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Research report

Labeling of olfactory ensheathing glial cells with fluorescent tracers for neurotransplantation

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ABSTRACT

Development of cell-based treatment strategies for repair of the injured nervous system requires cell tracing techniques to follow the fate of transplanted cells and their interaction with the host tissue. The present study investigates the efficacy of fluorescent cell tracers Fast Blue, PKH26, DiO and CMFDA for long-term labeling of olfactory ensheathing glial cells (OEC) in culture and following transplantation into the rat spinal cord. All tested dyes produced very efficient initial labeling of p75-positive OEC in culture. The number of Fast Blue-positive cells remained largely unchanged during the first 4 weeks but only about 21% of the cells retained tracer 6 weeks after labeling. In contrast, the number of cells labeled with PKH26 and DiO was reduced to 51–55% after 2 weeks in culture and reached 8–12% after 4–6 weeks. CMFDA had completely disappeared from the cells 2 weeks after labeling. AlamarBlue™ assay showed that among four tested tracers only CMFDA reduced proliferation rate of the OEC. After transplantation into spinal cord, Fast Blue-labeled OEC survived for at least 8 weeks but demonstrated very limited migration from the injection sites. Additional immunostaining with glial and neuronal markers revealed signs of dye leakage from the transplanted cells resulted in weak labeling of microglia and spinal neurons. The results show that Fast Blue is an efficient cell marker for cultured OEC. However, transfer of the dye from the transplanted cells to the host tissue should be considered and correctly interpreted.

1. Introduction

In recent years, cell transplantation strategies have become an intriguing possibility for treatment of spinal cord injury. Among various types of tested cellular grafts, the most promising effects on axonal regeneration have been reported using cultured olfactory ensheathing glial cells [7,60–62], Schwann cells [20,53] and various types of stem cells [16,19,67]. Further development of transplantation-based therapies will require better understanding of the basic mechanisms underlying survival and migration of transplanted cells [27,55]. Since experimentally grafted cells may change their phenotype after differentiation and become undistinguishable from other cell types in the host tissue [2], they should be labeled with stable non-toxic cell tracers.

A series of cell tracing techniques have been tested in transplantation experiments [52,77]. However, all methods of cell labeling have their advantages and limitations. For example, labeling with Hoechst 33342 and BrdU is a very simple procedure and has been widely used to trace different cell types. However, both *in vitro* and *in vivo* studies have demonstrated that these markers can leak from the labeled cells into the host tissue producing secondary non-specific staining [10,17,29,44]. It has also been shown that viral vector-mediated expression of the green fluorescent protein in olfactory ensheathing cells [37,65] and neural stem cells [28,72,73] is down-regulated 2–3 weeks after transplantation into the injured spinal cord. Although lentiviral vectors are more stable than other retroviral vectors, they are not entirely resistant to silencing in certain cell types [11].

Fluorescent tracers for retrograde and anterograde neuronal labeling have been used in neuroanatomical studies for many years [31,32,71] and some of these dyes including Fast Blue, True Blue, Diamidino Yellow, Fluoro-Gold and fluorescent latex microspheres demonstrate remarkable stability and can be found in labeled neurons several weeks after tracer application [1,4,12,57,59,78]. Our previous reports also show that Fast Blue is an efficient long-term tracer for spinal motoneurons [49], different subpopulations of sensory dorsal root ganglion neurons [74] and rubrospinal neurons [51].

In the present study we tested the hypothesis that retrograde neuronal tracer Fast Blue could be used as a long-term marker for olfactory ensheathing glial cells *in vitro* and following transplantation into the spinal cord. For comparison, we also assessed efficacy and stability of well known fluorescent cell tracers PKH26 [43,44,46], DiO [22,35] and CMFDA [6,42].

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2. Materials and methods

2.1. Experimental animals

Experiments were performed on adult (10–12 weeks, $n = 17$) female Sprague–Dawley rats (Scanbur AB, Sweden). The animal care and experimental procedures were carried out in accordance with the European Communities Council Directive (86/609/EEC) and the NIH Guide for Care and Use of Laboratory Animals (National Institutes of Health Publications No. 86-23, revised 1985) and were also approved by the Northern Swedish Committee for Ethics in Animal Experiments. All surgical procedures were carried out aseptically under general anesthesia using a mixture of ketamine (Ketalar, Parke-Davis; 100 mg/kg i.v.) and xylazine (Rompun, Bayer; 10 mg/kg i.v.). Normal saline (2 ml s.c.) and benzylpenicillin (Boehringer Ingelheim; 60 mg i.m.) were given after each surgical procedure.

