of transplanted NPCs in the adult CNS (Minger et al., 1996; Svendsen et al., 1997) and often, when assessing the fate of NPC grafts, have not employed transplantation of well-defined populations of cells.

The objective of the present study was to extensively characterize the long-term fate of a defined population of NPCs following transplantation into the developing and adult CNS (brain and spinal cord). Specifically, a defined population of neuronal-restricted precursors (NRPs) and glial-restricted precursors (GRPs), NPCs with lineage restrictions for neurons and glia, respectively, was used in this study (Mayer-Proschel et al., 1997; Rao and Mayer-Proschel, 1997). Transplanted cells were derived from embryonic day (E)-13.5 fetal spinal cord of transgenic donor rats that express the marker gene human placental alkaline phosphatase (AP) under the control of the ubiquitous Rosa 26 promoter (Kisseberth et al., 1999; Mujtaba et al., 2002) for reliable tracking. Previous work has demonstrated that NRPs and GRPs, whether grafted individually or together, display survival, migration and differentiation following transplantation into both the intact (Yang et al., 2000; Herrera et al., 2001; Cao et al.,

2002b; Han et al., 2002) and injured (Han et al., 2004; Lepore et al., 2004) CNS, while the differentiation of NRPs alone is inhibited in the injured spinal cord (Cao et al., 2002b). However, these studies did not assess the fate of transplants beyond time points of 6 weeks or the potential for long-term adverse effects.

We examined the fate of transplanted NPCs up to 15 months *in vivo*, over half of an average laboratory rat's lifespan. Specifically, we asked if the transplanted NPCs survive, persist as dividing NPCs, localize away from sites of engraftment, produce various classes of differentiated progeny and integrate with host tissue, including the formation of synapses (verified at the ultra-structural level). In addition, we asked if grafted cells localize to inappropriate areas, form tumors or elicit a pronounced immune response. Answers to these questions should help to evaluate the long-term safety and potential effectiveness of grafted CNS NPCs.

We found that grafted cells, even at long periods of time *in vivo*, displayed a similar fate as short-term transplants, including robust survival, morphological maturation, distribution away from injection sites, differentiation into all three mature CNS cell types (neurons, astrocytes and oligodendrocytes) and the formation of synapses. Furthermore, the grafts did not result in any apparent deleterious outcomes, in that we did not detect tumor formation, cells were not found in unwanted locations and no pronounced immune response was present at the graft sites. The long-term fate observed in this study suggests that transplantation of defined populations of lineage-restricted NPCs derived from the developing CNS is a potentially useful therapeutic strategy for treatment of CNS injury and degeneration.

EXPERIMENTAL PROCEDURES

Cell isolation and culture

Isolation and culturing of NRPs and GRPs. Mixed NRPs/GRPs (Lepore et al., 2004., 2005; Lepore and Fischer, 2005) were simultaneously isolated from the embryonic day-13.5 spinal cord of transgenic Fischer 344 rats that express the marker gene AP. Briefly, embryos were isolated in a dish containing Dulbecco's modified Eagle medium (DMEM)/F12 (Invitrogen; Carlsbad, CA, USA). Trunk segments were incubated in a collagenase Type I (Worthington; Lakewood, NJ, USA; 10 mg/ml)/Dispase II (Roche; Nutley, NJ, USA; 20 ng/ml)/Hank's balanced salt solution (Cell-Gro; Herndon, VA, USA) for 8 min at room temperature to allow for peeling away of meninges from the cords. Cords were dissociated using a 0.05% trypsin/ethylenediaminetetraacetic acid (EDTA; Invitrogen) solution for 20 min at 37 °C. Cells were then plated in NRP complete medium [DMEM-F12, bovine serum albumin (1 mg/ml; Sigma; St. Louis, MO, USA), B27 (Invitrogen), basic fibroblast growth factor (10 μg/ml; Peprotech; Rocky Hill, NJ, USA), Pen-Strep (100 IU/ml; Invitrogen), N2 (10 μl/ml; Invitrogen) and neurotrophin-3 (10 μg/ml; Peprotech)] on poly-L-lysine- (13.3 μg/ml; Sigma) and laminin- (20 μg/ml; Invitrogen) coated dishes. Fresh medium was added to cultures every 48 h.

