

Autologous Olfactory Glial Cell Transplantation Is Reliable and Safe in Naturally Occurring Canine Spinal Cord Injury

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ABSTRACT

Intraspinal transplantation of olfactory glial cells (OGC) has produced well-defined beneficial effects in experimental rodent models of spinal cord injury (SCI) and therefore has considerable promise as a treatment for severe SCI in human patients. In this study, we used clinical canine cases of severe SCI to determine whether derivation and transplantation of OGC from an autologous source was feasible. From the nerve fiber layer of a single olfactory bulb, we were able to generate 5×10^6 cells from each patient within 3 weeks. Of this population, 72% were p75⁺ OGC, 20% were meningeal cells, and the remainder mainly astrocytes. Intraspinal transplantation was not associated with any observable long- or short-term complications.

Key words: canine; olfactory glial cell; tissue culture; transplantation

INTRODUCTION

INTRASPINAL OLFACTORY GLIAL CELL (OGC) transplantation has been shown to improve functional outcome following experimental spinal cord injury (SCI) in rats and is associated with evidence of axonal regeneration across the lesion site (Li et al., 1998; Ramon-Cueto et al., 2000; Lu et al., 2002; Keyvan-Fouladi et al., 2003; Li et al., 2003a; Plant et al., 2003; Verdu et al., 2003). These findings have raised hope that it may be possible to translate this experimental intervention into a therapeutic option for treatment of severe SCI in human patients (Fawcett, 1998; Raisman, 2001; Lu and Ashwell, 2002; Santos-Benito and Ramon-Cueto, 2003).

There are many issues to be addressed in making this transition, which can be divided into two categories. First, there is a conceptual difference in relevant outcome: successful laboratory studies provide “proof of principle” by demonstrating statistical differences in outcome between

small groups of transplanted and control animals that have highly uniform injuries. In contrast, translational studies must aim to determine whether intraspinal OGC transplantation can produce a medically useful effect in large patient populations that have some variability in the degree of injury severity. Second, translational studies must direct attention to the precise methods by which OGC transplantation will be applied to human patients and focus on detection and avoidance of potential deleterious effects. For instance, there are questions to be resolved regarding selection of suitable sites, stages and severities of injury, source of cells, methods of transplantation and how best to introduce transplanted cells into a region of glial scarring. Naturally occurring spinal cord injury in the domestic dog population provides an ideal opportunity to develop both aspects of translational research in OGC transplantation (Jeffery et al., 2001).

SCI is commonly encountered in domestic dogs for two reasons: first, because they are often involved in road

traffic accidents; and, second, because many breeds are susceptible to a type of intervertebral disc degeneration that frequently results in explosive expulsion of the degenerate nucleus causing acute cord contusion (Hansen, 1952; Bray and Burbidge, 1998). White matter injury is the predominant cause of neurological dysfunction in most affected dogs because both fracture/luxations and intervertebral disc lesions occur with greatest frequency in the thoracolumbar spinal cord rostral to the lumbar intumescence (Hansen, 1952; Selcer et al., 1991). Clinical signs in dogs are similar to those encountered in human patients, and dogs that suffer severe spinal cord injury have the same poor prognosis for neurological recovery as their human counterparts. Therefore, this group of dogs accurately models many aspects of the target human population for which intraspinal OGC transplantation would be considered.

In this study, we have investigated the critical translational issues of (1) the feasibility of OGC transplantation from an autologous source; (2) production and expansion of a defined and reproducible population of transplant cells; and (3) surgical procedures by which transplanted cells can be introduced into the injured spinal cord.

MATERIALS AND METHODS

Case Selection

Choice of injury site. Transplants to improve white matter function after severe spinal cord injury in humans may be required in either cervical or thoracic regions. Because of the greater ease in management, experimental models in rats generally focus on the thoracic spinal cord. In this study, we also chose to treat only thoracolumbar spinal cord injuries (see Appendix) in order to simplify the nursing care demanded of the dogs' owners.

Choice of severe injuries. We chose severely injured cases to minimize the impact of any potential deleterious effects—if any were to occur, it would be preferable that they be in animals that had a hopeless prognosis for recovery of locomotion with conventional therapies. The population of dogs that have very severe SCI is relatively easy to identify at an early stage, since it is well established that the loss of pain sensation in the pelvic limbs is always associated with a poor prognosis (Olby et al., 2003). Absence of pain sensation associated with spinal fracture/luxation or absent pain sensation and absence of voluntary tail wag beyond 6 weeks after any injury implies a hopeless prognosis (Olby et al., 2003).

A major objective of this study was to detect any deleterious consequences of the transplantation procedure. Although the choice of a severely injured group of dogs

may have reduced our ability to detect a beneficial effect of the intervention, it should not have impaired detection of the more severe deleterious effects that could reasonably be expected, such as the development of pathological pain at the transplant recipient site.

Choice of autologous grafts. We chose to use autologous grafts because, although it would have been possible to derive OGC for transplants from adult cadavers, the requirement for immunosuppression and uncertainty regarding the survival of allograft cells in their new location would have complicated interpretation of the results. The period of *in vitro* expansion of the harvested cells also provides an opportunity to characterize the population prior to transplantation. The disadvantage of autologous grafting is the need for two operations to be carried out on each patient and carries the surgical risks inherent in the harvesting procedure, such as infection or iatrogenic injury.

