Identification of a human olfactory ensheathing cell that can effect transplant-mediated remyelination of demyelinated CNS axons

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Summary
The olfactory ensheathing cell (OEC) has attracted much interest recently because of its potential for transplantation-based therapy of CNS disease. Rat OECs are able to remyelinate demyelinated axons and support regeneration of damaged axons. Although OECs can be grown readily from the rat, a macromastic species, it has been uncertain whether it would be similarly straightforward to obtain these cells from the human, a microsmatic species with a relatively poorly developed olfactory system. In this study, we have identified a human OEC which shares many properties with its rat counterpart, including expression of the low-affinity nerve growth factor receptor (L-NGFr) and similar growth factor requirements. Purified populations of human OECs obtained by selection with L-NGFr antibodies have extremely high viability in tissue culture, and are capable of remyelinating persistently demyelinated CNS axons following transplantation into experimentally induced demyelinating lesions in the rat spinal cord. Thus, the human OEC represents an important new cell for the development of transplant therapy of CNS diseases.

Keywords: rat; transplantation; demyelination; growth factors

Abbreviations: ACM = astrocyte conditioned medium; BrdU = bromodeoxyuridine; BS = Bottenstein and Sato; DMEM = Dulbecco’s modified Eagle’s medium; EB = ethidium bromide; FCS = foetal calf serum; GFAP = glial fibrillary acidic protein; hOEC = human olfactory ensheathing cell; L-NGFr = low-affinity nerve growth factor receptor; OEC = olfactory ensheathing cell; rOEC = rat olfactory ensheathing cell; VWF = von Willebrand’s factor; X-EB = X-irradiated ethidium bromide

Introduction
Since the time of Cajal and Tello, much effort has been invested in attempting to provide a favourable environment for the regeneration of damaged CNS axons by transplanting Schwann cells from the peripheral nervous system, where axon regeneration occurs with much greater efficiency. Another region of the nervous system where glia support axonal regeneration in adulthood is the olfactory system (Graziadei et al., 1978; Graziadei and Graziadei, 1979). Here, olfactory ensheathing cells (OECs) permit growing axons from neurons of the nasal cavity olfactory mucosa to re-enter the olfactory bulb of the brain and form synapses with second-order neurons (Doucette, 1984). Recent studies have shown that rodent OECs are able to support axonal regrowth when transplanted into experimental models of spinal cord injury (Li et al., 1997; Ramon-Cueto et al., 1998) and are also able to form myelin sheaths around regenerated or demyelinated axons, thereby permitting rapid saltatory conduction to occur (Franklin et al., 1996; Li et al., 1997; Imaizumi et al., 1998; Ramon-Cueto et al., 1998). It has therefore been proposed that OECs may be suitable cells for transplant-mediated repair of spinal cord trauma or non-repairing foci of demyelination (such as may occur in chronic multiple sclerosis). These data indicate that transplanted OECs have a repair repertoire that is similar to that of...
Schwann cells, but may have advantages over these because of their ability to migrate and integrate within areas of astrocytosis that are characteristic of damaged CNS (Franklin and Barnett, 1997; Ramon-Cueto et al., 1998).

A key issue regarding their therapeutic potential is whether cells similar to those obtained from rat exist in the human olfactory tissue and whether or not they can be grown in tissue culture. Although OECs can be grown readily from the rat (Ramon-Cueto and Nieto-Sampedro, 1992; Barnett et al., 1993), which has a highly developed olfactory system, it is by no means certain whether it is similarly straightforward to obtain these cells from humans, who have a relatively poorly developed olfactory system. In this study, we demonstrate (i) that OECs, isolated from resected adult human olfactory bulb, can be grown in tissue culture with a high viability, and (ii) that human OECs (hOECs) have the ability to form new myelin sheaths following transplantation into areas of persistent demyelination in the adult rat CNS.

