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Brief Communication

Comparison of Olfactory Bulbar and Mucosal Cultures in a Rat Rhizotomy Model

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In an ongoing clinical trial, a spinal injured patient who received a transplant of autologous cells cultured from the olfactory bulb is showing greater functional benefit than three previous patients with transplants of mucosal origin. Previous laboratory studies of transplantation into rat spinal cord injuries show that the superior reparative benefits of bulbar over mucosal cultures are associated with regeneration of severed corticospinal tract fibers over a bridge of olfactory ensheathing cells (OECs) formed across the injury site. In a rat rhizotomy paradigm, we reported that transplantation of bulbar cell cultures also enables severed axons of the C6–T1 dorsal roots to regenerate across a bridge of OECs into the spinal cord and restore electrophysiological transmission and forepaw grasping during a climbing test. We now report a repeat of the same rhizotomy procedure in 25 rats receiving cells cultured from olfactory mucosal biopsies. In no case did the transplanted cells form a bridging pathway. No axons crossed from the severed roots to the spinal cord, and there was no restoration of forepaw grasping. This suggests that the superior clinical benefit in the patient receiving bulbar cell transplants is due to regeneration of severed fibers across the injury site, and this correlates with imaging and the pattern of functional recovery. Using present culture protocols, the yield of OECs from bulbar biopsies is around 50%, but that from mucosal biopsies is less than 5%. Improving the yield of OECs from mucosal biopsies might avoid the necessity for the intracranial approach to obtain bulbar cells.

Key words: Transplants; Spinal cord injury; Regeneration; Dorsal roots; Olfactory ensheathing cells (OECs)

INTRODUCTION

In a recent clinical trial (23), three patients with functionally complete spinal cord injuries received transplants of unpurified autologous cells cultured from biopsies taken from the patients' olfactory mucosa. Compared to three unoperated control patients, the transplanted patients showed minor degrees of functional improvements. A subsequent patient, who was receiving intracranial surgery for an independent sinus condition, presented an opportunity to carry out an intraspinal transplant of cultures of autologous cells isolated from an olfactory bulb combined with bridging materials from peripheral nerve (24). Ongoing examination of this patient reveals a considerable and progressive enhancement of recovery compared with the previous three patients with mucosa-derived cultures.

There are a number of studies of the reparative effects of transplanted olfactory bulb tissue in spinal injuries in rats (13,26) and in primates (20). In a comparative study, cells cultured by a similar procedure from bulbar and mucosal biopsies were transplanted into unilateral upper cervical corticospinal tract (CST) lesions (10,13,25). The bulbar transplants mediated a return of function in a directed forepaw reaching test, and histological analysis showed regeneration of the cut CST axons across the lesion site. The rats with mucosal transplants also showed a degree of functional benefit, although no axons were seen to cross the lesion. This suggests that the benefits of the mucosal transplants were associated with enhancement of connectivity in the surviving tissue ("plasticity").

To confirm that the return of function mediated by bulbar transplants is due to regeneration of severed axons, we

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used an experimental paradigm in which the sensory input from the forelimb was totally abrogated on one side by a unilateral C6–T1 dorsal rhizotomy. Severing these four dorsal roots led to a permanent loss of the ability to use the forepaw of the operated side for grasping the bars in a grid climbing test (8). In this situation, there is no surviving adjacent tissue through which sprouting could restore the forelimb input. Transplantation of cultures of adult olfactory ensheathing cell/olfactory nerve fibroblast (OEC/ONF) mixtures derived from the olfactory bulb induced structural and functional regeneration of dorsal root fibers into the spinal cord in rats (7). The severed dorsal root axons regenerated across these bridges ensheathed by the OECs, entered the dorsal horn, and ascended in the dorsal columns to the dorsal column nuclei. This was accompanied by a restoration of electrical transmission of impulses from the peripheral nerve into the spinal cord and cuneate nuclei and restoration of directed forepaw grasping during a climbing test. This recovery, therefore, was due to regeneration of severed axons.

In the present series of experiments, we report that mucosal cultures transplanted into the same rhizotomy situation do not mediate axon regeneration, and there is no functional recovery. By analogy, this suggests that the superior beneficial effects seen in the patient with transplanted bulbar cultures are associated with axon regeneration.

