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Efficacy of olfactory ensheathing cells to support regeneration after spinal cord injury is influenced by method of culture preparation

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ABSTRACT

Olfactory ensheathing cells (OEC) have been shown to stimulate regeneration, myelination and functional recovery in different spinal cord injury models. However, recent reports from several laboratories have challenged this treatment strategy. The discrepancy in results could be attributed to many factors including variations in culture protocols. The present study investigates whether the differences in culture preparation could influence neuroprotective and growth-promoting effects of OEC after transplantation into the injured spinal cord. Primary OEC cultures were purified using method of differential cell adhesion (a-OEC) or separated with immunomagnetic beads (b-OEC). After cervical C4 hemisection in adult rats, short-term (3 weeks) or long-term (7 weeks) cultured OEC were transplanted into the lateral funiculus at 1 mm rostral and caudal to the transection site. At 3–8 weeks after transplantation, labeled OEC were mainly found in the injection sites and in the trauma zone. Short-term cultured a-OEC supported regrowth of rubrospinal, raphaespinal and CGRP-positive fibers, and attenuated retrograde degeneration in the red nucleus. Short-term cultured b-OEC failed to promote axonal regrowth but increased the density of rubrospinal axons within the dorsolateral funiculus and provided significant neuroprotection for axotomized rubrospinal neurons. In addition, short-term cultured OEC attenuated sprouting of rubrospinal terminals. In contrast, long-term cultured OEC neither enhanced axonal growth nor prevented retrograde cell death. The results suggest that the age of OEC in culture and the method of cell purification could affect the efficacy of OEC to support neuronal survival and regeneration after spinal cord injury. This article is part of a Special Issue entitled: Understanding olfactory ensheathing glia and their prospect for nervous system repair.

Introduction

In the adult mammalian spinal cord, damage to nerve fibers results in nearly complete failure of the severed axons to regenerate. Pathology of the spinal injury is determined not only by the initial mechanical insult, but also by secondary processes including ischemia and cell death, and eventually results in cyst formation and enlargement of the trauma zone. Cytokines, free radicals and nitric oxide generated by microglia stimulate reactive astrocytes to secrete proteoglycans and to form glial scar (Busch et al., 2010; Fitch and Silver, 2008). In addition, retrograde degeneration occurs in the lesioned spinal tracts (Deumens et al., 2005). Severed axons are limited by inhibitory nature of myelin and the glial scar, and only capable of sprouting with little functional recovery (Boulenguez and Vinay, 2009; Bunge, 2008; Tetzlaff et al., 2010).

To achieve regeneration after spinal cord injury, several challenging objectives must be met, such as minimizing inhibitory signals in the trauma zone, reducing secondary degeneration, scarring and cavitation, bridging spinal cord tissue gaps and stimulating guided axonal growth across the lesion site. Experimental data indicate that different treatment strategies including modification of inhibitory extracellular matrix molecules, transplants of embryonic nervous tissue and glial cells, bridging nerve grafts and neurotrophic factors could, in principle, stimulate regeneration of injured central nerve tracts (Bunge, 2008; Fortun et al., 2009; Kocsis et al., 2009; Lu and Tuszynski, 2008; Tetzlaff et al., 2010).

Remarkably promising effects on axonal regeneration and neuronal survival after spinal cord injury have been reported after transplantation of a certain glial cell type, the olfactory ensheathing cells (OEC) (Fouad et al., 2009a; Franssen et al., 2007; Kocsis et al., 2009; Lindsay et al., 2010; Munoz-Quiles et al., 2009; Radtke and Wewetzer, 2009; Raisman et al., 2011; Richter and Roskams, 2008). Although the mechanisms of growth-promoting and neuroprotective effects of OEC are not completely understood. It has been found that OEC express various neurotrophic factors (Lipson et al., 2003; Pastrana et al., 2007), secrete proteins which indirectly stimulate Schwann cells to support neurite outgrowth and enhance spinal cord repair (Au et al., 2007) and, in contrast to Schwann cells, intermingle with astrocytes without inducing their hypertrophy (Lakatos et al., 2000; Santos-Silva et al., 2007). However, it has been discussed that

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individual therapies are unlikely to provide a panacea for spinal cord repair and rather a combinations of strategies could lead to improvements in outcome after spinal injury (Bunge, 2008; Lu and Tuszynski, 2008). The growth-promoting effect of OEC could be significantly improved by combining OECs with Schwann cells and chondroitinase treatment to overcome inhibition of the glial scar (Bretzner et al., 2010; Fouad et al., 2009b). It also has been demonstrated that OEC can attenuate degeneration of axotomized descending neurons after spinal cord injury (Xiao et al., 2005) and retinal ganglion cells after optic nerve lesion (Wu et al., 2010).

Nevertheless, the growing number of reports had questioned the efficacy of OEC to promote spinal cord repair (Aoki et al., 2010; Barakat et al., 2005; Bretzner et al., 2010; Collazos-Castro et al., 2005; Lu et al., 2006; Steward et al., 2006). It has been proposed that functional recovery after OEC transplantation could be supported by collateral sprouting (Bradbury and McMahon, 2006; Toft et al., 2007) and migrating host Schwann cells (Boyd et al., 2005) rather than by axonal regeneration across the lesion site. The discrepancy in results could be attributed to many factors including the source of tissue used to obtain the OEC, the age and the purity of transplanted cells and the methodology used in culture preparation (Barnett and Riddell, 2007; Kawaja et al., 2009; Nieto-Sampedro, 2003; Raisman and Li, 2007). Moreover, the type of spinal cord injury, the timing and the location of grafts within the injured spinal cord could also influence the outcome of OEC transplantation.

The present study investigates whether OEC harvested from the adult rat olfactory bulbs and purified with a method of differential adhesion (Nash et al., 2001; Sasaki et al., 2004) or with immunomagnetic beads (Gudino-Cabrera and Nieto-Sampedro, 1996; Lopez-Vales et al., 2007) could stimulate axonal regrowth across the lesion site and support survival and sprouting of axotomized rubrospinal neurons after cervical spinal cord hemisection in adult rats. Since transplantation into the injured human spinal cord will require large quantities of OEC and, therefore, could increase the time in culture, we compared growth-promoting and neuroprotective effects of short-term and long-term cultured cells.

Materials and methods

Experimental animals

The experiments were performed on adult (10–12 weeks, $n=96$) female Sprague–Dawley rats (Taconic Europe A/S, Denmark). The animal care and experimental procedures were carried out in accordance with the European Communities Council Directive (86/609/EEC) and were also approved by the Northern Swedish Committee for Ethics in Animal Experiments. All surgical procedures were performed 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.).

Culture of olfactory ensheathing cells

Primary cultures of olfactory ensheathing cells (OEC) were obtained using previously described technique (Novikova et al., 2006; Pettersson et al., 2010; Ramon-Cueto and Nieto-Sampedro, 1994). Adult female rats ($n=25$) were killed with an overdose of sodium pentobarbital (240 mg/kg, Apoteks-bolaget, Sweden) and the olfactory bulbs (OB) were dissected into Hank's balanced salt solution (HBSS, Invitrogen AB, Täby, Sweden) and cleaned from blood clots and meninges. The olfactory nerve and glomerular layers were separated under the microscope, cut into small pieces and collected into Ca^{2+} and Mg^{2+}-free HBSS. After washing in HBSS, the tissue was dissociated using 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 Sweden AB, Stockholm, Sweden) 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 Ramon-Cueto and Nieto-Sampedro, 1994). 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 primary culture contained a mixture of OEC, fibroblasts, endothelial cells and astrocytes, purification of OEC was achieved using a method of differential adhesion (a-OEC) (Nash et al., 2001) or immunomagnetic beads (b-OEC) (Gudino-Cabrera and Nieto-Sampedro, 1996; Lopez-Vales et al., 2007).

