Experimental Neurology 216 (2009) 439–448

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Umbilical cord blood mesenchymal stromal cells are neuroprotective and promote regeneration in a rat optic tract model

Isabel Zwart [a,c], Andrew J. Hill [a,c], Faisal Al-Allaf [b], Mili Shah [a], John Girdlestone [c,d], Athirah B.R. Sanusi [a], Huseyin Mehmet [b], Roberto Navarrete [a], Cristina Navarrete [c,d], Ling-Sun Jen [a,*]

[a] Department of Cellular and Molecular Neuroscience, Imperial College London, UK
[b] Institute of Reproductive and Developmental Biology, Faculty of Medicine, Imperial College London, UK
[c] Histocompatibility and Immunogenetics Research Group, National Health Service Blood and Transplant (NHSBT), Colindale Centre London, UK
[d] Department of Immunology and Molecular Pathology, University College London, UK

ARTICLE INFO

Article history:
Received 26 August 2008
Revised 28 December 2008
Accepted 30 December 2008
Available online 14 January 2009

Keywords:
Mesenchymal stromal cells
Umbilical cord blood
Neuroprotection
Axotomy

ABSTRACT

Exploitation of the ability of stem cells to protect damaged neuronal tissue may be a more viable strategy than cell replacement for repair of the central nervous system (CNS). In this study we assessed the capacity of human umbilical cord blood (hUCB)-derived mesenchymal stromal cells (MSCs) to protect and promote regeneration of axotomised neurons within the rat optic system. The optic tract of neonatal rats was transected at the level of the lateral geniculate nucleus, and MSCs were introduced into the lesion site. MSCs survived well up to 2 weeks after grafting, and did not migrate significantly or differentiate. In the presence of MSC grafts, host axonal processes were found to be present in the lesion site, and there was stimulation of an endogenous neural precursor population. Four weeks after grafting, retrograde tracer experiments demonstrated that grafted MSCs, as well as cells of a human fibroblast line, exerted a neuroprotective effect, rescuing a significant percentage of axotomised retinal ganglion cells (RGCs). Further experiments with retrograde and anterograde tracers strongly indicated that MSCs could also promote re-growth of axotomised RGCs to their target, the superior colliculus (SC). Further analysis showed that hUCB-derived MSCs secreted several immunomodulatory and neurotrophic factors *in vitro*, including TGFβ1, CNTF, NT-3 and BDNF, which are likely to play a role in neuroprotection. Our data indicate that hUCB-derived MSCs may be an easily accessible, widely available source of cells that can contribute towards neural repair through rescue and regeneration of injured neurons.

Introduction

The damaged central nervous system has limited capacity for self renewal (Fawcett and Asher, 1999). Therapeutic strategies in which grafted stem cells functionally replace lost neuronal tissue have met with some success (Kim et al., 2002; MacLaren et al., 2006; Ogawa et al., 2002; Roy et al., 2004; Studer et al., 1998), but there are ethical and practical problems associated with the derivation and use of embryonic stem (ES) cells and neural stem cells (NSCs), which represent the most promising sources for this treatment. An alternative strategy to cell replacement is the protection of injured cells and promotion of endogenous cell regeneration. Several studies have suggested neural progenitor cells may be able to encourage damaged cell survival and regeneration through modulation of environmental factors, with their main contribution being to promote functional recovery rather than cell replacement (Llado et al., 2004; Lu et al., 2003; Ourednik et al., 2002). Recent reports indicate that these neuroprotective properties are not restricted to neural precursor cells, and that this phenomenon can be observed following grafts of other cell types, including mesenchymal stromal cells (MSCs) (Honma et al., 2006; Lu et al., 2005; Neuhuber et al., 2005). MSCs derived from bone marrow have been reported to reduce infarction size in ischaemia models (Chen et al., 2003; Honma et al., 2006; Li et al., 2001), aid host axonal growth, and in some cases improve functional recovery in spinal cord injury (SCI) models of CNS damage (Lu et al., 2005; Neuhuber et al., 2005). However the extent of regeneration in these studies remains unclear, with no reports of successful re-growth of injured neurons to their appropriate targets.

This study aimed to investigate further the potential neuroprotective and regenerative effects of MSCs. These cells have several advantages over ES or neural progenitor cells, including a lack of ethical controversy, immunosuppressive effects demonstrated in clinical settings (LeBlanc et al., 2004; Ringden et al., 2006), and the recent progress in developing readily available and non-invasive alternatives to adult bone marrow, in particular human umbilical cord

* Corresponding author. Department of Cellular and Molecular Neurosciences, Room 12L10 Lab block, Charing Cross Campus, St. Dunstan's Road London W6 8RP, UK. Fax: +44 2088467025.
E-mail address: l.jen@imperial.ac.uk (L.-S. Jen).

0014-4886/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.expneurol.2008.12.028

blood (hUCB) (Erices et al., 2000; Goan et al., 2000) or perivascular tissue from the cord itself (Sarugaser et al., 2005). We have assessed the capacity of hUCB-derived MSCs for neuroprotection and regeneration using a rat optic system model of neurodegeneration, wherein axotomy at the level of the lateral geniculate nucleus (LGN) in neonatal rats results in rapid loss of retinal ganglion cell (RGCs), with the effects of treatments on RGC survival and regeneration assessed by retrograde and anterograde tracer experiments (Chan et al., 1989; Chan and Jen, 1988; Jen et al., 1989; Jen and Lund, 1981). We have found that introduction of MSCs into optic tract lesions results in the rescue of a significant number of RGCs from cell death, and report for the first time that MSCs also induce RGCs to regenerate and project from the lesion to their appropriate target, the superior colliculus (SC). This indicates that hUCB-derived MSCs may provide a suitable source of cells for regeneration in CNS injury.

Materials and methods

MSC isolation, culture, and transduction

MSCs were generated from full-term hUCB as described previously (Manca et al., 2008). Briefly, hUCB was collected from donors (>37 weeks gestation) with mothers' consent by the NHSBT Cord Collection team, Oxford. Lineage-negative cells were separated from the hUCB using the RosetteSep MSC antibody cocktail (Stem Cell Technologies; Vancouver, Canada), according to the manufacturer's instructions. Isolated cells were resuspended in MesenCult complete medium (MCm; MesenCult Basal medium+10% MSC Stimulatory Supplements [Stem Cell Technologies]+1% antibiotic-antimycotic [Gibco; Paisley, UK]) and cultured at 37 °C, 5% CO_2. After 1 week the medium was removed from adherent cells and replaced with fresh MCm. Half the medium was replaced weekly until dense colonies or confluency was observed after 3–4 weeks; cells were then passaged (0.25% trypsin-EDTA; Gibco) and plated at $8\times10^3/cm^2$ in MCm. Half the medium was replaced every 3–4 days and cells were passaged when confluent. Cells of the human fibroblast line HS27 (ATCC, CRL-1634) were grown in DMEM/10% FBS (Gibco).

MSCs and fibroblasts were transduced with a lentiviral vector expressing enhanced green fluorescent protein (eGFP) as described previously (Demaison et al., 2002). Briefly, a total of 10 μg plasmid DNA was used for the transfection of a single dish: 2 μg of envelope plasmid (pMDG), 4 μg of packaging plasmid (pCMVR8.91), and 4 μg of the self-inactivating transfer vector plasmid encoding eGFP (pRRL-SIN-cPPT-SFFV-eGFP-WPRE). Viral plasmids were a kind gift from Prof. AJ. Thrasher (University College London). The plasmid mixture was complexed with 3 μl FuGENE 6 (Roche Diagnostics; Burgess Hill, UK)/1 μg DNA in 1 mL Optimem (Invitrogen; Paisley, UK) at room temperature for 45 min, and then added to 293T cells. After overnight incubation in DMEM/10% FCS at 37 °C in a 5% CO2 incubator, the medium was replaced by fresh culture medium. 72 h after transfection the medium was harvested, cleared by low-speed centrifugation (1200 rpm, 5 min), and filtered through a 0.22 μm filter. Virus particles carrying the eGFP gene were used freshly without further concentration. The MSCs and fibroblasts were transduced with a multiplicity of infection of 10, and after 12 h the medium exchanged for fresh growth medium. Transduction efficiency was determined using flow cytometry on a FACScan (BD; NJ, US) and found to be 96% for the MSCs and 88% for fibroblasts.