Material and methods

Isolation and culturing of human OECs

Human olfactory bulbs were collected during surgery where removal was a necessary part of normal surgical procedure. Surgery was generally for tumour removal, although in no case did the neoplastic process involve the olfactory bulbs. All tissue collection was performed with local ethical approval including informed consent from South Glasgow University Hospitals Ethics Committee. The maximum time between resection and tissue culture was usually no greater than 1 h. In one instance, viable cells were cultured from a sample with 15 to 66 years and OECs were obtained from all samples cultured. Tissue was cleared of blood and meninges, chopped into small pieces and incubated with collagenase (ICN Pharmaceuticals Ltd, Basingstoke, UK; 13.3 mg/ml) for 10 min at 37°C. After centrifugation and resuspension, cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco Life Technologies, Paisley, UK) containing 10% foetal calf serum (FCS), fungazone (Gibco, 0.625 μg/ml) and gentamicin (Gibco, 25 μg/ml) on poly-L-lysine (Sigma, Poole, Dorset, UK; 13 μg/ml) coated flasks and dishes. Routine media supplements were based on optimal conditions for rat OECs (rOECs) as described in Franceschini and Barnett (Franceschini and Barnett, 1996) and by Pollock and colleagues (Pollock et al., 1999). These were human-specific neuregulin heregulin (HRG) β1 (R&D Systems, Abingdon, UK; 100 ng/ml); DMEM containing 10% FCS or conditioned medium from rat type-1 astrocytes (astrocyte conditioned medium, ACM) (Noble and Murray, 1984). Mitogenic response of hOECs to PDGF (platelet-derived growth factor) AA or BB (R&D Systems), bFGF (basic fibroblast growth factor; Peprotech EC Ltd, London, UK) and insulin-like growth factor (IGF)-I and -II (Boehringer Mannheim, Mannheim, Germany) were also assessed. For immunochemistry, OECs were plated on poly-L-lysine-coated coverslips. Primary antibodies were: the O4 antibody (gift of I. Sommer; 1:2 supernatant, IgM), anti-low-affinity nerve growth factor receptor, p75 (L-NGFr) (Roche Diagnostics Ltd, Lewes, UK; 1:10, IgG1), anti-von Willebrand’s factor (VWF) (Dako Ltd, Cambridge, UK; 1:50, anti-rabbit), anti-vimentin (Boehringer Mannheim; 1:50, IgG1), anti-glial fibrillary acidic protein (GFAP) (Sigma; 1:100, IgG1), anti-laminin (Sigma; 1:20, anti-rabbit), anti-fibronectin (Gibco; 1:10, anti-rabbit), anti-olfactory marker proteins (gift of F. Margolis; 1:1000, anti-rabbit) and anti-S100 (Dako Ltd; 1:100, anti-rabbit). Antibodies were visualized with the appropriate fluorochrome-conjugated secondary antibody. All secondary antibodies were purchased from Southern Biotechniques (Cambridge Bioscience, Cambridge, UK) or Jackson Laboratories (Stratatex, Luton, Beds., UK).

Purification of hOEC using L-NGFr and magnetic beads

For magnetic bead purification of OECs, 0.5 μg of mouse anti-human L-NGFr was incubated with 10⁷ Dynabeads (Dynal, Wirral, UK) and rotated for 30 min at room temperature. Bead-bound antibody was collected with a magnet, washed three times and resuspended in phosphate-buffered saline 0.1% bovine serum albumin at 4°C. Since expression of L-NGFr is trypsin sensitive (our own unpublished observation), hOECs trypsinized from culture were incubated at 37°C for 1–5 h to allow re-expression of the L-NGFr. A total of 1 × 10⁶ OECs per ml were incubated with 1 × 10⁷ beads, rotated at 4°C for 5 min and bead-bound cells were separated using the magnet. After three washes in DMEM containing 1% FCS, cells were resuspended in 200 μl of DMEM + 1% FCS, treated with DNase (250 U) and purified cells were collected and plated on coverslips to assess purity using the antibody to L-NGFr.

Measurement of cell proliferation

Cells were washed twice in serum-free modified DMEM medium (DMEM-BS, where BS represents Bottenstein and Sato) (Bottenstein et al., 1979), followed by incubation for 4 days in the appropriate culture conditions. On day 3, the cells were incubated with 20 μM bromodeoxyuridine (BrdU, Boehringer Mannheim) overnight, followed by immunolabelling with the cell surface marker, anti-L-NGFr (Roche Diagnostics Ltd), and anti-BrdU on day 4 (Harlan Sera Laboratories, Loughborough, UK) as previously described (Pollock et al., 1999).