The method of differential adhesion is based on a very low adhesion rate of OEC to the surface of uncoated culture flasks (Nash et al., 2001). 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 slides (Nalge Nunc International Corp) and cultured at 37 °C with 5% CO_2. After 18 h, the supernatant was removed and re-plated on another uncoated Lab-Tek® slide and incubated for additional 36–48 h. The supernatants from three chamber slides (6 olfactory bulbs from 3 rats) were removed and re-plated on 25 cm^2 poly-L-lysine-coated culture flask with D/F-10S culture medium. During the procedure, most of the non-OEC cells attached to the uncoated chamber slides (Nash et al., 2001). The culture was incubated for another 48 h to allow OEC to adhere to the poly-L-lysine-coated surface of culture flask. 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 immonumagnetic purification of OEC was performed on 1-week-old primary cultures using sheep anti-rabbit Dynabeads (M280; Dynal A.S., Oslo, Norway) conjugated to low-affinity NGF receptor IgG (p75; Sigma-Aldrich Sweden AB, Stockholm, Sweden and Chemicon International, Ltd., Malmö, Sweden) according to manufacturer's recommendation. The Dynabeads were incubated with p75 IgG for 24 h at 4 °C (2.5 μg of IgG for 10^7 of Dynabeads) and then with OEC suspension for 1 h at 4 °C (about 10 Dynabeads per target cell). After washing and re-suspension, OEC were placed on 25 cm^2 poly-L-lysine-coated culture flasks and kept at 37 °C, 95% humidity and 5% CO_2. The resulting purity of b-OEC was about 80%.

The cultures were expanded using three different growth mediums: 1) D/F-10S (Ramon-Cueto and Nieto-Sampedro, 1994), 2) DMEM-10S with 2 μM forskolin and 20 μg/ml pituitary extract (Invitrogen AB, Täby, Sweden) as mitogens (Pastrana et al., 2006; Ramon-Cueto et al., 1998; Ramon-Cueto et al., 2000; Rubio et al., 2008) and 3) D/F-10S with 2 μM forskolin and recombinant human glial growth factor-2 (GGF2, 40 ng/ml, Acorda Therapeutics, Inc., Hawthorne, NY) as mitogens (Alexander et al., 2002; Chuah et al., 2000; Yan et al., 2001). The medium was changed every second day and for *in vitro* experiments cells were maintained in culture for 20 weeks. Population doublings and cumulative growth rate were calculated in a-OEC cultures according to the formula PD = log (number of cells harvested/number of cells seeded)/log 2 as described previously by Rubio et al., 2008). For *in vivo* experiments, the time from OB dissection to OEC transplantation was either 3 weeks (short-term cultured cells, OEC ST) or 7 weeks (long-term cultured cells, OEC LT) and cells were expanded using growth medium D/F-10S with forskolin and GGF2. The timing was based on our preliminary findings that OEC cultured for 7 and 20 weeks had the same neuroprotective and growth-promoting effects after spinal cord transplantation (unpublished observation). The purity of transplanted OEC was assessed using immunostaining for p75 receptors and S100 protein (see below). OEC were sub-cultured on Lab-Tek® 8-chambers slides at a density of 15,000 cells per well for 2 days and then fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH = 7.4) at room temperature for 20 min.

Labeling of OEC

For labeling with fluorescent dye Fast Blue (Sigma-Aldrich Sweden AB, Stockholm, Sweden), cells were detached from culture flasks with

Trypsin/EDTA and concentrated in the growth medium to $1–3\times10^6$ cells/ml. Fast Blue was added to cell suspension at concentration 5 μl/ml for 15 min at 37 °C. After labeling, the cells were gently washed, plated in 25 cm^2 poly-L-lysine-coated culture flasks with growth medium and kept for additional 72 h before transplantation into spinal cord (Pettersson et al., 2010).

To express green fluorescent protein (GFP) in OEC (Fig. 1E), we used retroviral expression system. Retroviral packaging cell line PT67 (Clontech, Palo Alto, USA) was propagated in high glucose DMEM (Invitrogen AB, Täby, Sweden) supplemented with penicillin, 100 U/ml, streptomycin 100 μg/ml (Invitrogen AB, Täby, Sweden) and 10% FCS and transfected with a retroviral expression vector pLEGFPpuro. The GFP expression vector pLEGFPpuro resulted from substitution of the neomycin resistance gene in the retroviral vector pLEGFP-N1 (Clontech, Palo Alto, USA) by the puromycin resistance gene from pMSCVpuro (Clontech, Palo Alto, USA). The transfection of PT67 cells was done by a lipofection method with Lipofectamin 2000 (Invitrogen, Carlsbad, USA) according to the protocol provided by the manufacturer. Successfully transfected cells were selected with puromycin and maintained (Clontech, Palo Alto, USA). For virus production, the packaging cells were seeded at confluence about 60%, incubated overnight at 37 °C and transferred into a 32 °C incubator with 95% humidity and 5% CO_2. After 72 h, virus containing medium was collected and filtered through 0.45 μm low-protein binding filter (Pall Corporation, Cornwall, UK). For transduction, OEC were seeded at a density of 5×10^3 cells per cm^2 and 2 days later their growth medium was replaced with the viral medium containing 8 μl/ml polybrene (Sigma-Aldrich, Milwaukee, USA). The cells were kept at 32 °C for 24 h. After transduction, the viral medium was changed to a fresh OEC growth medium. Transduction efficacy was about 40%. Following incubation at 37 °C for 48 h, the GFP-positive cells were separated from non-transduced cells by fluorescence activated cell sorter technique (FACS DiVa, BD Biosciences, San Jose, USA).

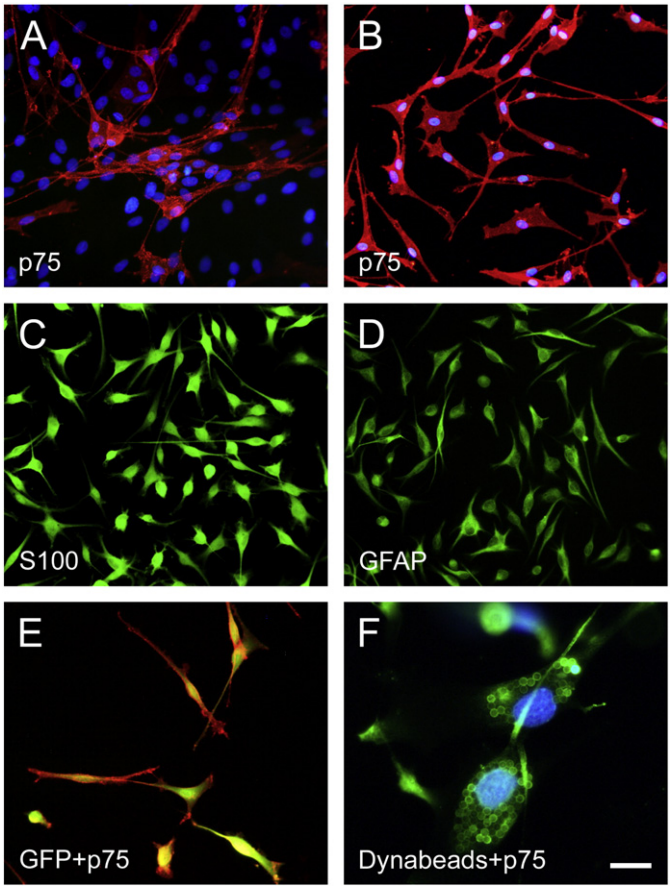

Fig. 1. Primary culture of olfactory ensheathing cells, OEC (A) and OEC purified with a method of differential adhesion (B–E) and immunomagnetic beads (F). Cell are immunostained for low-affinity NGF receptors p75 (p75), S-100 protein (S100) and GFAP. Cells in (E) labeled with GFP (green) and counterstained with p75 antibodies (red). Note numerous round profiles of immunomagnetic beads attached to OEC in (F). Blue nuclei are stained with DAPI. Scale bar, 50 μm (A–D), 80 μm (E) and 160 μm (F).