Patients

This study was approved by the Ethical Committee of the Department of Veterinary Medicine, University of Cambridge, and carried out under the jurisdiction of the Royal College of Veterinary Surgeons; informed written consent for diagnostic and surgical procedures was given by the owners in each case. During imaging and both surgical procedures, each dog was subject to routine intravenous monitoring and regulation of respiratory and heart rates, blood pressure, blood oxygenation (by pulse oximetry), and end-tidal carbon dioxide. Dogs entered into this prospective study had spinal cord injury (Fig. 1) at, or adjacent to, the thoracolumbar junction, of sufficient severity to prohibit recovery of acceptable function (either because of exceptional severity or because of previous failure to recover). This study did not evaluate transplantation into normal dogs, since such a procedure would require the use of experimental dogs, rather than the clinical cases included here. Moreover, aberrant axonal sprouting, a potential cause of deleterious effects, would more probably occur in animals that have previously suffered SCI. This report concerns nine dogs; eight of these animals retained no voluntary movement of the pelvic limbs nor any detectable perception of painful stimuli applied to the pelvic limbs or tail, and were urinary and fecally incontinent. Of these eight dogs, one (case 6) had neurological and magnetic resonance imaging (MRI) evidence of extensive malacia (Fig. 2a,b) spreading both cranially and caudally from an original site of injury at the thoracolumbar junction, causing loss of much of the panniculus and pelvic limb reflexes. One dog exhibited weak voluntary locomotor activity in the pelvic limbs, but was unable to bear weight normally on



FIG. 1. Dogs selected for this clinical trial had severe thoracolumbar spinal cord injuries resulting from fracture/luxations or extruded nuclei of intervertebral discs. (A) Fracture of T12 vertebra causing instability and narrowing of the vertebral canal. (B) Transverse T1-weighted magnetic resonance imaging (MRI) scan through intervertebral disc (IVD). The spinal cord is displaced by forcefully extruded nucleus pulposus (outlined by asterisks) causing a mixed contusive and compressive lesion.

the plantar aspect of the paws; this dog was both urinary and fecally continent.

Spinal cord injuries were caused by vertebral fracture/luxation (five dogs) resulting from road traffic accidents or as a consequence of intervertebral disc extrusion (four dogs). Six cases had previously had surgery for treatment of their spinal cord injuries: decompressive laminectomy and disc removal in three dogs, and stabilization of fracture/luxations in three dogs. There was variation in the time that had elapsed between the initial injury and presentation at our clinic, ranging from 5 days to 2 years, with a median of 25 weeks. There is also published data on locomotor recovery following similarly severe spinal cord injury in dogs (Olby et al., 2003; Naito et al., 1990) that permitted comparison with “historical controls.”

Imaging

Previously obtained radiographs of the vertebral column were available for all but one dog, together with MRI scans of the affected spinal cord in one case. Prior to transplantation, we obtained sagittal and transverse T1W, T2W, and post-gadolinium MRI images (Fig. 2) of the affected area in all but one dog (which contained a large ferromagnetic implant to stabilize a spinal fracture), using a 0.2-Tesla permanent open magnet system (Esaote).

Surgical Approach. 1. Cell Harvesting

Transnasal approach. The nasal bones were incised along the midline and laterally on the right hand side, extending caudally to include the margins of the frontal sinus (Fig. 3). The cortical bone of the outer table of the frontal bone was separated and elevated caudally, but the periosteum was left intact rostrally to act as a hinge. The inner table of the frontal bone and the cribriform plate

were excised using a combination of air-powered high-speed burr (3M) and rongeurs. Lateral exposure was sufficient to expose the fissure between the rostral aspect of the frontal lobe and the olfactory bulb. The right olfac-

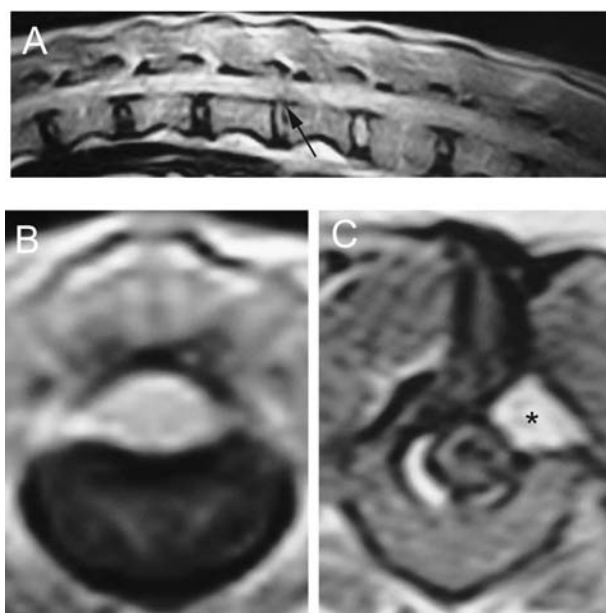


FIG. 2. Representative pre-operative magnetic resonance imaging (MRI) scans. (A) T2W mid-sagittal MRI image of acute spinal cord injury caused by extruded intervertebral disc nucleus (arrowed). (B) T2W transverse image of same spinal cord; the widespread cord hyperintensity visible on both sagittal and transverse images correlates with extensive myelomalacia. (C) T1W transverse image of another individual, showing extensive intramedullary fluid (syringohydromyelia) associated with cord injury caused by an extruded intervertebral disc 10 months previously. A free fat graft (*) was placed during previous decompressive surgery.