Transplantation into areas of persistent demyelination

A focal area containing demyelinated axons was created in the dorsal funiculus of the spinal cord of adult rats using the
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Results

Characterization of cells isolated from human olfactory bulbs

Cells from human olfactory bulb were cultured using a method previously developed for culturing rodent OECs with which it was possible to establish cultures of extremely high viability (Barnett et al., 1993). Cultured cells were obtained from all of seven samples taken (Fig. 1A). Initial immunolabelling of these unpurified cultures with a range of neural markers demonstrated that 66% of the cells expressed the L-NGFr. The remaining cell population included 15% VWF-expressing endothelial cells and 11% fibronectin-expressing fibroblasts. Cells were also identified which were immunolabelled with antibodies to GFAP (71%), S-100 (61%) and laminin (76%), and all of the cells expressed the intermediate filament vimentin (Fig. 1B–G). Purer populations of OECs were obtained by selecting those cells expressing L-NGFr using L-NGFr antibody coupled to magnetic beads. Immunolabelling of these purified cultures with a range of OEC markers demonstrated that an enrichment of the primary culture of up to 25-fold could be achieved depending on the proportion of L-NGFr in the starting population of cells.

Growth factor requirements of hOECs

We next identified tissue culture conditions that induced proliferation of the sorted L-NGFr-expressing OECs. The hOEC has similar tissue culture requirements to rOECs, responding to both defined medium lacking serum conditioned by cortical astrocytes (ACM) (Noble and Murray, 1984) and DMEM containing 10% FCS (Fig. 2A). We have shown previously using rOECs that the factor in ACM required for proliferation is a neuregulin isofrom (Pollock et al., 1999). We therefore tested HRG β1 for its capacity to induce hOEC proliferation and found that 100 ng/ml HRG β1 caused a 2-fold increase in the number of BrdU+/L-NGFr+ cells compared with culturing in defined serum-free medium alone (DMEM-BS) (Bottenstein et al., 1979). Addition of 2 × 10⁻⁶ M forskolin and 100 ng/ml HRG β1 resulted in a 2.3-fold increase (Figs 2B and 3). The increase in proliferation was statistically significant for the HRG β1- and forskolin-treated cells (P = 0.087) but less so for the ACM-treated (P = 0.48) and HRG β1-treated (P = 0.29) cells. This correlates with data obtained for human and rat Schwann cells, where neuregulin is a mitogen for these cells in both species (Casella et al., 1996). However, like human adult Schwann cells, this growth factor requirement could not maintain the cells in culture for more than five passages. In addition, it was only mitogenic for a proportion of hOECs, and prolonged exposure to heregulin β1 did not result in continued proliferation, indicating that other factors are required for maintenance of proliferation of hOECs. PDGF AA or BB, bFGF, NGF, IGF-I or GGF (glial growth factor) were not mitogenic for hOECs (data not shown). When cells purified to >68% L-NGFr positive were placed in DMEM containing 10% FCS for 4 days, only 15% of the hOECs (still identifiable as such on the basis of S-100 and GFAP immunoreactivity) expressed L-NGFr. Thus, L-NGFr expression by hOECs is lost rapidly in these conditions.

hOECs remyelinate demyelinated CNS axons following transplantation

Since transplantation of OECs has been proposed as a cellular therapy for both demyelination and axonal injury in the...
Fig. 1 Immunocytochemistry of freshly dissociated hOECs. (A) Phase of dissociated human olfactory bulbs after 2 days in culture. There are many viable cells. After 3 days, a few cells express the O4 antibody, which is weak and punctate (B). Some of these also express GFAP. After 1 week in culture, the cells express the L-NGFr (C), S-100 (D), diffuse GFAP (E) and vimentin (F), and therefore have a phenotype similar to rOECs. A small proportion of cells immunolabelled with endothelial (VWF) and fibroblast (fibronectin) markers. Magnification ×242.