The question of how these two types of culture differ and what might be done to improve the mucosal cultures will be discussed.

MATERIALS AND METHODS

Cell Culture

Mucosal cells were prepared according to the method previously described (11,15). Briefly, female 3- to 6-month-old Albino Swiss (AS) strain rats (bred locally) were decapitated under terminal anesthesia and the nasal septum exposed. The olfactory mucosa was transferred into ice-cold Hank's balanced salt solution (HBSS) without calcium and magnesium supplemented with 100 U/ml of penicillin and 100 µg/ml of streptomycin (all Invitrogen, Paisley, UK). It was washed twice in HBSS to remove excessive mucus. The tissue was then transferred to 35-mm dishes (Nunc, Thermo Scientific, Hemel Hempstead, UK) filled with culture medium consisting of Dulbecco's modified Eagle's medium/Ham's Nutrient Mixture (DMEM/F12 plus Glutamax; Invitrogen), 1% insulin–transferrin–selenium (1.0 mg/ml insulin, 0.67 mg/ml transferrin, and 0.55 mg/ml selenium; Invitrogen), 100 U/ml of penicillin and 100 µg/ml of streptomycin (Invitrogen), and 10% deactivated fetal calf serum (Invitrogen; DMEM/F12) and cut into small pieces (approximately 0.5 mm^2) with a scalpel, which were transferred and incubated in 2 ml of 5% collagenase type I solution (Sigma-Aldrich, Gillingham, UK) and 2.4 U/ml Dispase II (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) in DMEM/F12 at 37°C for 5 min. The pieces were triturated, and the enzymatic reaction was stopped by adding 8 ml of HBSS. After centrifuging at $300 \times g$ for 5 min and discarding the supernatant, the tissue pellet was further triturated into a cell suspension in the DMEM/F12. The resulting cells were seeded to a density of 20,000–25,000/cm^2 on 35-mm culture dishes coated with poly-L-lysine (0.1 mg/ml; Sigma-Aldrich), and medium was replaced every 3 days. At day 14, the cells were transfected with a green fluorescent protein (GFP) gene harboring a lentiviral construct (4,17,21). After a further 2 days, the cultures were rinsed, and the cell-secreted matrix containing GFP-labeled cells was scraped from the dish (Costar, Corning, NY, USA) (12).

Surgery and Behavioral Assessment

In 28 anesthetized (isoflurane, Abbott Laboratories Ltd., Maidenhead, Berkshire, UK) adult female AS rats (body weight 180–200 g, age 8–12 weeks), hemilaminectomies from C4 to T2 were carried out, the dura opened, and the dorsal roots from C6 to T1 were sectioned close to the surface of the spinal cord. The use of a semisolid transplant consisting of the matrix containing the cells was essential in order to retain the cells at the transplant site and bridge the gap. The matrix was applied between the cut ends of the dorsal roots and their original entry point on the spinal cord and retained in place with fibrin glue (Tisseel Kit, Baxter, Thetford, UK). The experiment was terminated in three rats that started to show self-mutilation of the affected digits. In the remaining 25 rats, from 3 weeks before surgery to 8 weeks after surgery, weekly video recordings were made during two successive climbs up a 1-m grid. All the recordings were analyzed by two independent observers. The forepaw ipsilateral to the lesion was assessed for accuracy in grasping the grid bars. A successful grasp was recorded whenever there was purposeful movement resulting in direct contact with the bar and ending with full flexion of the digits around the bar [for details, see Ibrahim et al. (8)]. A full neurological description of the complex deficits and compensatory changes in the affected and other limbs was beyond the scope of the study.

All animals were handled according to UK Home Office regulations ASPA 5(2) PIL 70/9580 for the care and use of laboratory animals, the UK Animals (Scientific Procedures) Act 1986 with the approval of the UCL Institute for Neurology.