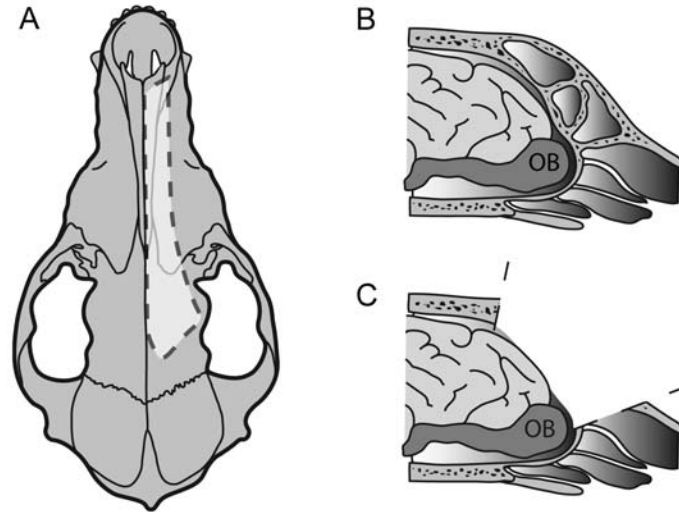


FIG. 3. Diagrammatic representation of surgical access for cell harvesting. (A) A bone flap overlying the frontal sinus and nasal cavity was elevated from caudal to rostral. (B) Sagittal section through cranium illustrating the olfactory bulb (OB) lying rostro-ventral to the frontal lobes of the cerebrum. (C) The inner table of the frontal bone was removed to expose the olfactory bulb, and the fissure between the olfactory bulb and the frontal lobe.

tory bulb was separated using a blunt probe, excised, and placed into tissue culture medium. The bone deficit overlying the frontal lobe was closed using a piece of temporal muscle fascia, and the bone flap replaced and sutured into position using PDS. Methadone (0.3 mg/kg four times daily) was administered to each dog for the first 48 h following surgery, followed by the non-steroidal anti-inflammatory drug carprofen (2 mg/kg twice daily) for a further 5 days.

Isolation and preparations of canine olfactory glial cells (OGC). Following excision of the olfactory bulb, the large and easily identifiable nerve fiber layer of the bulb was dissected free of connective tissue and blood vessels, and dissociated to a single cell suspension using enzymatic digestion and physical disruption. The cells were placed into culture without any further cell purification process.

For enzymatic treatment, the tissue was first chopped with a scalpel blade in a Petri dish into approximately 0.25–0.5-mm pieces to increase the reactive surface for enzymatic digestion. All tissue pieces were transferred into a 0.5-mL collagenase solution (6.66 mg/mL in L-15; 155 U/mg, Sigma, UK) and incubated for 15 min at 37°C followed by addition of 0.5 mL of trypsin (2.5 mg/mL, 10,000 BAEE; Sigma, UK) solution for a further 15 min. The enzymatic activity was then inhibited by the addition of 1 mL of soybean trypsin inhibitor solution (0.25 mg/mL, 10,000 BAEE; Sigma, UK) containing bovine pancreas DNase (0.04 mg/mL, 2000 Kunitz Unit; Sigma,

UK) and bovine serum albumin (3 mg/mL; Sigma, UK) diluted in L-15, followed by a mechanical trituration in the same solution.

The mechanical dissociation was carried out by triturating the tissue five times through a 5-mL blow-out pipette, then once through a 21-gauge needle and a 23-gauge needle using a 2-mL plastic syringe. The homogenate was centrifuged at 1000 rpm for 5 min in DMEM-based medium containing 1% FBS.

The cells were then plated (10,000–50,000/mL) onto PLL-coated 25-cm³ flasks and grown in DMEM containing 10% FBS (Gibco Life Sciences, Paisley, Scotland), 2 μM forskolin (Sigma, UK) and 20 ng/mL Heregulin (R&D Systems, UK; defined growth medium) to selectively induce proliferation of glial cells rather than the contaminating fibroblasts or meningeal cells.

After 7–10 days *in vitro*, a confluent monolayer of cells had been established, overlain with a population of small, loosely attached surface dwelling cells. A proportion of these loosely attached cells, predominantly contaminating cells with phenotypes resembling microglia and oligodendrocyte precursor cells inadvertently dissected along with the olfactory nerve fiber layer, had become detached from the monolayer whilst washing in Mg/Ca-free Hank's medium at their first passage due to their relatively low adhesiveness to the substrate compared to other cells. The cells from each flask were then passaged weekly into T75 flasks to encourage their proliferation and generate sufficient cells for transplantation, cryopreservation, and subsequent analysis.

Immunocharacterization of the canine OGC population. At weekly passages, aliquots of each canine culture were plated onto three separate PLL-coated coverslips and cultured for another day in growth medium, then processed for immunocytochemistry as described previously (Lakatos et al., 2000; Smith et al., 2002). Briefly, coverslips were gently removed from the 24-well plates and washed four times by dipping into universal tubes filled with PBS. For surface antigen-labeling, a series of primary antibodies were applied on living cells for 45 min. This was followed by the incubation with class specific goat anti-mouse fluorescein or rhodamin/Cy3 conjugated secondary antibodies diluted in the same buffer (1:100) for a further 45 min. Cultures were fixed in methanol (at 20°C) prior to intracellular antigen labeling (anti-GFAP, anti-fibronectin), followed by an incubation with secondary antibodies conjugated with a different fluorescein than those previously used for surface antigen labeling.