CNS, we examined whether hOECs were capable of remyelinating persistently demyelinated CNS axons by transplanting L-NGFr-purified hOECs into experimentally induced demyelinating lesions. These lesions were created using a standard model which involved creating focal areas of demyelination in the dorsal funiculus of the adult rat spinal cord by direct injection of a small volume of 0.1% EB (Blakemore and Crang, 1992). This procedure kills both astrocytes and oligodendrocytes in a clearly defined area, while leaving the axons largely intact and demyelinated. If EB injections are made into white matter that previously has been exposed to 40 Gy of X-irradiation, then the lesion is not repopulated by host-derived glia, and the demyelinated axons can only be remyelinated by transplanted cells (Fig. 4A). This feature of the lesion has been verified by injecting saline alone or inducing rejection of transplanted non-histocompatible cells by removal of an immunosuppressive regimen. In both situations, the lesions remain demyelinated (Crang and Blakemore, 1991; Crang et al., 1992). In an initial experiment, a suspension of cells which were 30% L-NGFr positive was injected into Sprague–Dawley rats, immunosuppressed by daily injection of cyclosporin to prevent rejection of the transplanted cells. The remaining non-L-NGFr-positive cells comprise the astrocyte-like OECs (Franceschini and Barnett, 1996), OECs that had lost L-NGFr and some endothelial cells, fibroblasts and meningeal cells. Three weeks after transplantation, hOECs were observed within the lesion associating with the demyelinating axons, but only on rare occasions were thin myelin sheaths identified. In a second experiment, a suspension containing 45% L-NGFr-positive hOECs was transplanted into T-cell-deficient athymic rats (used in preference to cyclosporin-immunosuppressed Sprague–Dawley rats, for which daily subcutaneous injections for survival periods longer than 3 weeks was not deemed acceptable) and the animals analysed after a survival period of 6 weeks. In three of the four animals killed at 6 weeks, there was evidence of more widespread remyelination of the demyelinated axons than at 3 weeks, at which time point there is extensive remyelination by rOECs (unpublished data). The extent of remyelination
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Fig. 3 Immunofluorescence of a proliferating hOEC. Human OECs were immunolabelled with anti-L-NGFr and anti-BrdU (B and D). A and C are 4',6-diamidino-2-phenylindole staining of B and D, respectively.

lesions, was the presence of numerous intermediate filament-containing cells that formed a loose network throughout much of the lesion area (Fig. 4F and G). The areas in which these cells were found frequently were rich in large diameter collagen and the axons generally remained demyelinated. The presence of these cells within the lesion identified them as being of transplant origin.

Discussion

In this study, we demonstrate firstly that OECs, isolated from resected adult human olfactory bulb, can be grown readily in tissue culture, and secondly that human OECs have the ability to form new myelin sheaths following transplantation into areas of persistent demyelination in the adult rat CNS.

Immunocytochemical profile of hOECs

To be useful in transplantation therapies, it will be necessary to grow relatively large numbers of pure hOECs and therefore purification procedures and the ability to identify these cells are very important. rOECs have been characterized by their O4, anti-GFAP, anti-L-NGFr and anti-polysialic acid (a specific epitope on the embryonic form of neural cell adhesion molecule E-NCAM) (Franceschini and Barnett, 1996) and have been termed astrocyte-like and Schwann cell-like according to their morphology and their antigenic phenotype (Pixley, 1992; Barnett et al., 1993). In serum-free media, the hOECs expressed a heterogeneous morphology.
which became more uniform with a predominantly flattened fibroblast-like morphology when cultured in DMEM-FCS. This property has been seen for rOECs (Barnett and Franceschini, 1999). The O4 antibody did not label the hOEC to the same extent as has been shown for rOECs, and cannot therefore be used to purify the cells. Although we were not able to detect E-NCAM-positive cells in these cultures, the antibodies to L-NGFr and GFAP can be used to define hOECs. hOECs can be selected for specifically using anti-L-NGFr antibodies. However, since expression of this receptor is rapidly lost, then selection based on its use must be carried out soon after cultures are prepared from olfactory bulb tissue.

**Growth factor requirements for hOECs**

The growth factor requirements for hOECs were similar to those of rOECs in that ACM and DMEM-FCS were mitogenic. hOECs respond to DMEM-FCS but lose L-NGFr rapidly, making continual analysis difficult. To characterize this property in detail, it would be necessary to carry out
clonal analysis of the hOEC. Since this was not the primary aim of this study, we have not addressed this point. Expression of the L-NGFr could be maintained longer for hOECs in ACM or medium containing HRG β1, but these cells stopped proliferating. We have found a similar cessation of growth for rOECs after 2–3 weeks in ACM or neuregulins (data not shown) that can be overcome by the addition of DMEM-FCS. It is clear that the growth factor requirements of hOECs are more complicated than those described for human Schwann cells. This observation is supportive of the growing body of evidence gleaned from rodent OECs that identify OECs as distinct from other glial cells types of the peripheral and central nervous systems.