Histology

After behavioral assessment, the rats were terminally anesthetized, and the tissues were fixed by transcardiac perfusion of 50 ml 0.01 M phosphate buffer solution (BDH, VWR, Lutterworth, Leicestershire, UK) followed

by 500 ml 4% phosphate-buffered paraformaldehyde (TAAB, Reading, Berkshire, UK). After hardening in fixative for 24–48 h, the spinal cord and the associated roots were gently dissected out, placed into 10–20% sucrose solution (Sigma-Aldrich), frozen in dry ice, and 16-μm sections were cut on the cryostat (GM3050; Leica, Wetzlar, Germany). For immunostaining, the sections were incubated in 2% milk (Oxoid Limited, Basingstoke, Hampshire, UK) containing primary antibodies: 1:1,000 anti-glial fibrillary acid (GFAP; mouse monoclonal; Sigma-Aldrich), 1:500 heavy chain polyclonal rabbit anti-neurofilament H200 antibodies (AHP245; AbD Serotec, Kidlington, UK), 1:500 anti-laminin antibodies (LN; rabbit polyclonal; Sigma), 1:250 mouse anti-low affinity nerve growth factor receptor (anti-p75; clone 192-IgG; Chemicon, Millipore, Chandlers Ford, Hampshire, UK), 1:200 mouse anti-thymocyte antigen 1 (Thy 1; IgG clone MRC OX-7; AbCam, Cambridge, UK), 1:500 rabbit anti-S100β Ig (Dako, Ely, Cambridgeshire, UK), 1:1,000 rabbit anti-fibronectin Ig (FN; Dako), and 1:200 mouse anti-nestin (clone rat-401; Chemicon, Millipore) overnight at 4°C. Secondary antibodies were Alexa Fluor 488, Alex Fluor 546 goat anti-mouse IgG, Alexa Fluor 546 goat anti-rabbit IgG (all 1:400; Molecular Probes, Invitrogen), for 2 h in the dark at room temperature.

RESULTS

The Cells

The olfactory mucosal cell cultures used for transplantation had a mixture of cell types. The point of confluence was reached at around 2 weeks in vitro. At this point, around 5% of cells were identified as OECs. They were p75, S100β, and nestin positive, with a central ovoid perikaryon around 5 μm across and a spectrum of shapes ranging from bipolar spindle around 10–15 μm in the long axis, to flattened multipolar (11). The remaining cells were identified as fibroblasts. They were flattened, more rectangular, Thy-1-positive, fibronectin-positive cells with less distinct outlines, and fewer processes with fibronectin immunofluorescence spreading outside the cell. When cultured beyond the 2-week point, the fibroblasts overgrew, and the OECs were gradually lost.

Forepaw Function

Weekly, for 3 weeks before and for up to 8 weeks after surgery, the 25 rats made two successive climbs up a 1-m grid. Before surgery, the rats accurately placed each of the fore- and hindpaws in regular sequence. On making contact with the bars, the digits closed in a grip. The number of grasps by each paw was assessed from video recordings at 25/s. Each forepaw carried out a mean of 7.1 ± 0.2 grasps in climbing the grid.

Previous results showed that unilateral C6 to T1 rhizotomy completely abolished the ability of the forepaw of the operated side to grasp the bars (8). Grasping was unaffected on the forepaw of the unoperated side, and the rats remained able to climb the grid. In a series of transplanted rhizotomized rats, the gap between the cut ends of the severed dorsal roots and the surface of the spinal cord was bridged by transplantation of a matrix containing the cells cultured from the outer layers of the olfactory bulb. Analysis of the use of the ipsilateral forepaw showed a progressive resumption of grasping, which reached 50% of normal by 8 weeks after operation (7).

In the present series, the same procedure was repeated in 25 rhizotomized rats with similar matrix transplants, but in this case, the cells were cultured from tissue samples taken from the olfactory mucosa. Observations of grasping by the forepaw of the unoperated side and the overall climb resembled that of the previous series of rhizotomized rats without transplants. However, in contrast to the rats with bulbar cell transplants, the video recordings showed that none of the 25 rats with mucosal cells used the forepaw of the operated side to grasp the bars during any climb at any time over the eight postsurgical weeks.