Spinal cord injury and transplantation

After cervical laminectomy, the lateral funiculus and adjacent gray matter of the C4 spinal cord segment were transected on the left side. The rats were randomly divided into experimental groups receiving different treatments: (i) spinal cord injury (SCI) without treatment (SCI, $n=17$), (ii) SCI followed by transplantation of short-term cultured a-OEC (a-OEC ST, $n=14$), (iii) SCI followed by transplantation of long-term cultured a-OEC (a-OEC LT, $n=17$) and (iv) SCI followed by transplantation of short-term cultured b-OEC (b-OEC ST, $n=13$). Transplantation of long-term cultured b-OEC was not performed since our results *in vitro* (see Results, Olfactory ensheathing cell culture) demonstrated that it was not possible to maintain 80% purity of b-OEC for more than 2–3 weeks due to contamination with non-OEC cells. Five rats at 1 week after Fast Blue application to the lumbar spinal cord and 5 rats at 1 week after anterograde labeling of rubrospinal axons with biotinylated dextran amine ("control" in histograms) served as baseline controls.

For transplantation, the cells cultured in D/F-10S with 2 μM forskolin and recombinant human GGF-2 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, 1.5–1.6 μl of the cell suspension (150,000–160,000 cells) was slowly (10 min) pressure-injected into the lateral funiculus (depths 1.0 mm) at approximately 1 mm rostral and 1 mm caudal to the lesion site using Stoelting's Lab Standard Stereotaxic Instrument (Stoelting Co., USA). The micropipette was left in place for additional 2–3 min. Dura mater was covered with stretched parafilm and Spongostan®, muscles and skin were closed in layers, and the rats were given analgesic Finadyne (Schering-Plough, Denmark; 2.5 mg/kg, i.m.), saline (2 ml s.c.) and benzylpenicillin (Boehringer Ingelheim; 60 mg i.m.).

Retrograde labeling of rubrospinal neurons

In the experiments dealing with neuronal survival, rubrospinal neurons were labeled with retrograde tracer 1 week before SCI (Novikova et al., 2002). Following a laminectomy, the L1 spinal cord segment was exposed and the dorsal portion of the left lateral funiculus including the rubrospinal tract was transected with a fine scissors under an operating microscope. A small pellet prepared from 1 to 2 μl of a 2% aqueous solution of the non-toxic fluorescent tracer Fast Blue (Sigma-Aldrich Sweden AB) was placed into the lesion cavity and covered with a thin sheet of parafilm and a small piece of Spongostan®.

Anterograde tracing of rubrospinal axons

Anterograde labeling of rubrospinal axons was performed as described by us previously (Novikova et al., 2002). In brief, at 12 weeks after spinal cord injury, the rats were mounted in a stereotaxic frame and a 2 mm hole was drilled in the skull to allow access to the red nucleus. A glass micropipette (outer tip diameter 40–50 μm) filled with a 10% solution of biotinylated dextran amine in saline (BDA; 10 000 M_W, lysine fixable, Molecular Probes) was used for stereotaxic insertions into the magnocellular and parvicellular regions of the red nucleus (6.6 and 6.1 mm caudal to the bregma, 0.7 mm lateral to the midline, and 7.2 mm ventral to the bregma)

using Stoelting's Lab Standard Stereotaxic Instrument (Stoelting Co., USA). BDA was injected iontophoretically by passing anodal current pulses of 10 μA (7 s on/7 s off) through the microelectrode for 10 min. The microelectrode was then left in place for additional 2–3 min.

Tissue processing

At 3, 8 and 13 weeks 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). In the experiments using anterograde BDA-labeling, the fixation consisted of a mixture of 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer (pH = 7.4). Spinal cord segments C3–C5 and the brain stem were then removed and transferred into the same fixative. For immunohistochemistry, spinal cord segments C3–C5 were post-fixed for 1–2 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. For fluorescence microscopy and cell counts, 50-μm-thick serial transverse sections from the midbrain 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 demonstration of anterogradely BDA-labeled rubrospinal axons and arborizations, serial 50-μm-thick transverse sections (C3 spinal cord segments) and longitudinal sections (C4–C5 spinal cord segments) were cut on a cryomicrotome or vibratome and processed according to a modified ABC method (Novikova et al., 2002). Briefly, free-floating sections were washed in PBS, incubated for 6 h at room temperature with avidin–biotin–peroxidase complex (1:1:100; Vector Laboratories, Burlingame, CA) in PBS containing 0.3% Triton X-100 and 1% bovine serum albumin, developed in a solution containing 0.05% of 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma), 0.06% $NiCl_2$ and 0.003% H_2O_2, mounted on glass slides, counterstained with cresyl violet and coverslipped in DPX.

Immunohistochemistry

Immunostaining was performed on longitudinal 16-μm-thick spinal cord sections and cells cultured on Lab-Tek® slides. After blocking with normal serum, the following primary antibodies were used: mouse anti-low-affinity NGF p75 receptor (1:200; Chemicon International, Ltd.), mouse anti-Thy 1.1 (1:1000; MAB1406 Chemicon), rabbit anti-S-100 protein (1:1000; Dakopatts AB, Älvsjö, Sweden), rabbit anti-GFAP (1:500; Dakopatts AB, Älvsjö, Sweden), rabbit anti-serotonin, (1:1000; Sigma-Aldrich Sweden AB), rabbit anti-CGRP (1:1000; Chemicon International, Ltd.), rabbit anti-GFP (1:500; Molecular Probes, Invitrogen), and a cocktail of monoclonal antibodies reacting with 68, 160 and 200 kDa neurofilament proteins (pan-NF; 1:200; Zymed; Labora AB, Sollentuna, Sweden). All primary antibodies were applied for 2 h at room temperature. After rinsing in PBS, secondary goat anti-mouse and goat anti-rabbit antibodies Alexa Fluor® 488 and Alexa Fluor® 568 (1:300; Molecular Probes, Invitrogen) were applied for 1 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.

Counts of Fast Blue-labeled rubrospinal neurons

The nuclear profiles of the retrogradely labeled neurons were counted in all sections through the red nucleus at 250× magnification. The total number of profiles was not corrected for split nuclei, since the nuclear diameters were small in comparison with the section thickness used (Novikova et al., 2000, 2002). We have previously demonstrated that the accuracy of this technique in estimation of retrograde cell death is similar to that obtained with physical disector method (Ma et al., 2001) and counts of neurons reconstructed from serial sections (Novikov et al., 1997).