All antibodies were diluted in the “washing-staining” solution containing 5% FBS, 0.01 M HEPES in DMEM (Gibco Life Sciences, Paisley, Scotland). All cell washes began with draining the coverslips on paper and were carried out following each staining step during the protocol four times by dipping the coverslips into universals containing a “washing-staining” solution. Following the final washes, the coverslips were rinsed once with distilled water and mounted with 4', 6-Diamidino-2-phenylindole (DAPI)-containing Vectashield mounting medium for fluorescence (Vector Laboratories Inc., CA). Finally, the edges of the glass coverslips were sealed using nail varnish.

1. *Primary antibodies.* Anti-GFAP (1:100, cow, polyclonal; DAKO, UK), anti-fibronectin (1:400; human, polyclonal; DAKO, UK), anti-p75 (1:100, mouse, monoclonal, IgG1; Chemicon, UK), anti-PSA-NCAM (1:200, mouse, monoclonal, IgM; Chemicon, UK), anti-A2B5 (1:1, mouse, monoclonal, IgM subtype; gift from M. Noble: Eisenbarth et al., 1979), O4 (1:1, mouse, monoclonal, IgM; gift from M. Noble: Sommer and Schachner, 1981), anti-CD11b (OX-42, 1:100, mouse, monoclonal, IgG2a; Serotec, UK)

2. *Secondary antibodies.* Anti-mouse IgG/IgM fluorescein-conjugated (1:100, goat; Southern Biotechnology, Cambridge, UK) and Cy3-conjugated antibodies (1:300, goat; Jackson Immunolaboratories), anti-rabbit Cy3-conjugated antibody (1:300, goat; Jackson Immunolaboratories).

Statistical analysis. The number of cells stained with each antibody was counted, and the results from three separate experiments were expressed as a mean percent-

age of the total number of cells. Two-tailed *t*-test was used for comparison in cell culture bioassays. For comparing more than one group, the one-way ANOVA test with Tukey's post hoc analysis was performed. The *p*-values below 0.05 are significant. In order to compare the values between groups, data was expressed as mean \pm standard error of the mean (SEM). All experiments were performed at least in triplicate.

Surgical Approach. 2. Transplantation

The affected area of spinal cord, as defined by MRI or implied by radiographic images, was exposed through a conventional dorsal laminectomy, using rongeurs and air-powered instruments. Along the length of the affected spinal cord, which was often apparent because of discoloration visible through the dura, a mid-sagittal myelotomy incision was made (Teague and Brasmer, 1978). In some cases, the cord contained a large fluid-filled cyst, and clear fluid (CSF) leaked through the incision. In one case (case 8), cells were injected into the cord without a prior myelotomy incision.

The transplant syringe (100 μ L Hamilton) was filled directly from the tissue culture medium containing the suspended cell population that had been triturated and treated with trypsin and DNase to produce a single cell suspension. The total volume of medium transplanted was 100 μ L, containing a total of 5×10^4 cells/ μ L and divided into 10 individual injections. This concentration of cells was chosen because it has previously been used successfully for transplants into rat spinal cord (Lakatos et al., 2003), and greater concentrations incur the risk of needle blockage; the volume was calculated by “scaling up” the number of cells used successfully in rats (Ramon-Cueto et al., 2000) to the larger volume of the cord lesions in dogs. At each injection site, the needle tip was introduced into the spinal cord parenchyma sufficiently deep to include the whole of the bevel. Each injection was made over a period of 60 sec, and the needle was left in place for a further 2 min at each site (Fig. 4). Injections were made at regular intervals at various dorsoventral sites throughout the length of the myelotomy incision on both sides, followed by a single injection of 10 μ L of cell suspension injected into the gap between the two sides of the myelotomised spinal cord.

Following cell injections and control of any remaining haemorrhage, the site was closed by placement of a free fat graft, taken from the lateral aspects of the incision, over the exposed spinal cord, followed by routine closure of the epaxial muscle fascia. Each dog received methadone by intramuscular injection for 48 h, followed by non-steroidal anti-inflammatory drugs for a further 5 days.

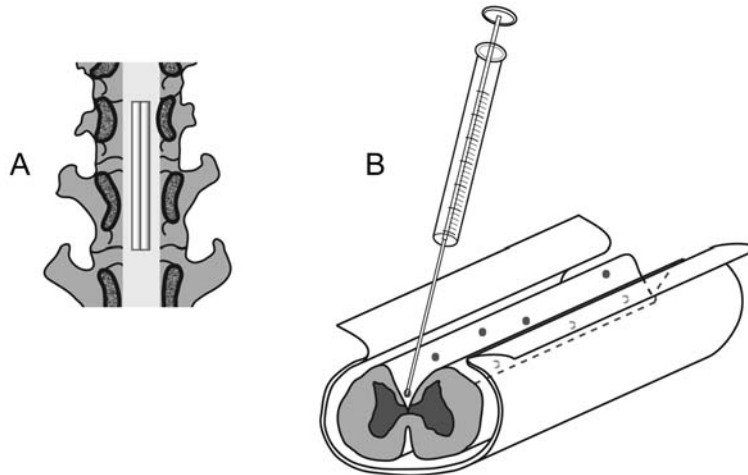


FIG. 4. Diagrammatic illustration of the transplant procedure. (A) Following a dorsal laminectomy, the dura was incised and retracted. (B) The previously injured region of spinal cord was incised to approximately half its depth using a razor blade. Cell suspension was injected into all funiculi of the spinal cord at various depths and several sites throughout the length of the myelotomy.