Animals and surgical procedures

Sprague–Dawley neonatal rats were obtained from Charles River laboratories (London, UK), with all maintenance and procedures carried out according to UK Home Office guidelines. Rats at postnatal day 1 (P1) were anaesthetised with intraperitoneal (IP) injections of Hypnorm (VetaPharma Ltd; Leeds, UK) (0.1 mg of fentanyl citrate and 0.27 mg fluanisone/100 g body weight) and Hypnovel (Roche; Welwyn Garden City, UK) (0.135 mg midazolam/100 g body weight) on ice. The optic tract at the level of the lateral geniculate nucleus (LGN) was transected and a lesion cavity created through aspiration (Chan et al., 1989; Chan and Jen, 1988; Jen et al., 1989; Jen and Lund, 1981). Cells were then implanted at 1×10^5 cells/2 μl DMEM/animal after injection onto a piece of gelfoam approximately 0.12 mm^3 in size (Nanking Pharmaceutical, Nanking, China) into the lesion site. Negative control animals received a lesion alone or a lesion and an implantation of gelfoam with DMEM. Positive control animals did not undergo surgery but were time-matched to experimental animals. After recovery from anaesthesia, the rats were given the pain killer Vetergesic (Reckitt-Benckiser Healthcare Limited; Hull, UK) at 0.03 mg/100 g body weight by subcutaneous injection before returning to their mothers. All rats were subsequently given IP injections of cyclosporine A (Novartis; Braintree, UK) (CsA; 1 mg/100 g body weight) and dexamethasone (Sigma; Gillingham, UK) (DXM; 10 μg/100 g body weight), 3 times weekly for the first two weeks after grafting, then once a week for subsequent weeks, irrespective of experimental group. The rats were sacrificed at 5 days or 2, 4 or 8 weeks post-grafting through overdose with IP injections of pentobarbital (0.2 ml Euthatal; Merial Animal Health Limited, Harlow, UK), then perfused with 4% paraformaldehyde (PFA) (for immunohistochemistry and Fast Blue labelling) or 1% PFA and 1.25% glutaraldehyde in PBS (for HRP labelling) and brains or retinas were collected for analysis. Brains were incubated in 30% sucrose (Sigma) in PBS overnight, and then embedded in OCT (VWR). Coronal sections (15 μm) were cut using a cryostat and kept at −20 °C until use for immunohistochemistry.

Immunofluorescence

Coronal sections were washed 3 times for 5 min each with PBST (PBS with 0.1% Triton X-100 [Sigma]), before blocking for 60 min with 10% goat serum (Sigma) in PBST. Primary antibodies were applied and incubated overnight. Primary antibodies: GFAP (1:1000; Dako, Ely, UK), rat nestin (1:1000; BD Pharmingen, Oxford, UK), Ox-6 (1:500; Abcam, Cambridge, UK), Ox-42 (1:1000; Caltag-Medsystems Ltd, Buckingham, UK), CD4 (1:1000; Serotec, Oxford, UK), CD8 (1:1000; Serotec), human-specific nestin (1:750; Chemicon-Millipore, Watford, UK), NF-200 (1:200; Novocastra VisionBiosystems, Newcastle, UK), MAP2 (1:500), β-tubulin-III (Tuj1, 1:400), vimentin (1:500) (all Sigma). Slides were washed and biotin-conjugated secondary antibody was applied and incubated for 60 min. Biotinylated secondary antibodies: goat anti-mouse IgG (1:400) and goat anti-rabbit IgG (1:600) (Sigma). Slides were washed and streptavidin-conjugated fluorochrome, diluted 1:100 (Texas Red, Vector Laboratories), was applied and incubated for 60 min. Slides were washed and mounted in aqueous mounting medium with DAPI (Vectashield+DAPI 1.5 μg/ml; Vector Laboratories, Peterborough, UK). All dilutions were made in PBST and all incubations were performed at room temperature. Negative controls omitted the primary antibody; cells or tissues known to express the relevant antigen were used as positive controls. The number of GFP-labelled cells was also counted, with the number of cells counted in 4 visual fields at ×10 magnification averaged for each animal, and then averaged across animals for each graft condition.

Tracer experiments

The retrograde label Fast Blue (EMS-CHEMIE (Deutschland) GmbH, Gross-Umstadt, Germany) was used to trace RGC survival. After 4 or 8 weeks, animals were anaesthetised with Ketamine chloride (0.75 ml/100 g) and zylaxine (11.66 ml/100 g) and received implants of surgical gelfoam containing Fast Blue (soaked in 10 mg Fast Blue/30 μl distilled water and then air-dried) (Jen et al., 1986; Liu and Jen,

1986) into either the lesion site or the superior colliculus. Animals were sacrificed 6 days later and the retina contralateral to the lesion site was removed and mounted flat. The numbers of Fast Blue-positive RGCs were counted in 16 fields of the retinal wholemounts, using ImagePro software (version 5, Media Cybernetics). The mean cell count per field ± S.E.M. was calculated for each group (including negative results), and statistical analyses were performed using SPSS software using the Kruksall–Wallis non-parametric test and one-way analysis of variance (ANOVA) Tukey's Multiple Comparisons test, whereby $p < 0.001$ was significant.

For horse radish peroxidase (HRP) labelling, animals were sacrificed at 4 or 8 weeks post-graft, 3 days after 3 µl of 30% HRP solution (Sigma type VI) was injected into the vitreous of the contralateral eye whilst under anaesthesia. Animals were perfused as above, and brains were removed, incubated overnight in 30% sucrose in PBS at 4 °C and sectioned coronally at 45 µm. Free-floating sections were washed in distilled water for 5 min, and then exposed to HRP reaction solution for 10 min on an orbital shaker. HRP reaction solution was a mix of two solutions. The first comprised 5% 0.2 M Na acetate buffer (pH 3.3) and 100 mg sodium nitroferricyanide (Sigma), made to 100 ml volume with distilled water. The second comprised 5 mg tetramethylbenzidine (Sigma) and 2.5 ml absolute alcohol. Then, 1 ml 0.3% H_2O_2 (Sigma) was added and the sections monitored for reaction product. The reaction was stopped with a 1:5 dilution of Na acetate buffer in water. Sections were then placed on slides and dehydrated in increasing concentrations of alcohol and xylene, and then mounted in DPX mounting medium.

Enzyme-linked immunosorbent assay (ELISA)

Cell-conditioned media were collected following 3 days incubation with cells plated at 1×10^5 cells/ml medium. Medium was concentrated from 2 ml to 500 µl using Centricon centrifugal filter devices (YM-10; Millipore (UK) Ltd, Watford, UK) and then frozen in aliquots at −20 °C until further use. Once thawed, supernatants were microfuged to remove particulates prior to assay.

Thawed supernatants were subjected to an acid pre-treatment step prior to assay to measure the total amount of factor present. The sample was diluted 1:2 in PBS, and then incubated with 1 µl 1N HCl/50 µl diluted sample for 15 min at room temperature. The reaction was stopped by neutralisation with 1 µl 1N NaOH/50 µl diluted sample. Treated samples were then diluted 1:2 in sample diluent buffer (Promega; Southampton, UK; or R&D Systems, Abingdon, UK) prior to addition to the plate. ELISAs were run as per the manufacturers' instructions using kits for the following factors: BDNF, GDNF, NT-3, TGF-β1 (all Promega), CNTF and IL-10 (R&D Systems). Subsequent serial 1:2 dilutions were performed for each sample and samples were run in duplicate. Plates were read at 450 nm in an iEMS Reader MF (Labsystems, Helsinki, Finland), and data analysed using Ascent software (Labsystems).