Histology

Continuous serial sections were taken through the entire length of the dorsal spinal cord and dorsal roots from C6 to T1. Use of the matrix method of transplantation effectively retained a high concentration of transplanted cells in the operation site. The transplanted cells were identified by the green fluorescence of the transduced GFP. They formed a cap of elongated cells, arranged in the rostrocaudal plane over the cut stump of the central branch of the dorsal root (Fig. 1A, B). The deafferented spinal cord surface consisted of a vertically orientated glial scar consisting of the flattened pial processes of the spinal cord astrocytes (green fluorescence of GFAP) and the associated meningeal fibroblasts. Although the cut dorsal roots and associated transplanted cells lay in direct contact along this surface (Fig. 1A–D; fibroblasts identified by the red fluorescence of LN; overlap with green GFAP shows as yellow), the transplanted cells were not orientated toward the cord, nor did the astrocytes of the cord surface show significant indication of outgrowth toward the transplant. Histologically, there were no indications of an immune response (cuffing of blood vessels) and no detectable infiltration of inflammatory cells.

The orientation of the transplanted mucosal cells was in contrast to the bridges observed in the previous study with transplanted bulbar OECs (7). In the animals with the bulbar cell transplants, the transplanted cells extended out from the cut stump of the dorsal root and mingled with a massive outgrowth of astrocytes from the spinal cord (Fig. 1E). Over this intermingled mass of aligned cell processes, regenerating dorsal root axons, identified by neurofilament immunostaining extended from the