Counts of rubrospinal axons and arborizations

Rubrospinal sprouting was studied in 20 randomly selected transverse sections from the C2 spinal cord segment of normal rats and at 13 weeks after SCI and cell transplantation as described previously (Novikova et al., 2002). Rubrospinal arborizations in lamina V of the C2 spinal segment were captured in four non-overlapping areas at 2000 final magnification with a Nikon DXM1200 digital camera and imported into Matrox Inspector 3.1 software (Matrox Electronic Systems Ltd., Quebec, Canada). The final image size was 1280 × 1024 pixels and corresponded to the area of 54.18 × 43.35 μm. The number of labeled profiles and the relative tissue area occupied by labeled profiles were quantified for each image at a constant discrimination level and an average value was calculated for each section. After counting the number of labeled rubrospinal stem axons in the lateral funiculus in five randomly selected sections at 250× final magnification, values describing the mean number of arborizations per labeled stem axon were calculated.

Image processing

Preparations were photographed with a Nikon DXM1200 digital camera attached to a Leitz microscope. The captured images were resized, grouped into a single canvas and labeled using Adobe Photoshop software. The contrast and brightness were adjusted to provide optimal clarity.

Statistical analysis

One-way analysis of variance (ANOVA) followed by a *post hoc* Newman–Keuls Multiple Comparison Test was used to determine statistical differences between the experimental groups (Prism®, GraphPad Software, Inc; San Diego, CA).

Results

Olfactory ensheathing cell culture

Primary OEC cultures in the present study contained about 35–40% of cells immunopositive for low-affinity NGF receptor p75 (Fig. 1A). After purification with the method of differential adhesion, 93–95% of cells in cultures were immunostained for p75 receptors, S-100 protein and GFAP (Fig. 1B,C,D). The majority of non-OEC cells were Thy 1.1-positive cells. In our attempts to maintain pure long-term cultures of a-OEC for intraspinal transplantation, we followed well known protocols using growth medium D/F-10S alone or with DMEM-10S supplemented with forskolin and pituitary extract. With D/F-10S medium cultures became contaminated with non-OEC cells already after 1 week (Fig. 2) and the proportion of p75-positive and S-100-positive cells was reduced to 42% ($P<0.001$) and 35% ($P<0.001$), respectively. With DMEM-10S medium supplemented with forskolin and pituitary extract the proportion of p75- and S-100-positive cells remained within 80–90% during the first week after purification, but after 2 weeks in culture it reduced drastically to 15–18% ($P<0.0001$; Fig. 2). Surprisingly, D/F-10S medium supplemented with forskolin and recombinant human GGF2 maintained 90–95% pure a-OEC for at least 11 weeks (Fig. 2). Purification of primary cultures with immunomagnetic beads resulted in most cases in 80% OEC purity. Immediately after purification, numerous Dynabeads remained attached to the cell surface (Fig. 1F). The number of attached

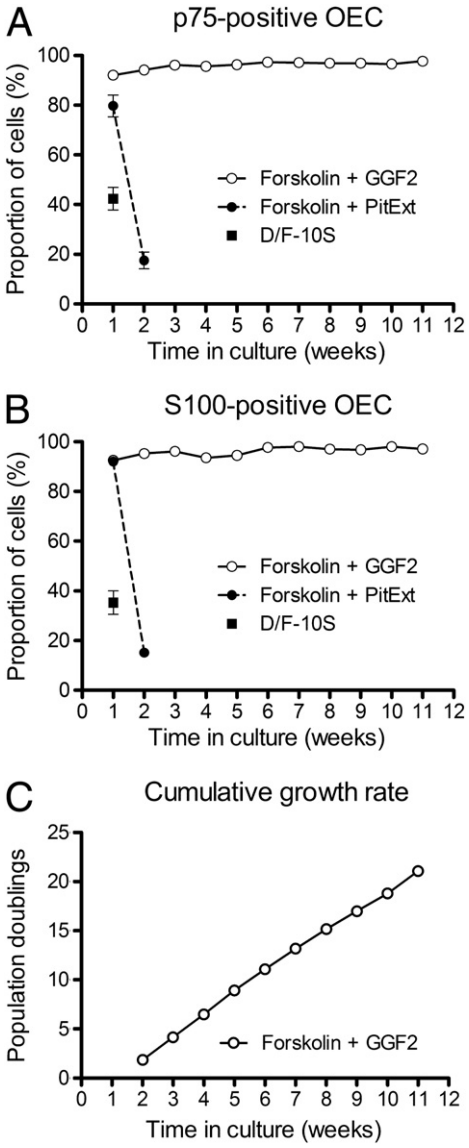

Fig. 2. Graphs showing the proportion of cells immunostained for low-affinity NGF receptors p75 (A) and S-100 protein (B) in OEC cultures purified with a method of differential adhesion and kept in D/S-10S medium (D/F-10S), DMEM-10S medium supplemented with forskolin and pituitary extract (Forskolin + PitExt), and D/F-10S medium supplemented with forskolin and recombinant human glial growth factor 2 (Forskolin + GGF2). Error bars show S.E.M. There are no statistical differences between OEC cultured with forskolin and GGF2 for 3 and 7 weeks ($P>0.05$). Graph in (C) shows cumulative growth rate and is based on Population Doublings per week (Rubio et al., 2008).

Dynabeads was reduced after 2 weeks in culture but they still can be found in spinal cord sections 13 weeks after transplantation (data not shown). In contrast to a-OEC, we found that with all growth mediums tested, it was not possible to maintain 80% pure b-OEC cultures for more than 2–3 weeks due to contamination with Thy 1.1-positive and p75-/S100-negative non-OEC cells.

Distribution of olfactory ensheathing cells

Three weeks after transplantation, numerous GFP-labeled OEC (Fig. 1E) were present in the injection sites rostral and caudal to the spinal cord hemisection and in the trauma zone (Fig. 3A). The trauma zone was connected with both injection sites by narrow tract formed by transplanted OEC. However, we did not find any OEC migration rostral and caudal from the injection sites. The number of GFP-OEC was markedly reduced at 8 weeks post-operatively (Fig. 3A and B). The changes where mainly seen in the trauma zone where only single GFP-positive cells could be detected (compare Fig. 3C and D). Since the reduction in GFP-OEC number could be due to down regulation of GFP expression (Vroemen et al., 2005), we also transplanted Fast Blue-labeled OEC (Pettersson et al., 2010) and found that distribution of these cells at 8 weeks after SCI was similar to GFP-OEC (Fig. 3B and E) with a predominant concentration of labeled cells at the injection sites (Fig. 3F). Immunostaining with p75 antiserum revealed that p75-immunoreactivity coincided with labeled cells in the transplantation sites but in the trauma zone it was beyond the areas occupied by GFP- and Fast Blue-positive OEC (Fig. 3A,B,E). In addition, animals transplanted with short-term cultured OEC (OEC ST) had larger areas of p75-immunostaining in the injection sites (Fig. 3G,H) than animals with long-term cultured OEC (OEC LT; Fig. 3I).

Survival of rubrospinal neurons

In the control animals 1 week after Fast Blue application to the lumbar spinal cord, the red nucleus contained 1704 ± 58 labeled neuronal profiles with nucleus (mean ± S.E.M.). Most of the labeled neurons were located in the ventral portion of the magnocellular and parvicellular regions of the red nucleus. At 8 weeks after cervical C4 SCI only 54% of labeled rubrospinal neurons remained in the nucleus ($P<0.001$; Fig. 4A) and most of them exhibited significant cell atrophy (data not shown). Transplantation of b-OEC ST increased survival rate to 84% ($P<0.001$; b-OEC ST vs. SCI; Fig. 4A) whereas transplantation of a-OEC ST protected 69% of the rubrospinal neurons ($P<0.05$; a-OEC ST vs. SCI; Fig. 4A). In contrast, transplantation of a-OEC LT had no effect on retrograde cell death after SCI (Fig. 4A).