Results

MSC survival, differentiation and migration

The survival, migration and differentiation of GFP-labelled MSCs in the brains of immunosuppressed neonatal rats were assessed by immunohistochemistry at 5 days, and 2 and 4 weeks after grafting. At 5 days, numerous GFP+MSCs were seen within the graft site ($n = 10$; Fig. 1A). GFP+MSCs did not adopt a neuronal morphology, and maintained their fibroblast-like shape for the first 5 days, though some cells developed multiple small processes. After 2 weeks, GFP+ MSCs were less abundant, with 50% of the number present at 5 days ($n = 10$; Fig. 1B), and the cell bodies became less elongated with few

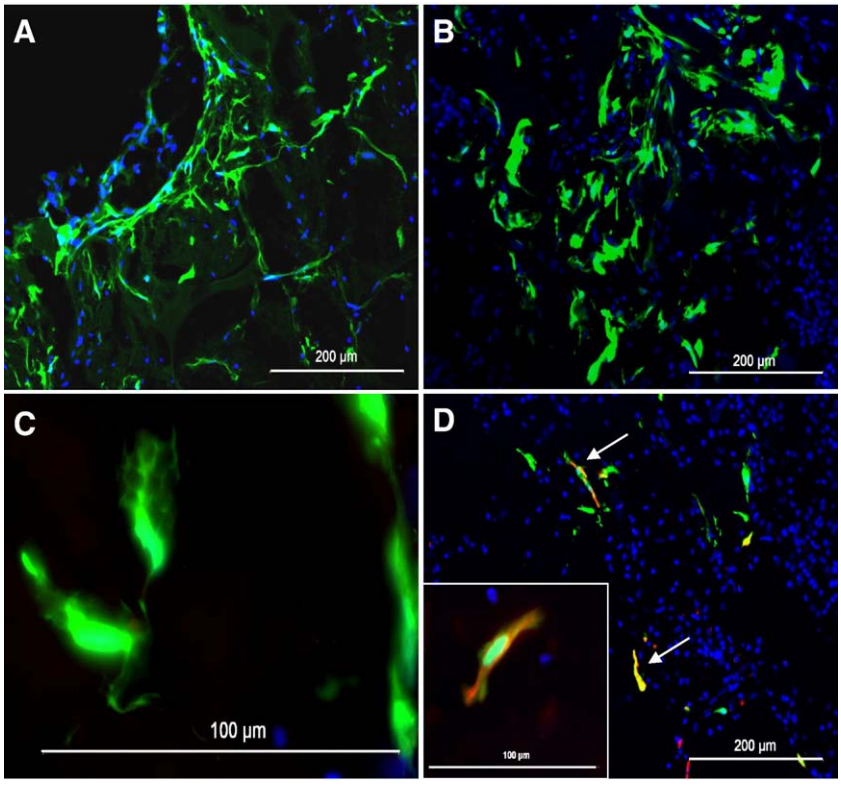


Fig. 1. MSC survival within the lesion. (A) MSCs (green) survive within the graft site for 5 and (B) 14 days ($N = 10$). (C) Few cells exhibited fine processes. (D) A minority of MSCs expressed nestin (red). $N = 3$; nuclei are indicated by DAPI (blue). Scale bars: 200 µm (A, B, D) or 100 µm (C, inset D).

processes seen (Fig. 1C). A small proportion of MSCs maintained expression of the neural precursor marker nestin for up to 2 weeks (Fig. 1D), which was expressed in around half the cells *in vitro* prior to grafting (Zwart et al., 2008). In contrast, vimentin expression which was strong prior to transplantation was not evident at 5 days (data not shown). No GFP+ cells were detected in the brain after 4 weeks (data not shown), suggesting an absence of MSCs, although the possibility of GFP expression being switched off in extant MSCs was not excluded. GFP+ cells were rarely observed outside the lesion site, suggesting a lack of appreciable migration of the grafted cells from the lesion cavity.

Host response

When the lesion sites were examined for markers of neural differentiation, it was found that they were surrounded by a glial scar of GFAP+ reactive astrocytes (Figs. 2A, B), also observed in negative control animals (data not shown). GFAP was not found in GFP+ MSCs. At both 5 days and 2 weeks post-grafting, the lesion site itself contained both GFP+ MSCs and separate β-tubulin III+ neuronal processes (Fig. 2C), suggesting the MSCs supported neuronal growth and/or survival in the lesion area for at least 2 weeks. There was no evidence of β-tubulin III expression in GFP+ MSCs, and expression was not seen in the lesion site of control animals that received only the gelfoam support (Fig. 2D). The proliferation marker Ki-67 was also found within the graft site at 5 days (Fig. 2E) but not 14 days (Fig. 2F). However, Ki-67 did not co-localise with GFP, suggesting the proliferating cells were endogenous (Fig. 2E, insert). Only limited Ki67 expression was seen in control lesions (Fig. 2G) which may represent the proliferation of infiltrating immune cells, as indicated by the presence of CD11b+ cells (Fig. 2H). These cells, likely to be microglia or macrophages were evident at 5 days but not at 2 weeks, indicating a transient inflammatory response to damage. In MSC-grafted animals there was no evidence of immune infiltration into or around the lesion by either T lymphocytes, as indicated by a lack of CD4 or CD8 expression, or macrophages or microglia, as shown by the absence of HLA Class II (Ox6) or CD11b expression at either 5 days or two weeks (not shown). Another distinction between the grafted and control animals was the expression of rat nestin in lesion sites provided with MSCs. Nestin was seen at 5 days in MSC-grafted animals (Fig. 2I), but was absent by 14 days (Fig. 2J), and was not seen at either time point in gelfoam-only control animals.

MSCs exert a neuroprotective effect after RGC axotomy

The ability of grafted MSCs to improve the survival rates of axotomised RGCs 4 and 8 weeks after lesion was assessed by introducing the retrograde label Fast Blue into the lesion site. The number of Fast Blue+ RGC cell bodies present in the retina contralateral to the lesion was then counted 6 days later. Of 10 positive control animals that received no lesion, 9 possessed Fast Blue+ RGCs 4 weeks after P1 (Fig. 3A), with a mean cell count of 88.3±4.1 (inclusive of negative case) (Fig. 3E). This value was considered to represent 100% survival. Of 11 negative control animals that received a lesion but only gelfoam+DMEM with no cells, 10 displayed no Fast Blue+ RGCs 4 weeks after lesion, with the remaining case exhibiting sparse labelling. Overall this group had a mean cell count of 0.2±0.1 (Fig. 3E).

In contrast, 9 of 13 animals that received an MSC cell graft to the lesion displayed Fast Blue+ RGCs 4 weeks later (Figs. 3B and C), with an overall survival rate for the whole group of 24.8% compared to positive controls (range: 0–68.2%; mean cell count of 21.9±1.9) which was significantly higher than negative controls ($p<0.001$) (Fig. 3E). It is remarkable that 3 retinas from this group showed survival rates between 60–70%. At 8 weeks post MSC graft the survival rate had dropped to 1.8%, not significantly different from negative controls, with only 3 of 8 exhibiting Fast Blue+ RGCs (range: 0–5.8%; mean cell count of 1.6±0.3; Fig. 3E).

At 4 weeks after lesion, the majority of retinas from animals that had received cell grafts exhibited a distribution of RGC soma similar to that seen in positive control retinas, with a higher density in the centre compared to the periphery of the retina (data not shown). However, there was evidence of a pattern whereby the average soma size was higher in retinas with lower numbers of surviving RGC (cf. Figs. 3B, C).