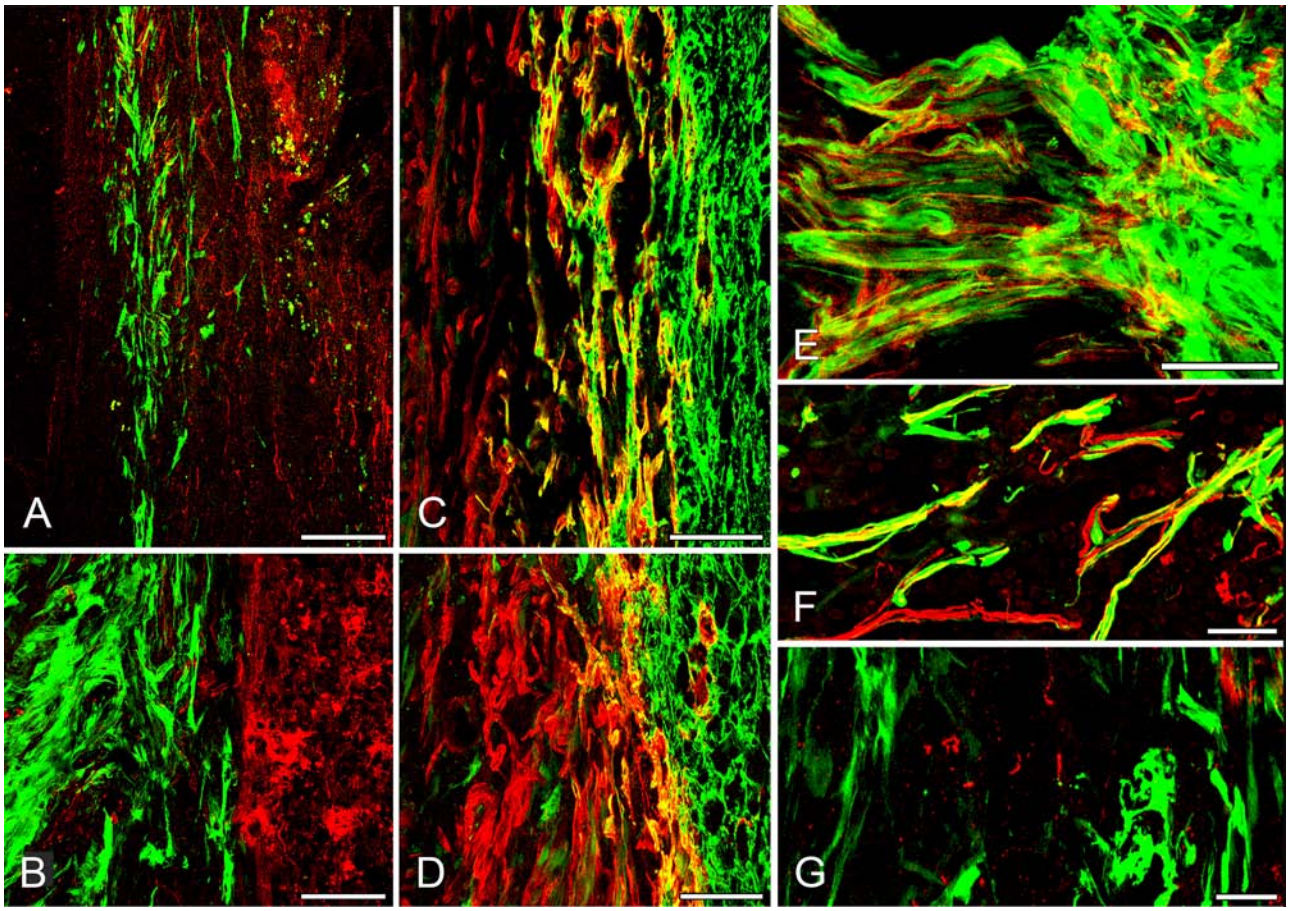

Figure 1. Arrangement of transplanted cells and nerve tissue 8 weeks after transplantation. (A, B) Vertical arrangement of transplanted mucosal cells [green, green fluorescent protein (GFP) label] with neurofilament-positive CNS tissue (red). (C, D) Adjacent sections showing minimal outgrowth of CNS astrocytic processes [glial fibrillary acidic protein (GFAP), green] from the spinal cord with laminin (LN)-positive peripheral nerve tissue (red; overlap in yellow) staining. (E) Contrasting response to transplanted bulbar olfactory ensheathing cells (OECs), showing outgrowth of astrocytic processes (green, GFAP) intermingled with laminin-positive peripheral nerve tissue (red; overlap in yellow). (F) High-power views showing the regenerating dorsal root axons [red, neurofilament (H200)] ensheathed by the transplanted bulbar OECs [green, green fluorescent protein (GFP), overlap in yellow] crossing the interface between the dorsal root and the spinal cord. (G) Similar interface with transplanted mucosal cells (green, GFP) and axons (red, neurofilament) but with no fibers taking a horizontal course toward the spinal cord and no ensheathment. Survival times, 8 weeks. Horizontal cryostat 16-μm-thick sections, rostrocaudal axis from above to below. Scale bars: 100 μm (A–D), 25 μm (E–G).

dorsal root across the bridge. The regenerating dorsal root axons were ensheathed by the transplanted OECs (Fig. 1F). After entering the astrocytic territory of the spinal cord, the regenerating axons arborized in the dorsal horn and ascended in the dorsal columns. In contrast to the axons regenerating across the bulbar cell transplants, no axons of the severed dorsal roots crossed the mucosal cell transplants (Fig. 1G).

DISCUSSION

In several studies (10,13,14), we have reported the effect of transplantation of suspensions of cultured bulbar cells into unilateral C1/2 level corticospinal tract (CST) lesions. The transplanted cells "opened" the configuration of the glial scar and ensheathed the regenerating CST axons as they crossed the lesion. The regenerating fibers reentered the caudal tract and terminated by arborizing in the medial gray matter. This resulted in functional recovery of a directed forepaw retrieval task.

Transplants of cultured olfactory mucosal cells did not bridge the CST lesion, and we did not observe any signs of regeneration of the cut axons. Despite the absence of fibers crossing the lesions, there was a degree of functional recovery of forepaw retrieval, although less than in the rats with bulbar cell transplants (25). While we do not have a formal demonstration of the anatomical basis of this recovery, the most likely cause would seem to be that, in the absence of reconnection of the severed fibers, the transplanted

mucosal cells have induced local sprouting in surviving intact tissue—"plasticity"—and that this has established adventitious pathways (1,18), which substituted functionally, at least in part, for the severed tract (22).

To determine whether the additional benefits of bulbar cells are due to regeneration, we therefore sought a lesion/transplant situation where a specific functional outcome was known to be dependent on regeneration. This was a complete unilateral section of the four dorsal roots from C6 to T1 in the rat. This injury completely abolished all categories of sensory input from the ipsilateral forepaw and resulted in a complete and permanent loss of the use of the deafferented forepaw for grasping the bars in a climbing test (8). For the performance of this test, plasticity in the remaining parts of the nervous system was unable to compensate functionally for the absence of the specific proprioceptive sensory information needed for grasping.