Axonal sprouting and regeneration

Quantification of BDA-labeled axonal profiles in the C2 cervical dorsolateral funiculus at 13 weeks after SCI revealed that the density of rubrospinal axons was significantly reduced to 30% of normal control ($P<0.001$; Fig. 4B). In comparison with SCI, transplantation of a-OEC LT had no effects ($P>0.05$; Fig. 4B–D). In contrast, transplantation b-OEC ST induced moderate increase of the density of rubrospinal stem axons within the dorsolateral funiculus ($P<0.05$; Fig. 4B) and, at the same time, reduced the density of terminal arborizations in the gray matter ($P<0.05$; Fig. 4C). Transplantation of a-OEC ST restored the density of rubrospinal stem axons to 84% of control values ($P<0.001$; Fig. 4B) and increased by 156% the density of rubrospinal terminations in lamina V ($P<0.001$; a-OEC ST vs. control; Fig. 4C). However, after relating the number of labeled arborizations in the gray matter to the number of labeled rubrospinal axons in the dorsolateral funiculus, a significant decrease in the number of arborizations per stem axon was found after transplantation of both b-OEC ST ($P<0.001$; Fig. 4D) and a-OEC ST ($P<0.05$; Fig. 4D). It seems, therefore, that despite a significant neuroprotective effect on red nucleus neurons, transplantation of short-term cultured OEC is accompanied by diminished sprouting of rubrospinal terminals rostral to the injury site.

At 13 weeks after SCI, pan-neurofilament (panNF) immunolabeling revealed only single nerve fibers in the lesion site. BDA injections into the red nucleus demonstrated that labeled rubrospinal axons approached the rostral border of the trauma zone but then suddenly changed their direction and grew in parallel to the scar tissue towards the contralateral gray matter (data not shown). Surprisingly and despite the marked survival effect on rubrospinal neurons, transplantation of b-OEC ST stimulated regrowth neither of panNF-immunolabeled nerve fibers nor rubrospinal axons (Fig. 5A,C). The same negative results were also obtained with a-OEC LT (Fig. 5B,D). In contrast, at 8–13 weeks after transplantation of a-OEC ST, numerous panNF-positive fibers bridged the lesion site (Fig. 5E,G) and sprouted within the trauma zone (Fig. 5F,H). BDA-labeled rubrospinal axons

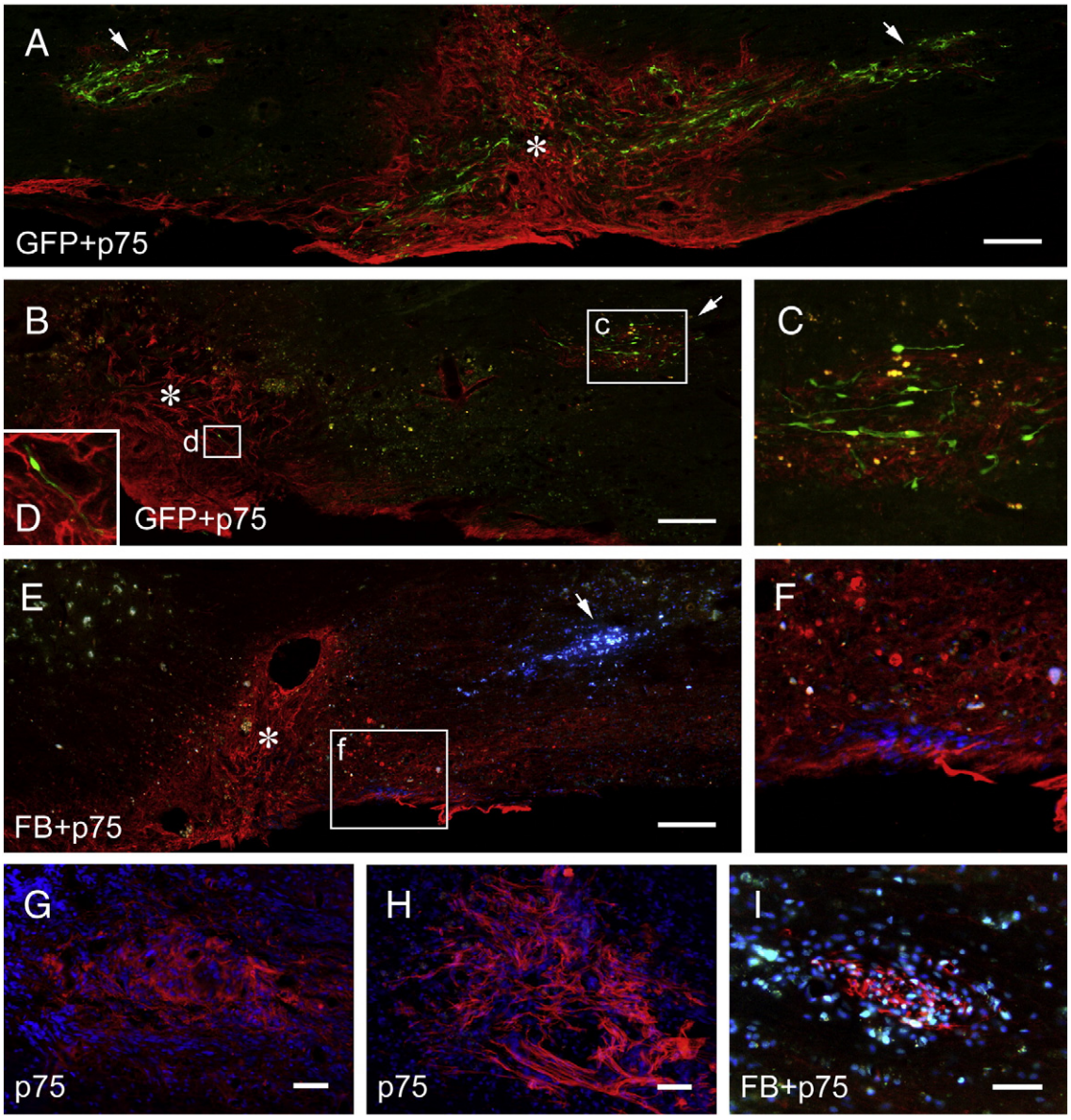

Fig. 3. Horizontal spinal cord sections immunostained for low-affinity NGF receptors p75 (A–F; p75, red) showing GFP-labeled OEC (A–D, green) and Fast Blue-labeled OEC (E–F, blue) at 3 weeks (A) and 8 weeks (B–F) after transplantation. Images in (C–D) and (F) are enlarged views of the boxed areas in (B) and (E), respectively. Injection sites (G–I) immunostained for p75 receptors at 8 weeks after transplantation of b-OEC ST (G), a-OEC ST (H) and a-OEC LT (I). Note significant reduction of p75-immunoreactivity in (I). Arrows indicate injection sites. Asterisks indicate trauma zones. FB in (I) indicates Fast Blue labeling. Blue nuclei in (G) and (H) labeled with DAPI. Scale bar, 200 μm in (A, B, E), 50 μm in (G–H) and 25 μm in (I).

(Fig. 6A–C) and serotonin-positive raphaespinal axons (Fig. 6E–I) crossed the injury site along the lateral border but grew for only about 1500 μm in the white and gray matter of the distal spinal cord. Sensory axons immunostained for calcitonin gene-related peptide (CGRP) were found mainly within the trauma zone (Fig. 6D). Thus, the method of culture preparation could influence growth-promoting effect of OEC after intraspinal transplantation.