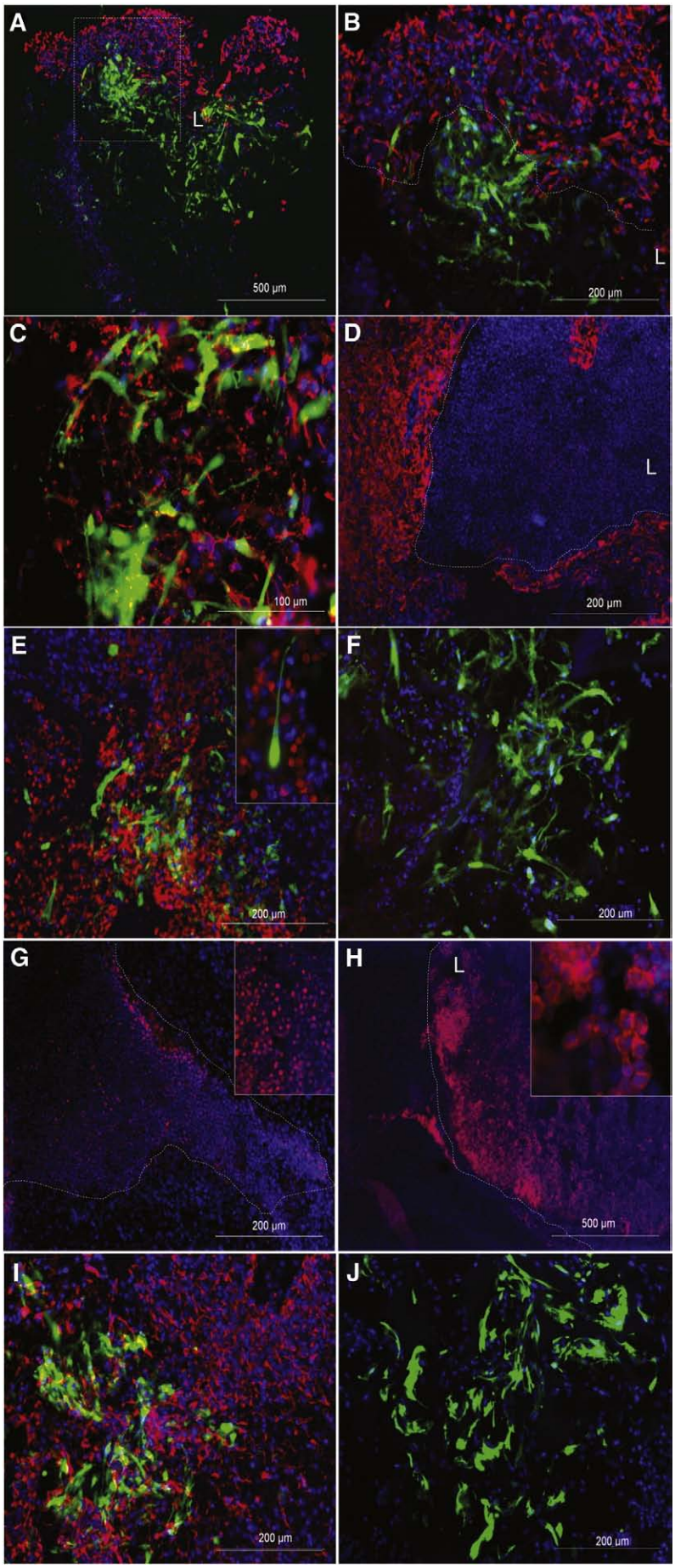
MSCs promote regeneration of axotomised RGCs

The above data indicate that MSCs possess the ability to protect a significant percentage of axotomised RGCs from cell death. To investigate whether MSCs could also facilitate regeneration of axotomised RGCs beyond the lesion, Fast Blue was applied to the superior colliculus (SC) 4 or 8 weeks after grafting. Successful labelling of the contralateral retina was thus contingent on axonal re-growth towards the SC beyond the lesion site at the LGN. All 5 positive control animals that received Fast Blue to the SC 4 weeks after P1 possessed Fast Blue+ RGCs (Fig. 3E), with a mean cell count of 88.9±2.2, which was considered to represent 100% survival in animals that received Fast Blue to the SC; no RGCs were present in 5 negative controls which received Fast Blue to the SC (Fig. 3E).

In remarkable contrast, 16 of 17 lesioned animals that received an MSC graft possessed Fast Blue+ RGCs after the label was applied to the SC. In 4 cases the RGC survival rate was in the range of 40–50%, with an overall average of 20.9%, (range 0–49.2%; mean cell count of 18.6±1.1; Figs. 3D and E). This value was significantly different from relevant negative controls ($p<0.001$). 8 weeks after lesion and MSC graft, only half of 10 cases displayed Fast Blue+ RGCs, with a much-decreased mean survival rate of 4.8% (range 0–26.1%; mean cell count of 4.3±0.7; (Fig. 3E) which was not significantly different from negative controls.

Anterograde HRP labelling was used to confirm that axotomised RGCs did indeed re-grow towards their original target of the SC in the presence of grafted MSCs. HRP was injected into the vitreous of eyes contralateral to the lesion 4 or 8 weeks post-lesion, and HRP labelling of the SC was then assessed 3 days later. All positive control animals showed strong staining of the visual section of the SC (Fig.4A; $n=5$), whilst HRP staining was absent in all negative control animals with no cell grafts at 4 weeks (Fig. 4B; $n=6$). In contrast, 7/15 animals that received MSCs showed HRP staining in the SC after 4 weeks (Fig. 4C). Staining in this group was not as dense as in positive controls, but the area stained was typically as large. At longer times of 8–10 weeks after MSC grafting, only 1/6 brains was positive for HRP in the SC (Fig. 4D). The staining observed was sparse compared to staining performed after 4 weeks. This data supports the assertion that MSCs can facilitate re-growth of axotomised RGC axons to their original target, although as seen with the Fast Blue labelling the regeneration is evident at 4 weeks, but not 8 weeks.

Fig. 2. Host response to MSC grafting. (A) GFAP+ cells (red) surround lesions (L) containing MSCs (green). (B) Higher magnification of box in A (dotted line=lesion boundary). (C) MSC-grafted animals showed neuronal process stained with β-tubulin III (red) that were not evident in (D) gelfoam-only control lesions. (E, F) Ki-67 staining (red) of MSC-grafted animals revealed large numbers of proliferating endogenous cells in the lesioned area at 5 days (E) but not at 14 days (F). Inset shows a higher magnification in E. (G) Fewer Ki-67+ cells were seen in gelfoam-only controls. (H) CD11b+ cells (red) in a gelfoam-only control animal. (I, J) Nestin staining (red) in MSC-grafted animals after 5 (I) and 14 (J) days. N≥3; nuclei are indicated by DAPI (blue); Scale bars: 500 μm (A, H), 200 μm (B, D–G, I–J) or 100 μm (C).


A
L
500 μm
B
L
200 μm
C
100 μm
D
L
200 μm
E
200 μm
F
200 μm
G
200 μm
H
L
500 μm
I
200 μm
J
200 μm