Preparation of cells for grafting

Following embryonic dissection, NRPs and GRP were co-cultured in complete medium on poly-L-lysine- and laminin-coated dishes for 5–7 days prior to transplantation. A mixed population of NRPs and GRPs was dissociated from culture flasks using 0.05% trypsin/EDTA (Invitrogen), washed and re-suspended at a concentration of 12,500 cells/μl (in basal media) for transplantation. Cells were placed on ice throughout the grafting session. After the completion of the grafting session, cell viability was assessed using the Trypan Blue assay. Viability was always found to be greater than 90%. The composition of the NRP/GRP cultures, with respect to the presence of undifferentiated NPCs and the absence of mature cells, was verified before grafting by staining for the immature neural marker nestin, for markers of mature neurons (NeuN), astrocytes (GFAP) and oligodendrocytes (RIP), as well as for markers of NRPs (E-NCAM) and GRPs (A_2B_5).

Animal surgery/transplantation

Adult female Fischer rats received NPC transplants into both the intact striatum and spinal cord during the same grafting session. Spinal cord transplants were conducted first, followed subsequently by grafting into the striatum. No immune suppression was utilized.

Adult brain transplants. Adult female Fischer ($n=19$) rats (approximately 200 g) received i.p. injections of anesthetic cocktail [acepromazine maleate (0.7 mg/kg; Fermenta Animal Health, Kansas City, MO, USA), ketamine (95 mg/kg; Fort Dodge Animal Health; Fort Dodge, IA, USA), and xylazine (10 mg/kg; Bayer, Shawnee Mission, KS, USA)]. Each animal received a single unilateral graft of 25,000 cells in 2 μl into the striatum. The coordinates used for grafting were as follows: striatum anterior/posterior (A/P), +0.6; lateral (L), ±2.8; dorsal/ventral (D/V), −4.8/−4.2. The tooth bar was set at −2.3, and D/V coordinates were taken from the dura. Briefly, cells were delivered using a 10 μl Hamilton (Hamilton; Reno, NV, USA) Gastight syringe with a 33-gauge beveled needle attached. Cells were injected at both the deepest and shallowest D/V coordinates. The tip was held in place both before and after each of the two injections for 2 min. Cells were delivered at a rate of 0.5 μl/min.

Adult spinal cord transplants (intact). Adult female Fischer ($n=9$) rats (approximately 200 g) received i.p. injections of anesthetic cocktail (same as above). The back musculature was separated and retracted, and a laminectomy was performed at the cervical (C)3/4 level. The dura was cut and retracted above the injection site using a 30-gauge needle. Each animal received a single unilateral graft of 25,000 cells in 2 μl into the dorsal column (right side) at the C4 level. Briefly, cells were delivered using a 10 μl Hamilton Gastight syringe with an attached borosilicate glass tip (50–100 μm tip diameter). The injection pipette was secured to a manual micromanipulator (World Precision Instruments; Sarasota, FL, USA) attached to an 80° tilting base. The tip was lowered to a depth of 1 mm below the surface of the cord and was held in place for 2 min before and after cell injection. Cells were delivered under the control of a microsyringe pump controller (World Precision Instruments) at a rate of 1 μl/min. The dura was closed with 9-0 suture; muscle was re-apposed; skin was closed with wound clips. Animals received Bupranorphin (Reckitt Benckiser; Richmond, VA, USA) postoperatively.

Neonatal brain transplants. Postnatal day-2 Sprague–Dawley ($n=11$) rats (both male and female) were anesthetized via hypothermia. Each animal received a single unilateral graft of 25,000 cells in 2 μl into the striatum. Transplants were delivered using a neonatal rat stereotax. The coordinates used for grafting are as follows: striatum A/P, 0.0; L, ±2.4; D/V, −3.4/3.0. D/V coordinates were taken from the surface of the brain. Briefly, cells were delivered using a 10 μl Hamilton Gastight syringe with a 33-gauge beveled needle attached. Cells were injected at both the deepest and shallowest D/V coordinates. The tip was held in place both before and after each of the two injections for 2 min. Cells