Behavioral observations. It was not an aim of this study to accrue detailed quantitative observations, and those reported here may be subject to bias because of the impossibility of “masking” the observer and, especially, the owners of the treated dogs. However, at monthly re-examinations, we made observations regarding possible deleterious effects, such as (i) donor site pain, (ii) pathological pain at the recipient site, and (iii) sepsis. Pain at the donor site was estimated by pressure during manual examination; pain at the transplantation site was evaluated using careful needle pressure on the skin of the dorsum; cardinal signs of infection were evaluated routinely.

Locomotor activity of each dog was examined by two methods: (i) observation as it walked “overground,” permitting a Basso-Beattie-Bresnahan (BBB) score to be attributed; and (ii) observation of treadmill locomotion with hindquarter support so that pelvic limb stepping could be observed more closely.

RESULTS

Surgical Outcome

During the first 2–3 days following harvesting surgery, a small volume of serosanguinous fluid flowed from the right nostril. Each dog was keen to eat within a few hours of surgery, and there was no discernible effect on olfactory acuity at any stage after this procedure (i.e., dogs continued to explore their environment by frequent sniffing and did not become anorexic). As expected, there was mild discomfort on deep palpation of the nasal incision

until bone healing was complete. However, the dogs did not appear to suffer undue pain following surgery and did not exhibit signs of frontal lobe (or other neurological) deficits. Sutures were routinely removed at 10 days, and the cosmetic outcome was acceptable throughout all phases of healing. There was an uneventful recovery following the transplant procedure, similar to following laminectomy for other spinal problems. None of the operated cases showed weight loss or any evidence of either donor or recipient site pain, during a prolonged follow-up period (up to 2 years). Three dogs (two of which had sustained their spinal injuries during road traffic accidents) exhibited seizures within 3 months of the harvesting procedure, which were easily controlled using a routine anticonvulsant (phenobarbital). A repeat MRI examination of the transplanted region was obtained on one dog, revealing that an intramedullary fluid accumulation at the site of injury (interpreted as a syrinx on the pre-operative scan) had been obliterated (Fig. 5).

OGC Cultures of Reproducible Composition Can Be Obtained from Adult Dog Olfactory Bulb Biopsy

Initially the harvested cell population contained mainly myelin debris and some strongly adhesive cells such as flat fibroblasts. OGCs only began to attach to the substrate a few days prior to establishment of the culture at around day 7 *in vitro*; therefore, the unpurified OGC cultures (Lakatos et al., 2003; Li et al., 2003b) were characterized only at days 7, 14, and 21. Previously plated sister cells of transplanted cells were characterized by

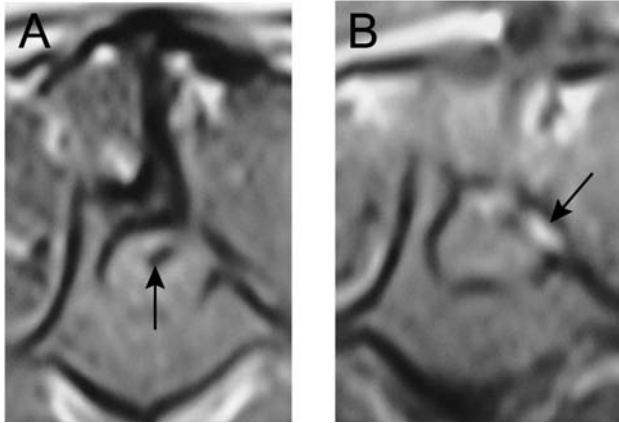


FIG. 5. Obliteration of a pre-operative syrinx was observed after cell transplantation in one dog. (A) Pre-operative transverse T1W magnetic resonance (MR) image of dog 8 showing a syrinx (arrow) in the damaged region of cord. The vertebral spinous process is still visible, but the pedicle has been excised on the right hand side during previous decompressive surgery. (B) Post-operative transverse T1W magnetic resonance (MR) image of the same dog 2 months after intraspinal olfactory glial cell (OGC) transplant. The spinous process has been excised to expose the dorsal aspect of the cord for intraspinal injection of OGCs and a free fat graft is visible (arrow).

morphology, and by qualitative and quantitative measures of immunostaining, using a series of antibodies identifying astrocytes (GFAP⁺⁺⁺), olfactory ensheathing cells (p75⁺⁺/GFAP^{diffuse}), microglia (OX42⁺/GFAP⁻), oligodendrocyte progenitor cells A2B5⁺/psa-NCAM⁺, and meningeal cells/fibroblasts (FN⁺⁺⁺/GFAP⁻; Fig. 6).

Analysis revealed that cultures obtained from different individual dogs were very similar in their cell constituents and did not change significantly during the

whole period *in vitro*. The transplanted populations consisted mainly of OGCs ($72.55 \pm 5.38\%$) and meningeal cells/fibroblasts ($19.26 \pm 3.28\%$) with a small proportion of contaminant cells, such as astrocytes (6.37 ± 1.6), oligodendrocyte progenitor cells (2.1 ± 1.28), microglia ($<1\%$), and a very small proportion of unidentified cells (Fig. 6). Following a 3-week period in culture, sufficient numbers of cells ($5.22 \pm 0.42 \times 10^6$) were available for autologous transplantation.