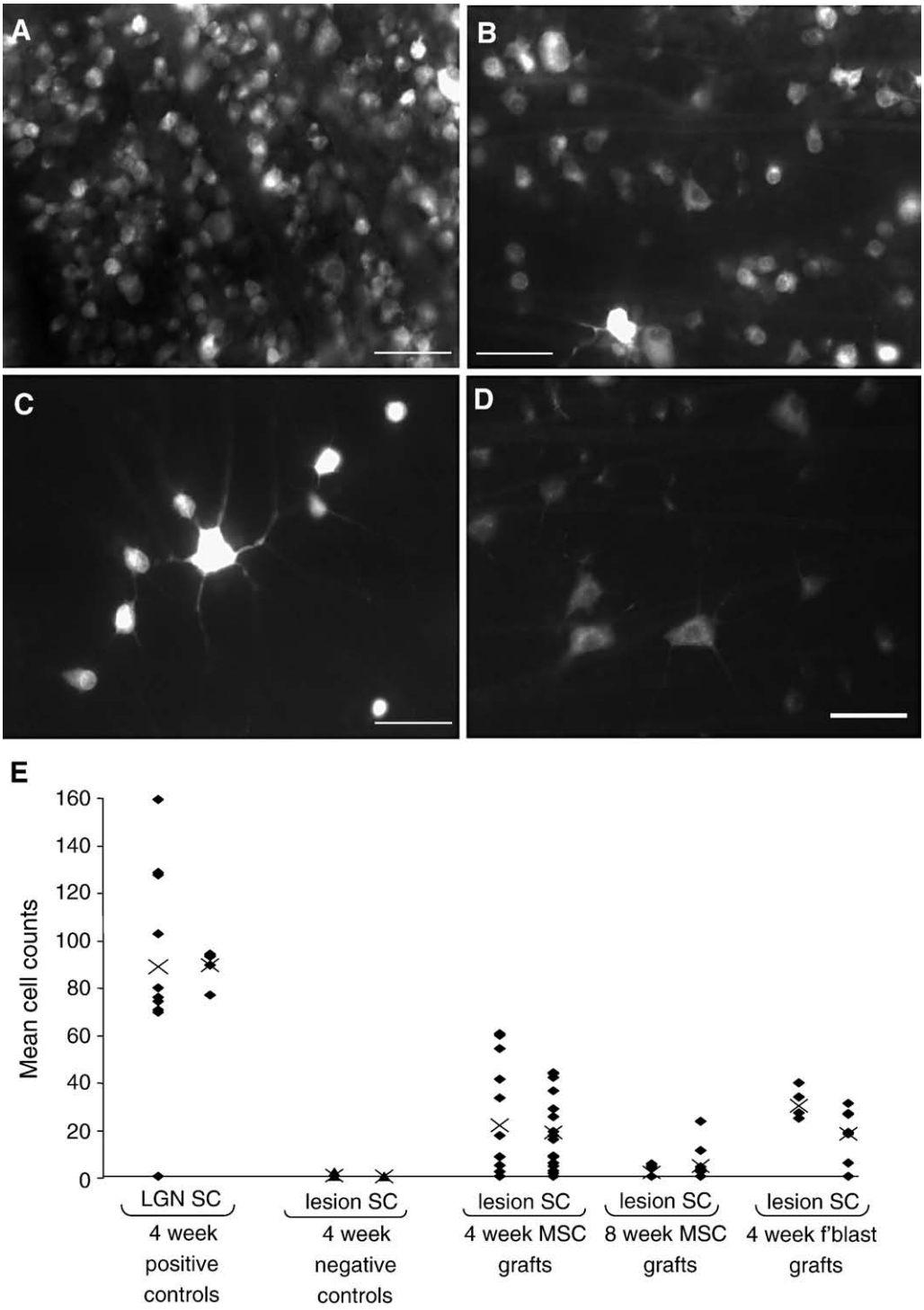


Fig. 3. Neuroprotection and regeneration of RGCs by MSCs. (A) Unlesioned animals displayed Fast Blue in the retina when the dye was introduced at the level of the lateral geniculate nucleus. (B, C) Patterns of RGC survival at 4 weeks in MSC-grafted animals after application of Fast Blue to the lesion site. (D) Fast Blue applied to the SC labels RCG in MSC-grafted animals at 4 weeks. Scale bars: 50 μm. (E) Comparison of survival rates of RGCs under different graft conditions, with Fast Blue applied at the lesion (LGN for the control) or SC. Mean cell counts for each retina are shown by filled diamonds, with the overall mean of each group shown by crosses.

Fibroblasts are neuroprotective but do not activate endogenous neural precursor cells

To determine whether these neuroprotective and regenerative effects were unique to the MSCs and dependent on their stem cell status, we grafted a non-multipotent cell population of human fibroblasts, line Hs27. At 4 weeks after grafting, application of Fast Blue to the lesion site indicated that significant numbers of RGCs survived in 5/5 animals with a mean survival rate of 33.6% (range: 0–49.2%; mean cell count of 29.65 ± 1.1; Fig. 3E). This value was significantly different to negative controls but not MSC-grafted animals, although higher individual survival rates were seen in MSC-grafted animals. Significant regeneration of RGCs was also seen in fibroblast-grafted animals (7/8) at 4 weeks post-lesion following Fast Blue application to the SC, with an average RGC survival of 20.6% (mean cell count of 18.2 ± 1.2; Fig. 3E). Despite no significant difference between the means for MSC- and

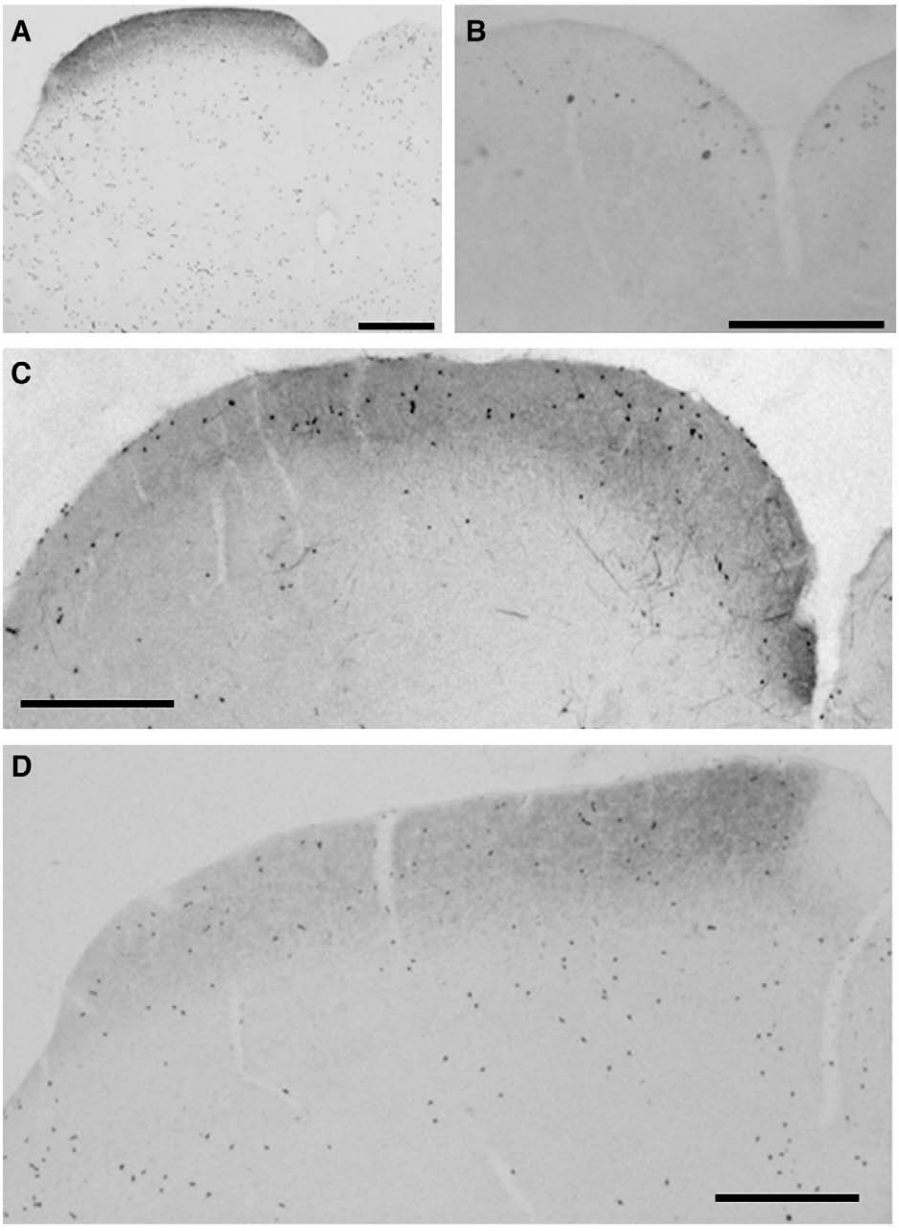


Fig. 4. Anterograde labeling of RGC. (A) HRP labelling is evident in the SC of an unlesioned animal, but (B) absent in lesion-only animals. (C) HRP staining at 4 weeks and (D) 8 weeks, in the SC of MSC-grafted animals. Scale bars: 250 μm.

fibroblast-treated groups, the highest individual survival rates were again seen in the MSC group.

Grafted fibroblasts survived well up to 5 days (Fig. 5A), decreasing in number by 2 weeks (data not shown), comparable to MSCs. In contrast to the MSCs, the fibroblasts elicited a notable immune response at 5 days post-graft, with a large number of CD4+ and MHC Class II+ cells seen in the graft site (Figs. 5B and C). However, these immune cells could no longer be detected 2 weeks after grafting, suggesting a transient rather than prolonged response. No significant migration was seen, and signs of neural differentiation as indicated by neural marker expression were absent. Fibroblast-grafted lesion sites contained endogenous cells expressing the neuronal marker β-tubulin III (Fig. 5D), and grafting again elicited a glial scar identical to that seen in MSC-grafted animals (data not shown). However, the activation of endogenous neural precursors and proliferation observed in MSC-grafted cases was absent, as indicated by a lack of endogenous nestin (Fig. 5E) and Ki67 expression (Fig. 5F).

MSCs and fibroblasts secrete neurotrophic factors in vitro

The secretion of neurotrophic factors by grafted cells is a potential mechanism of both neuroprotection and regeneration. We therefore investigated the secretion *in vitro* of several of these factors by MSCs and fibroblasts using ELISAs. MSCs secreted TGFβ1, CNTF, NT-3 and BDNF (Fig. 6). These factors were also secreted by fibroblasts, but to differing degrees (Fig. 6). Neither cell type secreted detectable amounts of GDNF or IL-10. These results show that MSCs and fibroblasts produce neurotrophic and immunomodulatory factors, but with distinct patterns of expression.