Discussion

The present study shows that the age of cells in culture and the method of cell purification could influence the neuroprotective and growth-promoting effects of OEC transplants. Although purified OEC can be maintained for several months in culture without losing their p75 and S100 immunoreactivity, only short-term cultured cells attenuated massive retrograde cell death in the red nucleus and decreased sprouting of rubrospinal terminals after cervical spinal cord injury. Long-term cultured OEC had no effect on neuronal survival and regeneration, and p75 immunoreactivity was markedly reduced in the injection sites. Unexpectedly, short-term cultured OEC purified with a method of differential cell adhesion and with immunomagnetic beads had different effects on axonal growth. OEC separated with Dynabeads (b-OEC) failed to promote regrowth across the lesion site but increased the number of rubrospinal fibers in the white matter rostral to the hemisection. In contrast, OEC purified with a method of differential adhesion (a-OEC), stimulated growth from the descending rubrospinal and raphaespinal tracts and from the sensory CGRP-positive fibers. In addition, after intraspinal transplantation, labeled OEC showed very limited migration and were found mainly at the injection sites and in the trauma zone. However, in accordance with previous report (Lu et al., 2006), the trauma zone was connected with both injection sites by narrow tract formed by transplanted OEC.

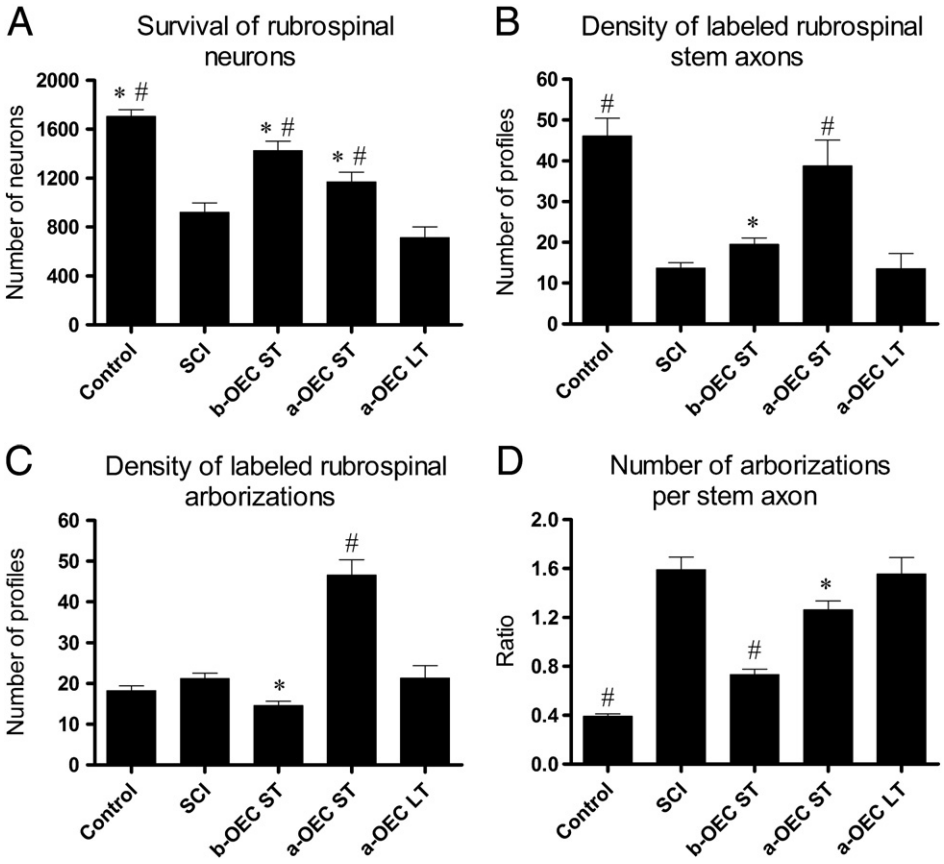

Fig. 4. Histogram showing survival of Fast Blue-labeled rubrospinal neurons at 8 weeks after cervical spinal cord injury (A) and the density of BDA-labeled rubrospinal stem axons in the dorsolateral funiculus (B), the density of rubrospinal terminal arborizations in the gray matter (C) and the ratio between the density of rubrospinal arborizations and the axons in the dorsolateral funiculus (D) at 13 weeks after cervical spinal cord injury (SCI) and following transplantation of OEC. Control indicates untreated rats at 1 week after Fast Blue-labeling (A) or normal uninjured rats at 1 week after BDA-labeling (B–D). Error bars show S.E.M. In (A): $P<0.05$ is indicated by * (control vs. b-OEC ST; a-OEC ST vs. SCI and b-OEC ST) and $P<0.001$ is indicated by # (control vs. SCI, a-OEC ST and a-OEC LT; b-OEC ST vs. SCI and a-OEC LT; a-OEC ST vs. a-OEC LT). In (B): $P<0.05$ is indicated by * (b-OEC ST vs. SCI) and $P<0.001$ is indicated by # (control and a-OEC ST vs. SCI, b-OEC ST and a-OEC LT). In (C): $P<0.05$ is indicated by * (b-OEC ST vs. control, SCI and a-OEC LT) and $P<0.001$ is indicated by # (a-OEC ST vs. control, SCI, b-OEC-ST and a-OEC LT). In (D): $P<0.05$ is indicated by * (a-OEC ST vs. SCI and a-OEC LT) and $P<0.001$ is indicated by # (control vs. experiment; b-OEC ST vs. SCI, a-OEC ST and a-OEC LT).

Factors determining the outcome of OEC transplantation

Transplantation of OEC has been shown to support axonal regeneration, myelination and neuronal survival in different experimental models of spinal cord injury (Fouad et al., 2009a; Kocsis et al., 2009; Lindsay et al., 2010; Munoz-Quiles et al., 2009; Radtke and Wewetzer, 2009). However, reports from different laboratories have demonstrated considerable variations in results ranging from significant axonal regeneration across the lesion site and functional recovery (Gorrie et al., 2010; Keyvan-Fouladi et al., 2003; Lopez-Vales et al., 2007; Ramon-Cueto et al., 2000; Takeoka et al., 2010) to very little or no effect after spinal cord repair (Aoki et al., 2010; Barakat et al., 2005; Bretzner et al., 2010; Collazos-Castro et al., 2005; Lu et al., 2006; Steward et al., 2006). The conflicting data regarding the ability of OEC to support axonal regeneration have been reviewed (Barnett and Riddell, 2007; Nieto-Sampedro, 2003; Raisman and Li, 2007; Richter and Roskams, 2008) and the factors which could potentially determine the outcome of OEC transplantation have been discussed. Although there is no consensus in the ongoing discussion about significance of these factors of importance for OEC transplantation into spinal cord injury (Barnett and Riddell, 2007; Deumens et al., 2006; Lu et al., 2006), the source of tissue used to obtain the OEC (neonatal and adult olfactory bulb and olfactory mucosa), the senescence and the purity of transplanted cells and the methodology used in the culture preparation seem to be the most important variables (Pastrana et al., 2006, 2007; Richter et al., 2005; Santos-Benito and Ramon-Cueto, 2003). However, direct comparisons made in the same experimental model between differently composed cell grafts revealed that the donor age, the purity of the cells and the presence of an endogenously produced matrix are not related to the discrepancy in results found in OEC transplant studies (Andrews and Stelzner, 2004; Deumens et al., 2006; Toft et al., 2007). Also the initial findings that OEC from the lamina propria are more beneficial for spinal cord repair than the cells from the olfactory bulb (Richter et al., 2005) have not been confirmed in a subsequent study (Lu et al., 2006). However, in studies dealing with demyelination in the rat and porcine spinal cord, it has been found that the purity of cell cultures and the senescence of OEC could significantly influence remyelination (Lakatos et al., 2003; Radtke et al., 2010). The results of the present study, however, confirm previous observations that prolonged cell cultures reduce stimulatory effect of OEC and, in addition, demonstrate that the technique of cell purification could modulate growth-promoting effect of OEC after transplantation into spinal cord.