Transplanted hOECs differentiate into a myelinating phenotype in the presence of demyelinated axons and behave in a manner similar to that of rOECs

The encouraging data on the CNS repair potential of transplanted OECs that have been generated thus far have involved the use of rodent cells grafted into rodent models (Franklin et al., 1996; Li et al., 1997; Imaizumi et al., 1998; Ramon-Cueto et al., 1998). A critical question in assessing the clinical relevance of this work is whether the rodent OEC bears any resemblance to the human OEC. In this study, we show that OECs derived from the human olfactory system behave in a similar fashion to the rodent cell. This has achieved two objectives. First, the ability of transplanted hOECs to differentiate into a myelinating phenotype that remyelinates demyelinated axons means that one can be sanguine about the feasibility of OEC grafts to repair demyelinated foci in the human CNS disease. Secondly, the similarity in the behaviour of OECs derived from human and rodent nervous systems validates the relevance of studies using rat cells for human disorders.

It is clear from our results that not all transplanted cells adopted a myelinating phenotype since in some areas cells that did not engage in remyelination were also observed. The identity of these cells is not clear at present. Their fine-process-bearing morphology and their association with bundles of collagen are reminiscent of meningeal cell contaminants (Franklin et al., 1993). On the other hand, they contain more intermediate filament than is commonly associated with meningeal cells suggesting that these cells may be more like astrocytes (Franklin et al., 1992). While it is possible that these cells may have been a contaminant population co-transplanted with the OEC suspension, it is worth noting that cells with both astrocyte-like and meningeal cell-like features were observed following transplantation of a clonal rodent OEC line into an X-EB lesion (Franklin et al., 1996). Thus, these cells could be of OEC origin and indicate that transplanted hOECs adopt a diversity of morphologies that reflect their phenotypic plasticity in tissue culture. The extent to which the recipient environment or the composition of the transplant suspension determines the phenotypes assumed by the transplanted cells is an important issue that remains to be resolved. For example, in the context of repairing an area of demyelination, the myelinating phenotype is clearly more desirable than a non-myelinating phenotype. In which case, in order to optimize the extent of remyelination, it may be necessary to select for OEC phenotypes that give rise to the remyelinating cell rather than the alternative phenotypes, prior to transplantation.

The cells used in this study were obtained from adults using invasive neurosurgical procedures. However, the olfactory epithelium and nerve located outside the cranium, in which glial cells have been identified, may provide an alternative source of OECs. Moreover, this area can be biopsied in humans with no adverse effect (Lanza et al., 1994; Feron et al., 1998). This would be especially true if unilateral biopsies were undertaken, which may at worst induce a hemianosmia, a mild debility when set against the possibility of ameliorating the profound neurological dysfunction associated with the target diseases for transplantation therapy. A more intriguing possibility however, is that OECs capable of remyelination following transplantation can be obtained from embryonic stem cells, the feasibility of which has been demonstrated recently for oligodendrocyte lineage cells (Thomson et al., 1998; Brustle et al., 1999). Regardless of their source, human OECs can be regarded as a feasible candidate cell in formulating transplantation-based clinical trials for the treatment of traumatic and demyelinating diseases of the CNS.

In this study, we have taken the promising data on the therapeutic potential of the OEC a step closer to clinical application, by identifying and characterizing the hOEC and demonstrating that it exhibits many of the properties of the rodent OEC. In particular, we have shown that hOECs will form new myelin sheaths around demyelinated axons. These are encouraging results in the context of using OECs for cellular therapy of CNS disease in humans.

Acknowledgements

The authors wish to thank the neurosurgery theatre staff for collecting the samples, and C. Ready and M. Peacock for their excellent technical assistance. This work was supported by the Wellcome Trust, the Myelin Project and the Multiple Sclerosis Society for Great Britain and Northern Ireland.

References


Received February 11, 2000. Revised March 6, 2000. Accepted March 9, 2000