Reapposition of the central ends of the cut roots to the spinal cord via a matrix containing cultured bulbar cells resulted in a recovery of forepaw grasping function on the climbing frame. This was associated with recovery of electrical transmission from the peripheral nerves to the spinal cord and brain stem. Histology showed that transplanted OECs interleaved with an outgrowth of astrocytes from the surface of the dorsal horn of the spinal cord. This formed a complete cellular bridge between the cut ends of the dorsal roots and the spinal cord. Along this bridge, dorsal root axons regenerated through OEC tubes, reentered the CNS arborized in the gray matter of the dorsal horn, and ascended in the dorsal columns to reach the cuneate nuclei (7).

In the present study, we report a repeat of this experiment but used cells cultured from mucosal biopsies. The culture procedure was similar to that used to obtain the cells from the olfactory bulb biopsies. This clearly showed that transplants of cells cultured in this way from the olfactory mucosa did not exert the same reparative effect as those from the olfactory bulb. Cells from both preparations survived transplantation and attached themselves to the cut surface of the central stump of the severed dorsal root. However, histology showed considerable differences. In the case of the mucosal tissue, the transplanted cells, identified by GFP fluorescence, formed a closed cap over the dorsal root stump. The astrocytes covering the surface of the spinal cord failed to respond. No cellular bridges were formed, and the "door" to the CNS remained closed. None of the 25 rats with the mucosal transplants showed any sign of functional improvement in the climbing test.

These observations suggest that the superior benefits observed in the patient receiving bulbar cell cultures were due to regeneration of severed fibers across the injury site. This is supported by the clinical pattern of ongoing temporal and spatial recovery of movement and sensation (unpublished data).

Composition of the Cultures

There are a number of possible reasons for the difference between bulbar and mucosal cultures (7,13,23, 25,26). The most striking difference is the much lower proportion of OECs in the mucosal cultures. In both bulbar and mucosal cultures, the cells were harvested once the cultures achieved confluence [e.g. (2,19)]. The point of confluence for both bulbar and mucosal cultures is reached at 14–16 days. Beyond that point, the cultures become overgrown by fibroblasts, and the cells start to detach from the dish and degenerate. In the case of the bulbar cultures, the point of confluence is reached with approximately equal numbers of OECs and olfactory nerve fibroblasts (11). However, in the case of the mucosal cells, both the rat cultures and those of the three patients of the clinical study (23) achieve no more than 5% OECs.

In terms of accessibility for clinical use, the intranasal approach to the mucosa is less invasive than the intracranial approach to obtain bulbar tissue (5). The question therefore arises as to whether there is a procedure that can be used to obtain reparative cells from mucosal biopsies. Clearly, one of the first possibilities for improving the reparative effectiveness of mucosal transplants would be to enrich the number of OECs. This will entail further steps, such as cell separation, and added growth factors. Each added step will need to be controlled in a separate series of in vivo experiments, and each will necessitate individual safety validation before clinical application.

Our own studies suggest that, at least for bulbar tissues, purification of OECs [to the exclusion of fibroblasts (9)] greatly reduced their ability to engraft (Y. Li and G. Raisman, unpublished data). McKay-Sim and colleagues obtained a high degree of enrichment and purification of mucosal OECs using the growth factor neurotrophin-3 (NT3) in the medium (3,6). More recent studies have suggested that mucosal OECs purified using a transforming growth factor-α (TGF-α) protocol have benefit in a model of spinal cord injury in the rat (16). To the best of our knowledge, there has been no report of true recovery in patients transplanted with purified OECs.

CONCLUSION

Both laboratory observations and an ongoing clinical trial indicate that to obtain the optimal anatomical and functional repair the superiority of transplantation of cells obtained from the olfactory bulb over those obtained by current culture techniques from biopsies of the olfactory mucosa is due to their ability to mediate regeneration of severed axons across the injury site. There are several ongoing attempts to modify mucosal cells or indeed discover other cell types that can be used to repair spinal cord injuries.

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