Discussion

Stem cell populations, including neural progenitors and bone marrow-derived MSCs, have been reported to exert beneficial effects in various models of CNS injury by mechanisms other than replacement of damaged neurons (Charalambous et al., 2008; Honma

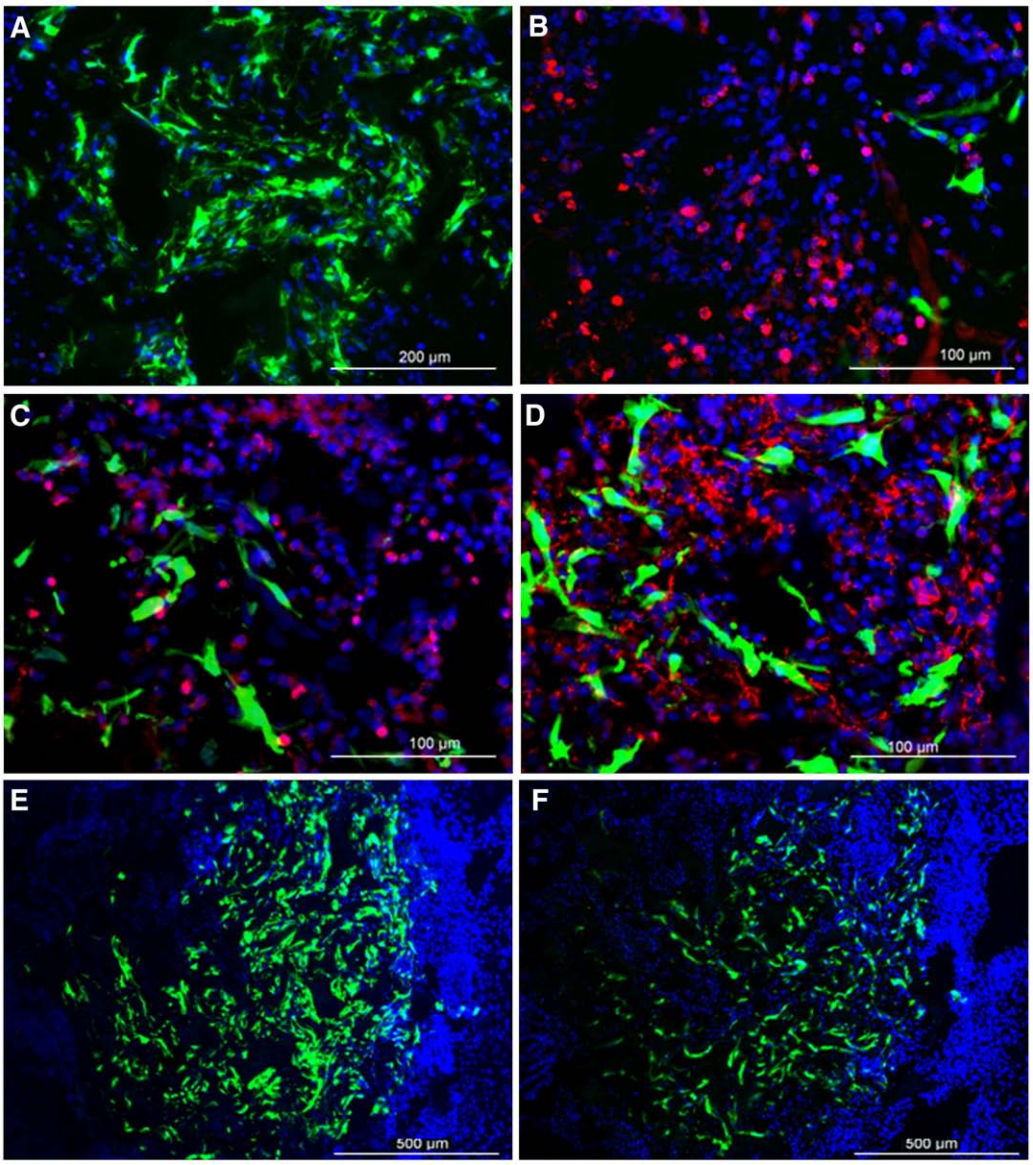


Fig. 5. Grafting of fibroblasts. (A) Fibroblasts (green) are present within the graft site at 5 days ($N = 10$). (B) Fibroblast-treated animals exhibited CD4+ (red) and (C) MHC Class II+ cells (red) within the lesion site. (D) β-tubulin III (red) was seen in fibroblast (green)-grafted lesions. Panels E, F show a lack of nestin (E) and Ki-67 (F) in fibroblast-grafted animals. N ≥ 3; nuclei are indicated by DAPI (blue); Scale bars: 200 μm (A), 100 μm (B–D) or 500 μm (E–F).

et al., 2006; Li et al., 2001; Lu et al., 2005). MSCs are characterized by their ability to differentiate into adipocytes, osteoblasts and chondrocytes (Dominici et al., 2006), and have also been reported to develop into neural cell types (Deng et al., 2006). We did not find evidence for such differentiation in the current study, but have shown that our hUCB MSC lines can exhibit limited astrocytic but not neuronal differentiation under certain conditions *in vitro* (Zwart et al., 2008). MSCs are also known to exert immunosuppressive effects, a property shown recently to be shared with fibroblasts (Jones et al., 2007), and to secrete neurotrophic factors (Chen et al., 2002; Crigler et al., 2006), and it is these properties that may account for their neuroprotective and regenerative effects seen in our model of optic nerve degeneration.

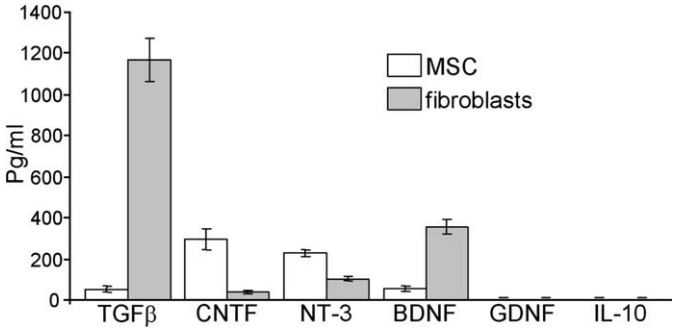


Fig. 6. Production of neurotrophic factors by MSCs and fibroblasts. ELISA data for the *in vitro* secretion of cytokines by cultures of the MSC and fibroblast lines used in the grafting experiments (mean pg/ml ± S.D.).

The hUCB MSCs survived in the lesion site for at least two weeks post-grafting, but were lost by 4 weeks. This limited survival was not unique to the MSCs, since human fibroblasts also exhibited poor long-term survival. Cell loss occurred despite immunosuppression of the recipients, a procedure found to be required to inhibit a xenogeneic response (data not shown). This survival period is similar to that reported by others (Coyne et al., 2006; Li et al., 2004; Neuhuber et al., 2005; Pfeifer et al., 2004), although syngeneic transplants have been reported to endure for longer periods (Chopp et al., 2000; Lu et al.,

2005). The frequency of immunosuppression was reduced two weeks after surgery, and it remains to be determined if increased rates or doses of suppressants would lead to longer survival of the grafted cells, and also to longer regenerative effects. Emigration from the graft site did not appear to account for a significant loss of the MSCs or fibroblasts, similar to other reports of limited movement (<1–2 mm) (Lu et al., 2003, 2005; Neuhuber et al., 2005). The glial scar around the lesion and/or the implantation of the cells in a coagulant-containing gelfoam might provide barriers to migration. However, MSCs have been shown to home to sites of injury in response to factors released as a result of tissue damage (Ji et al., 2004), so implantation of cells into the lesion itself may obviate their migration.

Despite immunosuppression there was still a strong infiltration of immune cells in the negative control animals, but this was strikingly inhibited in the presence of the MSCs. It is not know whether the human MSCs are eventually lost due to immune surveillance by the host, despite immunosuppression by drugs and the immunomodulatory capacity of the MSCs, or if the lesion environment does not support their survival. The mechanism(s) by which MSCs suppress immune reactions, and the relative contributions of cell–cell and secreted factors remain unclear, but it will be of great interest to elucidate their roles in neuroprotection in this model.

In addition to inhibiting inflammation around the optic tract lesion, an observation seen with the MSC, but not the fibroblast grafts, was the presence of proliferation and nestin expression by host cells in the graft site at 5 days. This indicates that MSCs have the ability to induce activation of endogenous neural precursor cells in the site of injury. A similar phenomenon has been reported following MSC grafts in both healthy rats and animals with induced stroke, whereby proliferation of endogenous neural precursor cells was seen either in the grafted brain region or in the subventricular zone (Chen et al., 2003; Munoz et al., 2005). Whether these endogenous precursor cells differentiated into mature neural cells was not examined in our study, but an absence of proliferation and nestin expression at 2 weeks indicates the cells had either died, differentiated, reverted to a quiescent state or migrated away from the site. Since the mean RGC survival was similar in the MSC and fibroblast-treated animals, it is not clear if the observed proliferation and nestin induction contribute to regeneration of the optic tract.