Behavioral/Functional Observations

BBB score. Upon admission to the study, eight dogs showed no motor or sensory function to the pelvic limbs (the equivalent of ASIA grade A for human patients), correlating with a BBB score of 0; the remaining dog, being able to move two joints on both hind limbs, scored 3 (Basso et al., 1995). During the period of follow-up, there was improvement in pelvic limb function in all except one operated dog, as summarized in Table 1; two dogs were scored at 10 on the BBB scale (occasional weight-supported plantar steps; no forelimb/hindlimb coordination). Four other operated cases scored between 1 and 8 during the 6–12-month period following transplantation. The remaining transplanted dog, which did not improve, had very severe and extensive injury to the lumbar spinal cord including the grey matter of the lumbar intumescence that caused loss of pelvic limb reflexes and absence of the panniculus reflex on the skin caudal to the thoracic limb girdle. Case 9, which originally had some movement of two joints of the pelvic limbs, attained a BBB score of 10 by 2 months post-operatively.

Stepping movements. In seven of the eight transplanted dogs the owners described increased stepping movements in the pelvic limbs that could be observed during swimming or walking. Notably, several cases showed air-step-

TABLE 1.

Dog	Age (years)	Weight (Kg)	Interval	Injury type	Injury location	BBB-initial	BBB-post	Notes
1	6	10.2	5 days	Fracture	T12/L13	0	10 (six months)	Transected spinal cord–pelvic limb swimming
2	7	11.2	2 years	Fracture	T11/L12	1	8 (one year)	Bilateral orthopedically impaired hip motion
3	4	29.6	10 weeks	Fracture	T13/L1	0	4 (three months)	
4	6	9.6	10 months	Disc	T13/L1	7	10 (three months)	
5	13	23	8 months	Disc	T12/L13	0	1 (two months)	Return of pain perception
6	5	4.4	6 days	Disc	L1/2	0	0 (11 months)	Extensive malacia
7	9	22	10 weeks	Fracture	T12/L13	0	2 (two months)	
8	7	8.2	10 months	Disc	T13/L1	3	10 (two months)	Continent before and after surgery

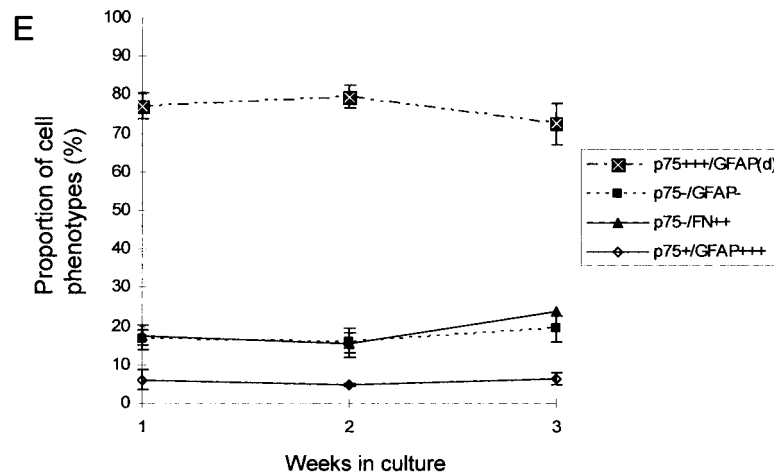
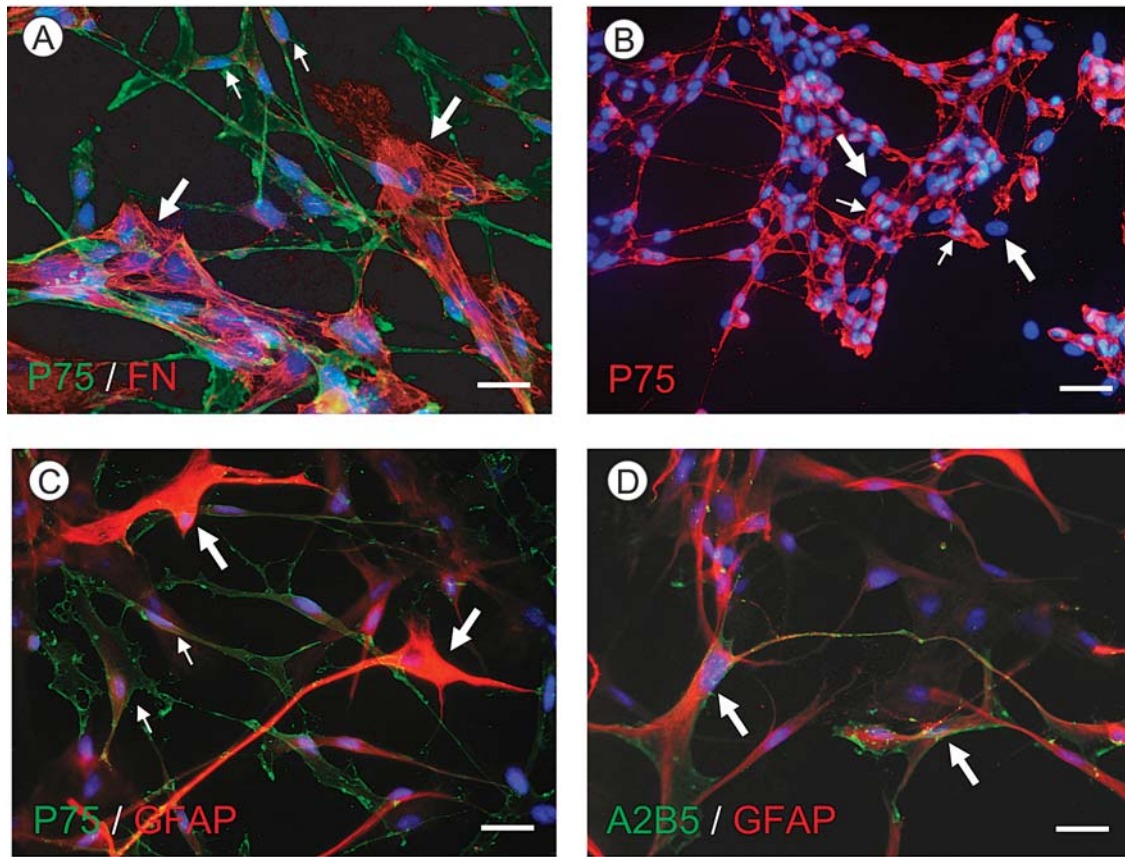