Long-term cultured OEC failed to support regeneration and neuronal survival

The mechanisms underlying the lack of neurotrophic and growth-promoting effects of long-term cultured OEC are not clear. They could be attributed to the low survival rate of transplanted OEC (Pearse et al., 2007) and to the loss of certain classes of surface receptors which are important for interaction with surrounding glial cells, macrophages and regenerating axons. In this respect, the results show that although OEC retain their p75 immunoreactivity for several months *in*

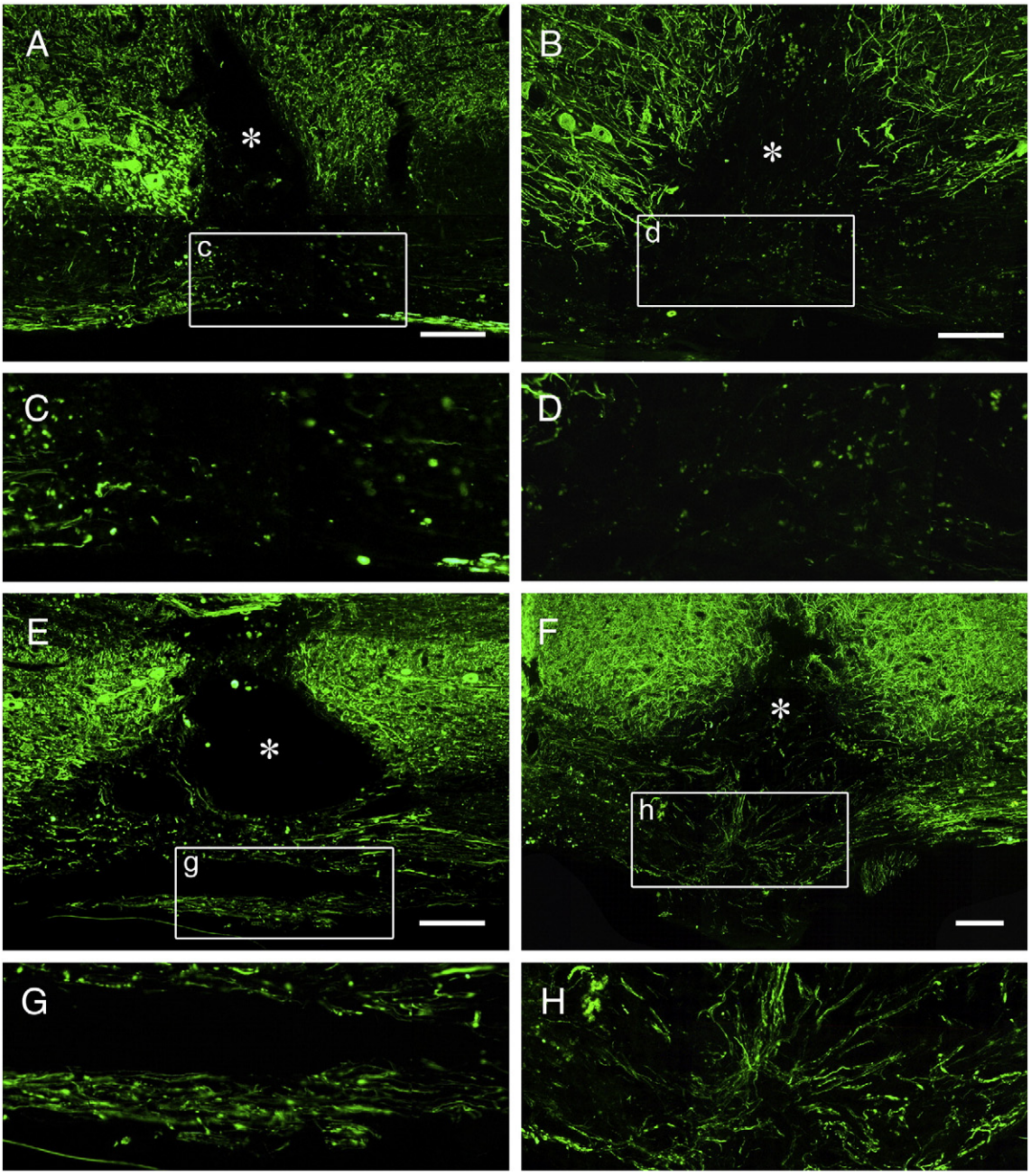

Fig. 5. Horizontal spinal cord sections immunostained for pan-neurofilament and showing regeneration in the trauma zone (asterisks) at 13 weeks after cervical SCI and following transplantation of b-OEC ST (A), a-OEC LT (B) and a-OEC ST (E and F). Boxed areas in (A), (B), (E) and (F) are enlarged in (C), (D), (G) and (H), respectively. Note numerous regenerating fibers in (E–F) after a-OEC ST transplantation. Scale bar, 200 μm.

vitro, transplantation sited with long-term cultured OEC have lower levels of p75 expression than with short-term cultured cells. Also, it has been recently demonstrated that in contrast to primate OEC, adult rat and porcine OEC could become senescent already after 3–5 weeks in culture and lose their phenotypic properties (Radtke et al., 2010; Rubio et al., 2008). Moreover, it has been found that if culture period is extended for several weeks beyond senescence, OEC can resume division and undergo spontaneous immortalization (Rubio et al., 2008; Sonigra et al., 1996).

Another possible explanation could be related to the reports that OEC express and naturally secrete several classes of neurotrophic molecules including NGF, NT-4/5, BDNF and GDNF (Lipson et al., 2003; Pastrana et al., 2007; Sasaki et al., 2006) which are known to promote neuronal survival and axonal regeneration after spinal cord injury (Deumens et al., 2005) However, after prolonged time in culture OEC might stop to produce sufficient amounts of neurotrophic factors and lose their neuroprotective effect after intraspinal transplantation. This hypothesis is supported by findings that extracellular BDNF is produced in significant amounts by the early passage OEC whereas the neurotrophin is absent in the extracellular medium collected from the late passage cells (Pastrana et al., 2007). Moreover, long-term cultured OEC have very low levels of matrix metalloproteinase-2 which seems to be an important modulator of OEC-mediated axonal regeneration (Pastrana et al., 2006). The latter results are in line with observations that long-term cultured OEC are less efficient in inducing neurite outgrowth *in vitro* (Au et al., 2007; Pastrana et al., 2006, 2007).