The unique aspect of this study is the observation that the MSC grafts not only increased RGC survival significantly, but also allowed for re-growth to their original target area in the SC. This was demonstrated by Fast Blue retrograde labelling and was confirmed with HRP anterograde tracing of some MSC graft cases, although it remains to be determined whether functional connections are re-established. Several studies with SCI models reported survival and growth of host neurons at the graft site in animals that received bone marrow-derived MSCs (Lu et al., 2005; Neuhuber et al., 2005), however they did not show evidence of neuronal regeneration and growth past the lesion to the target. Indeed, it was shown that regenerating axons entering the graft never leave the graft area (Lu et al., 2005), although this was seen with MSCs genetically modified to express BDNF (Lu et al., 2005), indicating that excessively high levels of neurotrophins may prevent axons from exiting the graft.

Variability in the survival of particular subpopulations of RGCs between animals was noted in the graft experiments. Retinas with a high RGC survival rate were largely populated with small RGCs, whilst those with a low RGC survival rate mainly displayed RGCs with large cell somas and multiple distinct processes. It is possible that larger RGCs are more responsive to neurotrophic factors secreted by the graft cells and therefore are better suited to survival. As there may be competition between RGCs for factors, larger cells may possess an advantage over smaller cells. Similar observations of differential survival of larger RGCs has been made in rats that received exogenous neurotrophic factors after transection of the optic nerve (Mey and Thanos, 1993), and in a murine model of glaucomatous neuropathy (Filippopoulos et al., 2006). Further examination of this phenomenon should provide insights into the mechanisms of RGC rescue and how they might be applied to optimizing functional regeneration.

There are several possible mechanisms by which the grafted cells could have exerted their neuroprotective and regenerative effects. The absence of any indication of neural differentiation by MSCs, and the ability of fibroblasts to mimic their beneficial effects on RGC survival and regeneration, suggests that stem cell multipotency is not required for the beneficial effects seen in this model. More likely mechanisms include immunosuppression, and secretion of trophic factors by the grafted cells. MSCs prevented the appearance of host immune cells at the lesion, which may have reduced the response to damage that prevents neuronal re-growth (Fawcett and Asher, 1999). Although an immune infiltration was seen transiently with fibroblast grafts, these cells produce high levels of TGFβ1 which is known to be immunomodulatory as well as neuroprotective in the CNS (Flanders et al., 1998; Krieglstein et al., 1998). RGCs are dependent on various neurotrophins for their survival *in vivo* (Harvey et al., 2006), and studies have shown that administration of exogenous BDNF, GDNF, CNTF or NT-3 can promote RGC survival following injury (Logan et al., 2006; Mey and Thanos, 1993; Yan et al., 1999). Both MSCs and fibroblasts secreted TGFβ1, CNTF, NT-3 and BDNF *in vitro*, similar to previous reports of neurotrophic factor production by MSCs and fibroblasts (Chen et al., 2002; Neuhuber et al., 2005). The secretion patterns differed between MSCs and fibroblasts, which may account for the differences in the ability of the graft cells to stimulate endogenous neural precursors. However, it must be noted that secretion *in vitro* may not reflect secretion *in vivo*, as an injured environment can alter MSC secretory patterns (Chen et al., 2002).

Conclusion

This study has shown that hUCB MSCs can protect axotomised neurons, and facilitate a degree of re-growth of injured RGCs to their target in the CNS. Although significant neuroprotection was also observed using fibroblast grafts, MSCs exerted a greater immunomodulatory effect within and around the graft site, and also stimulated endogenous proliferation and appearance of neural precursors within the graft site. Whilst the importance of endogenous precursors in this model is not currently clear, this phenomenon, combined with the evidence of superior immune response suppression, suggests that MSCs may be a better population of cells with which to protect and regenerate injured neurons. Despite the significant effects of the grafted cells, they provided for only a transient survival of the RGCs, and it will be important to determine if improved graft survival or complementary treatments can lead to a more durable repair. Our findings, along with the widespread availability and accessibility of these cells, make them strong candidates for the development of cell-based therapies for CNS injury and disease.

Acknowledgments

The authors would like to thank the NHS Blood and Transplant Oxford Cord Blood Collection Team for the supply of umbilical cord blood for research projects. The research was supported by NHS Blood and Transplant, NHS R&D (Grant number PG03/1 RP40), and the EU Framework VI Thercord Programme.

References

Chan, S.O., Jen, L.S., 1988. Enlargement of uncrossed retinal projections in the albino rat: additive effects of neonatal eye removal and thalamectomy. Brain Res. 461, 163–168.