FIG. 6. Antigenic properties of olfactory glial cells (OGCs) *in vitro*. Bar = 25 μ m (A,C,D), 50 μ m (B). (A) OGCs (small arrows) express p75 receptor (fluorescein) and show faint immunoreactivity to fibronectin antibody (Cy3); they can be clearly differentiated from strongly fibronectin immunoreactive/p75-negative meningeal fibroblasts (large arrows). (B) 4', 6-Diamidino-2-phenylindole (DAPI) staining demonstrates the small ovoid nuclei of OGCs (small arrows, p75⁺), which can be differentiated from the large, less dense nuclei of the meningeal cells (large arrows). (C) OGCs (small arrows) exhibit faint perinuclear glial fibrillary acidic protein (GFAP) immunopositivity (Cy3), but this is clearly distinguished from the strong, generalized GFAP positivity of the astrocytes (large arrows). (D) Other contaminant cells, such as A2B5⁺/GFAP⁺ oligodendrocyte progenitor cells (large arrows), were observed in small numbers at all stages of olfactory bulb culture. (E) The proportion of different cells at different time points during olfactory nerve layer cell culture. No significant changes were seen over the 3-week culture period in the relative number of various phenotypes; the proportion of OGCs (p75^{+/+}/perinuclearGFAP) was constant over the culture period and also similar in all preparations derived from different animals (~76%). Thus, OGCs were not overgrown by the major contaminants such as meningeal cells (~17%, FN^{+/+}/p75⁻, or p75⁻/GFAP) or astrocytes (~5%, p75^{+/+}/GFAP⁺⁺⁺).

ping movements in the pelvic limbs within several days of surgery. On a treadmill, 7/8 cases were able to make stepping movements with the pelvic limbs, although these were weak in two dogs and in all animals appeared to be poorly coordinated with forelimb movements (although this will require detailed quantification). All but one dog in this series showed very prominent shifting of weight onto the thoracic limbs, which was frequently accompanied by stepping movements of the pelvic limbs. With prolonged time after surgery, these walking movements became more prominent and more easily elicited by stimuli such as brushing the dorsum of the paws, or placing the animal onto a treadmill.

Bladder/Bowel Function

Two owners reported that their dogs would bark, and one further case would seek to go outdoors, when they required their bladders to be emptied. There was no return of true fecal or urinary continence in any animal.

Pain Sensation

Although all but of the dogs that presented with total sensory loss recovered "normal" paw knuckling responses (i.e., correcting weight-bearing from the dorsal to the plantar surface of the paw), only one dog regained any pelvic limb or tail sensation. At two months following transplantation, crushing stimuli to the digits of both pelvic limbs and the tail were clearly perceived in this individual (the dog cried in pain). However, there was no evidence of alteration in sensory level on the dorsal skin of any dog (i.e., the panniculus reflex was initiated at the same cranio-caudal level following surgery).

DISCUSSION

Harvested Autologous OGC Can Be Maintained in Vitro and the Population Expanded without Changes in its Constituents

For OGC transplantation to have a future as a realistic clinical therapy, it is necessary that sufficient numbers of cells can be readily and reliably generated. We demonstrate here that use of an autologous source is feasible, since the harvesting and proliferation techniques proved very reliable and reproducible. In each patient we were able to harvest small volumes of nerve fiber layer from one olfactory bulb and expand the population *in vitro* to generate ample cell numbers for transplantation. We calculated the required cell number on the basis of "scaling up" from that used successfully in rodent experiments to the larger volume of the canine spinal cord lesions. Although only small numbers of OGC were de-

tectable initially, during the 3-week *in vitro* period the total cell number expanded to $\sim 5 \times 10^6$. The transplant cell population was readily definable using immunocytochemistry and proved to be remarkably similar between individual dogs, with the proportion of OGCs remaining constant at $\sim 72\%$. Such reproducibility provides a strong basis for accurate and reliable functional analysis following transplantation.

There Is No Evidence of Detrimental Effects following OGC Transplantation

There are several detrimental effects that could be hypothesised to occur following autologous OGC transplantation depending both on the nature of the procedure itself and as a result of the axon growth enhancing effects attributed to OGC. A myelotomy incision was made (in 7/8 dogs) as a means of mechanically disrupting the mature glial scar that was present in many of these cases and to facilitate accurate cell injection. This procedure did not appear to be unduly injurious in that there was no evidence of a change in sensory level in the dorsal skin. Direct injection of OGC into the spinal cord in one dog (case 8) also appeared to be well-tolerated; this case retained both urinary and fecal continence and exhibited locomotor improvement during the following 2-month post-operative period.

Pathological pain has often been suggested as a possible consequence of interventions that evoke axonal sprouting, since that could lead to aberrant connections being made in the pain-processing pathways. However, there has been no evidence of such an effect generated by transplanted OGC in previous rodent models and in this clinical model there was also no evidence of development of pathological pain, even during a prolonged period of follow-up. Therefore, we consider that the procedure is safe.