2.2. Culture of olfactory ensheathing cells

Primary cultures of olfactory ensheathing cells (OEC) were obtained using previously reported protocols [50]. Adult female rats ($n = 9$) were killed with an overdose of sodium pentobarbital (240 mg/kg) and the olfactory bulbs (OB) were dissected into Hank's balanced salt solution (HBSS, Invitrogen) and cleaned from blood clots and meninges. The olfactory nerve and glomerular layers were separated under the dissecting microscope, cut into small pieces and collected into Ca^{2+} and Mg^{2+}-free HBSS. After washing in HBSS, the tissue was dissociated with 0.1% trypsin for 15 min at 37 °C. The enzymatic activity was stopped with a mixture of DMEM and Ham's F-12 medium (1:1; Sigma–Aldrich) supplemented with 2 mM glutamine, 10 U/ml penicillin, 10 μg/ml streptomycin, 50 μg/ml gentamycin and 10% fetal calf serum, FCS ("D/F-10S medium" from [63]). The digestant was centrifuged at 800 rpm for 3 min, re-suspended and triturated 5–7 times through a fire-polished, siliconized Pasteur pipette and 5–7 times through a 21 G needle. Since the resulting cell suspension contained a mixture of OEC, fibroblasts, endothelial cells and astrocytes, purification of OEC was achieved using method of differential cell adhesion [45]. The method is based on a very low adhesion rate of OEC to the surface of uncoated culture flasks. The cell suspension obtained from each pair of OB was re-suspended in D/F-10S culture medium, seeded on uncoated Lab-Tek® one-chamber slide (Nalge Nunc International Corp.) and cultured at 37 °C with 5% CO_2. After 18 h, the supernatant was removed and re-plated on a second uncoated LabTek® slide and incubated for additional 36–48 h. During this procedure, most of the non-OEC cells attached to the uncoated chamber slides [45]. The supernatants from three chamber slides were removed and re-plated on 25 cm^2 poly-L-lysine-coated culture flask with D/F-10S culture medium and incubated for 48 h. After removing the medium the cells were washed with Ca^{2+} and Mg^{2+}-free HBSS. The resulting purity of a-OEC was 93–95%. The cultures were expanded using D/F-10S [63] with 2 μM forskolin and recombinant human glial growth factor-2 (GGF2, 40 ng/ml, Acorda Therapeutics, Inc., Hawthorne, NY) as mitogens [2,13,76].

2.3. Labeling of OEC

After washing with HBSS, the cells were detached from culture flasks with trypsin/EDTA and concentrated in the corresponding medium to 10^6 cells/ml. Suspensions of OEC were labeled with Fast Blue (2% aqueous solution; 5 μl/ml of cell suspension for 15 min at 37 °C, Sigma–Aldrich), PKH26 (2 μl/ml of cell suspension for 5 min at room temperature, Sigma–Aldrich), Vibrant DiO (5 μl/ml of cell suspension for 15 min at 37 °C, Molecular Probes) and Cell Tracker™ Green CMFDA (5 μM final concentration in the medium for 60 min at 37 °C, Molecular Probes). The cells were washed and cultured on Lab-Tek® 8-chambers slides at a density of 10,000 cells per well for 24, 48 or 72 h or plated in culture flasks and then sub-cultured on Lab-Tek® slides for analysis at 2, 4, 6 and 8 weeks after initial labeling.

2.4. Immunocytochemistry and cell quantification

Cell cultures were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at room temperature for 20 min. For cell quantification, OEC were additionally immunostained for their specific marker low-affinity NGF receptor p75 [50,63]. In this way, non-OEC cells labeled with fluorescent tracers could be excluded from analysis. After blocking with 5% normal goat serum for 15 min, antisera against p75 receptor raised in mouse (1:200; Chemicon) was applied for 2 h at room temperature. After rinsing in PBS and additional incubation in 5% normal serum, secondary goat anti-mouse highly cross-adsorbed Alexa Fluor® 488 (PKH26-labeled OEC) and Alexa Fluor® 568 (Fast Blue, DiO and CMFDA) antibodies (1:300; Molecular Probes) were applied for 2 h at room temperature in the dark. The Lab-Tek® slides were coverslipped with Vectashield® mounting medium (Vector Laboratories). DAPI was used to counter stain nuclei of OEC labeled with PKH26, DiO and CMFDA. Labeled cells were counted in 5 randomly selected chambers of Lab-Tek® slides at 250× final magnification using 400 μm × 400 μm frame. The results were expressed as ratio between p75-positive cells labeled with fluorescent tracer and total number of p75-labeled cells in culture (Table 1).

Table 1
Relative number of labeled olfactory ensheathing cells (%).