Chan, S.O., Chow, K.L., Jen, L.S., 1989. Postnatal development of the ipsilaterally projecting retinal ganglion cells in normal rats and rats with neonatal lesions. Brain Res. Dev. Brain Res. 49, 265–274.
Charalambous, P., Hurst, L.A., Thanos, S., 2008. Engrafted chicken neural tube-derived stem cells support the innate propensity for axonal regeneration within the rat optic nerve. Invest. Ophthal. Visual Sci. 49, 3513–3524.
Chen, X., Li, Y., Wang, L., Katakowski, M., Zhang, L., Chen, J., Xu, Y., Gautam, S.C., Chopp, M., 2002. Ischemic rat brain extracts induce human marrow stromal cell growth factor production. Neuropathology. 22, 275–279.
Chen, J., Li, Y., Katakowski, M., Chen, X., Wang, L., Lu, D., Lu, M., Gautam, S.C., Chopp, M., 2003. Intravenous bone marrow stromal cell therapy reduces apoptosis and promotes endogenous cell proliferation after stroke in female rat. J. Neurosci. Res. 73, 778–786.
Chopp, M., Zhang, X.H., Li, Y., Wang, L., Chen, J., Lu, D., Lu, M., Rosenblum, M., 2000. Spinal cord injury in rat: treatment with bone marrow stromal cell transplantation. NeuroReport 11, 3001–3005.
Coyne, T.M., Marcus, A.J., Woodbury, D., Black, I.B., 2006. Marrow stromal cells transplanted to the adult brain are rejected by an inflammatory response and transfer donor labels to host neurons and glia. Stem Cells 24, 2483–2492.
Crigler, L., Robey, R.C., Asawachaicharn, A., Gaupp, D., Phinney, D.G., 2006. Human mesenchymal stem cell subpopulations express a variety of neuro-regulatory molecules and promote neuronal cell survival and neuritogenesis. Exp. Neurol. 198, 54–64.
Demaison, C., Parsley, K., Brouns, G., Scherr, M., Battmer, K., Kinnon, C., Grez, M., Thrasher, A.J., 2002. High-level transduction and gene expression in hematopoietic repopulating cells using a human immunodeficiency [correction of imunodeficiency] virus type 1-based lentiviral vector containing an internal spleen focus forming virus promoter. Hum. Gene Ther. 13, 803–813.
Deng, J., Petersen, B.E., Steindler, D.A., Jorgensen, M.L., Laywell, E.D., 2006. Mesenchymal stem cells spontaneously express neural proteins in culture and are neurogenic after transplantation. Stem Cells 24, 1054–1064.
Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., Deans, R., Keating, A., Prockop, D., Horwitz, E., 2006. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy. 8, 315–317.
Erices, A., Conget, P., Minguell, J.J., 2000. Mesenchymal progenitor cells in human umbilical cord blood. Br. J. Haematol. 109, 235–242.
Fawcett, J.W., Asher, R.A., 1999. The glial scar and central nervous system repair. Brain Res. Bull. 49, 377–391.
Filippopoulos, T., Danias, J., Chen, B., Podos, S.M., Mittag, T.W., 2006. Topographic and morphologic analyses of retinal ganglion cell loss in old DBA/2NNia mice. Invest. Ophthalmol. Vis. Sci. 47, 1968–1974.
Flanders, K.C., Ren, R.F., Lippa, C.F., 1998. Transforming growth factor-betas in neurodegenerative disease. Prog. Neurobiol. 54, 71–85.
Goan, S.R., Junghahn, I., Wissler, M., Becker, M., Aumann, J., Just, U., Martiny-Baron, G., Fichtner, I., Henschler, R., 2000. Donor stromal cells from human blood engraft in NOD/SCID mice. Blood 96, 3971–3978.
Harvey, A.R., Hu, Y., Leaver, S.G., Mellough, C.B., Park, K., Verhaagen, J., Plant, G.W., Cui, Q., 2006. Gene therapy and transplantation in CNS repair: the visual system. Prog. Retin. Eye Res. 25, 449–489.
Honma, T., Honmou, O., Iihoshi, S., Harada, K., Houkin, K., Hamada, H., Kocsis, J.D., 2006. Intravenous infusion of immortalized human mesenchymal stem cells protects against injury in a cerebral ischemia model in adult rat. Exp. Neurol. 199, 56–66.
Jen, L.S., Lund, R.D., 1981. Experimentally induced enlargement of the uncrossed retinotectal pathway in rats. Brain Res. 211, 37–57.
Jen, L.S., Dai, Z.G., Liu, Z.H., 1986. The cells of origin of the aberrant recrossed retinotectal projection in the rat. Brain Res. 377, 368–373.
Jen, L.S., Cheung, Y.M., Chow, K.L., 1989. The origin and terminal arbors of individual uncrossed retinogeniculate fibers in rabbits. Neuroscience 29, 479–493.
Ji, J.F., He, B.P., Dheen, S.T., Tay, S.S., 2004. Interactions of chemokines and chemokine receptors mediate the migration of mesenchymal stem cells to the impaired site in the brain after hypoglossal nerve injury. Stem Cells 22, 415–427.
Jones, S., Horwood, N., Cope, A., Dazzi, F., 2007. The antiproliferative effect of mesenchymal stem cells is a fundamental property shared by all stromal cells. J. Immunol. 179, 2824–2831.
Kim, J.H., Auerbach, J.M., Rodriguez-Gomez, J.A., Velasco, I., Gavin, D., Lumelsky, N., Lee, S.H., Nguyen, J., Sanchez-Pernaute, R., Bankiewicz, K., McKay, R., 2002. Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease. Nature 418, 50–56.
Krieglstein, K., Henheik, P., Farkas, L., Jaszai, J., Galter, D., Krohn, K., Unsicker, K., 1998. Glial cell line-derived neurotrophic factor requires transforming growth factor-beta for exerting its full neurotrophic potential on peripheral and CNS neurons. J. Neurosci. 18, 9822–9834.
LeBlanc, K., Rasmusson, I., Sundberg, B., Gotherstrom, C., Hassan, M., Uzunel, M., Ringden, O., 2004. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. Lancet 363, 1439–1441.
Li, Y., Chen, J., Wang, L., Lu, M., Chopp, M., 2001. Treatment of stroke in rat with intracarotid administration of marrow stromal cells. Neurology 56, 1666–1672.
Li, S., Hu, B., Tay, D., So, K.F., Yip, H.K., 2004. Intravitreal transplants of Schwann cells and fibroblasts promote the survival of axotomized retinal ganglion cells in rats. Brain Res. 1029, 56–64.
Liu, Z.H., Jen, L.S., 1986. Displaced retinal ganglion cells in normal rats and rats with one eye enucleated at birth. Neurosci. Lett. 67, 239–244.
Llado, J., Haenggeli, C., Maragakis, N.J., Snyder, E.Y., Rothstein, J.D., 2004. Neural stem cells protect against glutamate-induced excitotoxicity and promote survival of injured motor neurons through the secretion of neurotrophic factors. Mol. Cell. Neurosci. 27, 322–331.
Logan, A., Ahmed, Z., Baird, A., Gonzalez, A.M., Berry, M., 2006. Neurotrophic factor synergy is required for neuronal survival and disinhibited axon regeneration after CNS injury. Brain 129, 490–502.
Lu, P., Jones, L.L., Snyder, E.Y., Tuszynski, M.H., 2003. Neural stem cells constitutively secrete neurotrophic factors and promote extensive host axonal growth after spinal cord injury. Exp. Neurol. 181, 115–129.
Lu, P., Jones, L.L., Tuszynski, M.H., 2005. BDNF-expressing marrow stromal cells support extensive axonal growth at sites of spinal cord injury. Exp. Neurol. 191, 344–360.
MacLaren, R.E., Pearson, R.A., MacNeil, A., Douglas, R.H., Salt, T.E., Akimoto, M., Swaroop, A., Sowden, J.C., Ali, R.R., 2006. Retinal repair by transplantation of photoreceptor precursors. Nature 444, 203–207.
Manca, M.F., Zwart, I., Beo, J., Palasingham, R., Jen, L.S., Navarrete, R., Girdlestone, J., Navarrete, C.V., 2008. Characterization of mesenchymal stromal cells derived from full-term umbilical cord blood. Cytotherapy 10, 54–68.
Mey, J., Thanos, S., 1993. Intravitreal injections of neurotrophic factors support the survival of axotomized retinal ganglion cells in adult rats in vivo. Brain Res. 602, 304–317.
Munoz, J.R., Stoutenger, B.R., Robinson, A.P., Spees, J.L., Prockop, D.J., 2005. Human stem/progenitor cells from bone marrow promote neurogenesis of endogenous neural stem cells in the hippocampus of mice. Proc. Natl. Acad.Sci. U. S. A. 102, 18171–18176.
Neuhuber, B., Timothy, H.B., Shumsky, J.S., Gallo, G., Fischer, I., 2005. Axon growth and recovery of function supported by human bone marrow stromal cells in the injured spinal cord exhibit donor variations. Brain Res. 1035, 73–85.
Ogawa, Y., Sawamoto, K., Miyata, T., Miyao, S., Watanabe, M., Nakamura, M., Bregman, B.S., Koike, M., Uchiyama, Y., Toyama, Y., Okano, H., 2002. Transplantation of in vitro-expanded fetal neural progenitor cells results in neurogenesis and functional recovery after spinal cord contusion injury in adult rats. J. Neurosci. Res. 69, 925–933.
Ourednik, J., Ourednik, V., Lynch, W.P., Schachner, M., Snyder, E.Y., 2002. Neural stem cells display an inherent mechanism for rescuing dysfunctional neurons. Nat. Biotechnol. 20, 1103–1110.
Pfeifer, K., Vroemen, M., Blesch, A., Weidner, N., 2004. Adult neural progenitor cells provide a permissive guiding substrate for corticospinal axon growth following spinal cord injury. Eur. J. Neurosci. 20, 1695–1704.
Ringden, O., Uzunel, M., Rasmusson, I., Remberger, M., Sundberg, B., Lonnies, H., Marschall, H.U., Dlugosz, A., Szakos, A., Hassan, Z., Omazic, B., Aschan, J., Barkholt, L., Le Blanc, K., 2006. Mesenchymal stem cells for treatment of therapy-resistant graft-versus-host disease. Transplantation 81, 1390–1397.
Roy, N.S., Nakano, T., Keyoung, H.M., Windrem, M., Rashbaum, W.K., Alonso, M.L., Kang, J., Peng, W., Carpenter, M.K., Lin, J., Nedergaard, M., Goldman, S.A., 2004. Telomerase immortalization of neuronally restricted progenitor cells derived from the human fetal spinal cord. Nat. Biotechnol. 22, 297–305.
Sarugaser, R., Lickorish, D., Baksh, D., Hosseini, M.M., Davies, J.E., 2005. Human umbilical cord perivascular (HUCPV) cells: a source of mesenchymal progenitors. Stem Cells 23, 220–229.
Studer, L., Tabar, V., McKay, R.D., 1998. Transplantation of expanded mesencephalic precursors leads to recovery in parkinsonian rats. Nat. Neurosci. 1, 290–295.
Yan, Q., Wang, J., Matheson, C.R., Urich, J.L., 1999. Glial cell line-derived neurotrophic factor (GDNF) promotes the survival of axotomized retinal ganglion cells in adult rats: comparison to and combination with brain-derived neurotrophic factor (BDNF). J. Neurobiol. 38, 382–390.
Zwart, I., Hill, A.J., Girdlestone, J., Manca, M.F., Navarrete, R., Navarrete, C., Jen, L.S., 2008. Analysis of neural potential of human umbilical cord blood-derived multipotent mesenchymal stem cells in response to a range of neurogenic stimuli. J. Neurosci. Res. 86, 1902–1915.