Recently a new growth-promoting factor, the SPARC (secreted protein acidic rich in cystein) (osteonectin), has been identified in OEC conditioned medium (Au et al., 2007). It has been demonstrated that SPARC is an OEC-derived matricellular protein which could

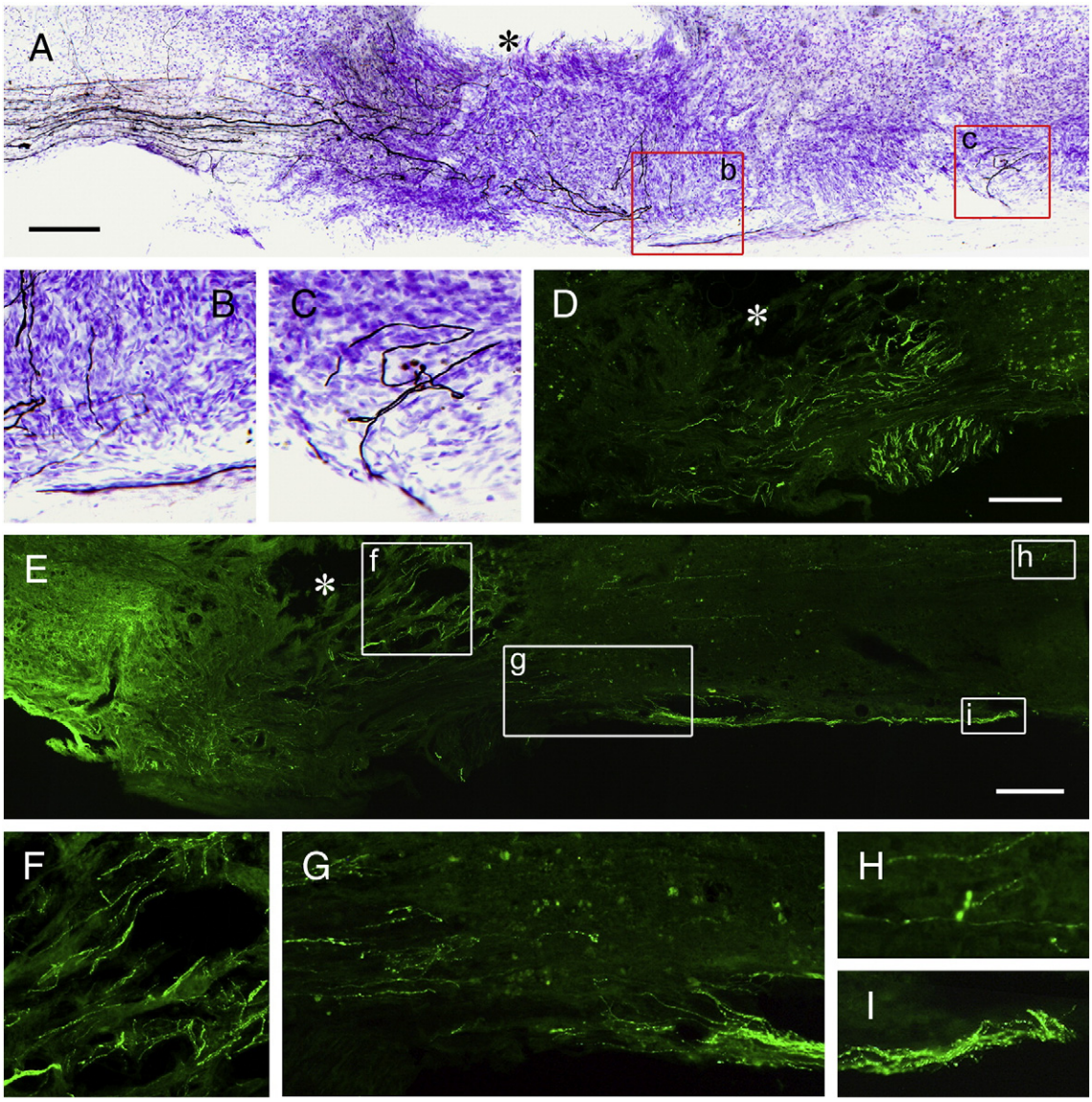

Fig. 6. Horizontal spinal cord sections showing BDA-labeled rubrospinal axons (A–C), CGRP-immunolabeled axons (D) and raphaespinal axons immunostained for serotonin (E–I) at 13 weeks after cervical SCI and following transplantation of a-OEC ST. Boxed areas in (A) and (E) are enlarged in (B–C) and (F–I), respectively. Note labeled rubrospinal and raphaespinal axons crossing the lesion site. Asterisks indicate trauma zones. Images in (A–C) are counterstained with cresyl violet. Scale bar, 200 μm.

indirectly enhance the ability of Schwann cells to stimulate axonal regeneration via laminin-1 and TGFβ signaling. However, it also has been shown that early P2 passage OEC have 30–50% higher levels of SPARC expression than late P6 passage cells (Au et al., 2007).

With respect to neuroprotective effect of the short-term cultured OEC, our findings are in line with previous reports that OEC transplantation into the injured spinal cord could protect descending corticospinal neurons from retrograde degeneration (Sasaki et al., 2006). Biochemical analysis performed in the latter study demonstrates significantly elevate levels of BDNF in the spinal cord injury zone at 1 week after OEC transplantation but no difference is detected in NGF and NT-3 levels. Moreover, olfactory neural progenitors from the olfactory mucosa, with the capacity to generate both neurons and OEC, have been found to produce BDNF and to rescue axotomized red nucleus neurons after SCI (Xiao et al., 2005).

It has previously been shown that neurotrophic factors could not only prevent retrograde degeneration but also support regeneration of rubrospinal neurons in different models of spinal cord injury (Kobayashi et al., 1997; Novikova et al., 2000; Storer et al., 2003; Tobias et al., 2003). However, varying outcomes of rubrospinal regeneration have been reported after OEC transplantation. Following complete spinal cord transection (Cao et al., 2004) or moderate contusion injury (Plant et al., 2003), significant increase in the number of retrogradely labeled neurons have been found in the red nucleus. The present study also demonstrated growth-promoting effect of short-term cultured a-OEC on rubrospinal neurons. In contrast, after less-severe injury to the dorsolateral funiculus, anterograde tracing reveals no regeneration of rubrospinal axons across the lesion site into the distal cord (Bretzner et al., 2010; Ruitenberg et al., 2003).

Method of cell purification could affect growth-promoting effect of OEC transplant

The limited effects of b-OEC on regeneration were unexpected because it has been previously shown that the same method of purification with immunomagnetic Dynabeads results in OEC grafts capable of inducing significant regenerative response after transplantation into completely transected rat spinal cord (Lopez-Vales et al., 2006, 2007). However, immunomagnetic beads separate only OEC

with sufficiently strong expression of p75 receptors leaving all other OEC subtypes in the supernatant. It has also been demonstrated that the major OEC population in primary cells suspensions does not initially express p75 receptors but upregulates them after several days in culture (Wewetzer et al., 2005). Thus, it is possible that in contrast to previous reports (Lopez-Vales et al., 2006, 2007), we separated different subtypes of OEC which had marked neuroprotective effect but failed to promote axonal regrowth. Another possibility is that remaining on the cell surface Dynabeads could interfere with the processes of interaction between transplanted OEC and surrounding host cells including reactive astrocytes.

However, it is not clear why short-term cultured a-OEC are less effective in rescuing rubrospinal neurons than b-OEC but are much more effective in stimulating axonal regrowth across the trauma zone. One possibility is that the signaling pathways promoting neuronal survival and axonal regeneration respond differently to neurotrophic factors and that efficient neuroprotection of axotomized neurons is not a sufficient prerequisite for axonal regeneration (Lu et al., 2001; Novikova et al., 2002). Another interesting observation in the present study is that the neuroprotective effect of the short-term cultured OEC is associated with reduced rubrospinal sprouting. The same effects on lesioned rubrospinal neurons have been described previously after prolonged intrathecal infusion of BDNF and NT-3 (Novikova et al., 2002).

In summary, the present study demonstrates that the age of cells in culture and the method of cell purification have influence on the growth-promoting and neuroprotective effects of OEC after transplantation into the injured spinal cord. The finding that methods of culture preparation might affect the outcome of OEC transplantation could be an important issue since human trials with OEC have been conducted in several countries (Dobkin et al., 2006; Kay-Sim et al., 2008).

Acknowledgments

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