Tracer	2 weeks, P2	4 weeks, P4	6 weeks, P6
Fast Blue	100[#]	100 (weak labeling)[†]	21.1 ± 4.8[*]
PKH26	55.0 ± 5.4[*]	11.0 ± 0.8	8.1 ± 0.7
DiO	51.4 ± 5.0[*]	12.3 ± 3.5	7.1 ± 1.5
CMFDA	No labeling	No labeling	No labeling

P2, P4 and P6 are cell passages.
[*] $P < 0.001$ (Fast Blue, 6w vs. Fast Blue, 2w and 4w; PKH26, 2w vs. PKH26, 4w and 6w; DiO, 2w vs. DiO, 4w and 6w).
[#] $P < 0.001$ (Fast Blue, 2w vs. PKH26, 2w and DiO, 2w).
[†] $P < 0.001$ (Fast Blue, 4w vs. PKH26, 4w and DiO, 4w).

2.5. Cell proliferation assay

AlamarBlue™ assay (Serotec) was used for detection of cells proliferation. The assay incorporates a colorimetric oxidation–reduction (REDOX) indicator which changes color in response to the chemical reduction of the growth medium resulting from changes in metabolic activity of the cells. It has been shown that the optical density of AlamarBlue is proportional to the number of cultured cells and that this method can be used as a conventional proliferation assay [33,50]. In addition, it is not toxic or sample-destructive. The optical density of AlamarBlue was obtained by measuring changes of absorbance at 570 nm and using a background correction at 600 nm. For the assay, OEC were transferred into 96-well culture plates (2500 cells in 250 μl of growth medium per well and 12 wells per tracer) and AlamarBlue™ was added to obtain 10% final concentration. The cultures were maintained under identical conditions and the plates were subsequently read on a microplate reader (Spectra Max 190, Molecular Device, Albertville, MN, USA) every day at the same time point for 4 consecutive days.

2.6. Transplantation of OEC into spinal cord

The cervical C4 spinal cord of normal rats ($n = 8$) was exposed by dorsal laminectomy. Based on the results of *in vitro* experiments, only Fast Blue-labeled OEC were used for transplantation. Labeled cells were detached with trypsin/EDTA, washed and concentrated to 10^5 cells/μl in the growth medium without serum. After transfer into a siliconized glass micropipette (outer diameter 100 μm) attached to a 5 μl Hamiltone syringe, 0.5–0.6 μl of cell suspension (50,000–60,000 cells) was slowly (10 min) pressure-injected into the left and right lateral funiculus (depth 1.0 mm from the surface of the spinal cord). The micropipette was left in place for additional 2–3 min. After covering the dura mater with stretched parafilm and Spongostan®, the muscles and skin were closed in layers and the rats given saline (4 ml s.c.) and benzylpenicillin (Boehringer Ingelheim; 60 mg i.m.).

2.7. Tissue processing and immunohistochemistry

At 2 and 8 weeks after transplantation, the animals were deeply anesthetized with an intraperitoneal overdose of sodium pentobarbital and transcardially perfused with Tyrode's solution followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Spinal cord segments C3–C5 were then removed and transferred into the same fixative. For fluorescence microscopy, 50-μm-thick serial longitudinal sections from the spinal cord were cut on a vibratome (Leica Instruments, Germany), mounted on gelatin-coated glass slides, air dried, shortly immersed in xylene and coverslipped in DPX (Kebo Lab AB, Sweden). For immunohistochemistry, spinal cord segments C3–C5 were post-fixed for 2 h, cryoprotected in 10% and 20% sucrose for 2–3 days and frozen in liquid isopentane. Serial longitudinal 16-μm-thick sections were cut on a cryomicrotome (Leica Instruments, Germany), thaw-mounted in pairs onto SuperFrost®Plus slides, dried overnight at room temperature and stored at −85 °C before processing. After blocking with 5% normal goat serum for 15 min, mouse anti-OX-42 (1:250; Serotec) and mouse anti-NeuN (1:200; Chemicon) primary antibodies were applied for 2 h at room temperature. Monoclonal mouse anti-rat OX-42 (Catalog Number MCA275G; www.serotec.com) recognizes the rat equivalent of human CD11b, the receptor for the iC3b component of complement. The antigen is expressed on most macrophages and microglial cells in the brain. NeuN antibody (NEUronal Nuclei; clone A60; Catalog Number MAB377; www.chemicon.com) specifically recognizes the DNA-binding, neuron-specific protein NeuN, which is present in most CNS and PNS neuronal cell types. After rinsing in PBS, secondary goat anti-mouse antibody Alexa Fluor® 568 (1:300; Molecular Probes) was applied for 2 h at room temperature in the dark. The slides were coverslipped with Vectashield® mounting medium (Vector Laboratories). The staining specificity was tested by omission of primary antibodies.

2.8. Counts of labeled cells in spinal cord sections

The extent of Fast Blue leakage from the labeled OEC was studied in experimental animals at 2 weeks after cell transplantation into the normal spinal cord ($n = 4$). Spinal cord sections were additionally immunostained with OX-42 and NeuN antibodies to reveal Fast Blue-labeled microglia and spinal neurons, respectively.