Is There Evidence of Transplantation-Mediated Benefit?

There is some evidence to suggest that there may be a beneficial effect on locomotion, since all but one operated cases showed an increase in stepping movements in the pelvic limbs as measured by the BBB scale from the date of transplantation. Although "spinal walking" (i.e., walking presumed to be generated from reflex movements in which the pelvic limbs "walk" without instruction from cranial structures) has been long reported in dogs, its true incidence is unknown since (a) few experiments have been carried out in spinalized dogs and (b) clinical cases of spinal cord injury do not necessarily have completely severed spinal cords. In a report on a series of dogs that had lost all pain perception and motor func-

tion following a variety of injuries there was no recovery of walking in any animal that did not show a voluntary tail wag at one month after injury (Olby et al., 2003). Similarly, in an experimental study spinalized dogs required a prolonged period of post-operative recovery (<10 months) before they were able to walk unsupported on a treadmill (Naito et al., 1990). Therefore, the recovery of locomotor activity in the transplanted animals in our current case series appears to be considerable quicker than that observed in historical controls. The restoration of the paw knuckling response, usually considered to be dependent on conscious proprioceptive pathways, appears to be a facet of the plastic responses that occur in the independent distal part of the spinal cord following severe injury. In contrast, the restoration of pain perception from the tail of one animal must be the result of sensory information crossing the spinal cord injury site and is therefore of great significance.

This study is a Phase I trial and has generated data that would suggest that there is sufficient justification for progressing to a Phase II trial, to seek evidence of efficacy in comparison with control cases. In the current trial we selected cases that were deemed to be beyond all hope of recovery with conventional therapy. This is a reasonable choice when carrying out a Phase I trial in which we seek evidence for deleterious effects. However, this population may not be the best to select when seeking evidence for efficacy. Thus, it would be rather unrealistic to imagine that such severely injured animals would be able to recover a normal repertoire of locomotor activity with this single intervention.

Functional recovery after spinal cord injury is very complex, depending on inherent plastic responses and environmental effects. Although it has been shown that a second spinal transection abolishes improvement of locomotion following intraspinal OGC transplantation (Lu et al., 2001), implying that they contribute to recovery by promoting axonal regeneration, they could also influence many other naturally occurring plastic responses. In addition repeated physiotherapy and treadmill walking may have beneficial effects on outcome independently of any transplant-mediated effect. Therefore in Phase II trials it will be necessary to modify the behavioural assessment to include (i) more sophisticated methods of quantification of locomotion; and (ii) inclusion of cases that may have a better chance of recovery than the population we have hitherto treated. It is well recognized that, in dogs, following acute extrusion of an intervertebral disc the loss of “deep pain” sensation (i.e., absence of any discernible response to severe crushing of the digits, tail or pelvic limb bones) is associated with a poor, though not hopeless, prognosis (Scott and McKee, 1999). Thus, this group of patients would be eminently suitable for in-

vestigation of a therapy that has been shown to be non-detrimental but has potential for improving outcome. Comparison of outcome between transplanted and non-transplanted dogs in this category would provide clear evidence of whether the procedure was efficacious.

Value of Naturally Occurring Canine Spinal Cord Injury Patients

This study provides evidence for the validity of using naturally-occurring canine spinal cord injury in evaluating potential therapies for human patients. The results of OGC transplantation into the canine patients reported here are less dramatic than those reported in experimental rodent models of spinal cord injury. Several explanations are possible, many of which have importance in translation from the laboratory to the clinic. One explanation is that the effect of transplantation is sufficiently small in magnitude that it is lost in the “noise” associated with clinical variability in injury—an effect that would have clear implications in treatment of human patient populations. Secondly, it is possible that the dog, despite sharing a quadrupedal gait with rodents, has gait control mechanisms that more closely resemble humans rather than rodents. This is an important issue with regard to modelling, since although it would be anticipated that spinal cord injury in primates may model human spinal cord injury most closely the use of primates is restricted in many countries and the use of dogs for “screening” purposes would be valuable.

Implications for Human Transplants

It is currently unlikely that autologous OGC transplants derived from the olfactory bulb would be considered for human patients, because of the risk of brain injury during the harvesting procedure. Nevertheless, by showing that autologous transplantation is feasible and that the transplantation procedure itself is non-injurious, this study provides information that will aid in design of future human trials.

APPENDIX

Important comparative features of canine spinal cord anatomy, physiology, and pathology:

- The dog has seven lumbar and thirteen thoracic spinal cord segments
- The spinal cord terminates in the fifth lumbar vertebra
- The patellar reflex is controlled through the fourth and fifth lumbar spinal cord segments and associated peripheral nerves

- Reflexes mediated by the sciatic nerve (such as the flexor reflex) are routed through spinal cord segments L5, L6, L7 and S1 and the associated peripheral nerves
- The panniculus reflex is mediated via segmental afferents of thoracic (13 segments) and 1st lumbar spinal cord segments, which innervate the skin over the dorsum; the efferent reflex response is mediated via C8 spinal cord segment and ventral nerve root.
- Intervertebral disc degeneration in chondrodystrophic breeds of dog leads to mineralisation of the nucleus and can result in nuclear extrusion. This is frequently an explosive event that causes severe cord contusion

Dogs in this series all had lesions between T3 and L3—which, since this region is cranial to the femoral and sciatic afferents and efferents, implies that the hindlimb reflexes were all intact.

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