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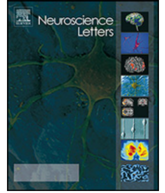

Transplanted olfactory ensheathing cells incorporated into the optic nerve head ensheathe retinal ganglion cell axons: Possible relevance to glaucoma

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ABSTRACT

A mixture of olfactory ensheathing cells and fibroblasts cultured from the adult rat olfactory mucosa was transplanted through a scleral incision into the retina. A major stream of transplanted cells migrated through the stratum opticum and penetrated for up to about 0.5 mm into the optic nerve head. This stream of transplanted cells consisted of a mixture of bipolar olfactory ensheathing cells with long processes which give rise to a non-myelinating ensheathment of single retinal ganglion cell axons, and olfactory nerve fibroblasts embedded in a dense fibronectin-positive extracellular matrix. A second stream of ovoid olfactory ensheathing cells with tufted processes and unaccompanied by fibroblasts or matrix migrate into the internal plexiform layer. The incorporation of olfactory ensheathing cells in the optic nerve head may suggest future possibilities for protection of the axons in this vulnerable region from mechanical damage, as in the raised intraocular pressure of glaucoma.

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There is considerable current interest in the use of olfactory ensheathing cells (OECs) to induce regeneration of severed axons in the spinal cord [2,16,18,19,21] and spinal roots [8], and cultured cells transplanted into total transections of the retrobulbar optic nerve induce regeneration of retinal ganglion cell (RGC) axons within the graft [10]. Clinically, the commonest cause of damage to RGC axons is the glaucomatous increase in intraocular pressure [1,14,17,25,27] acting on the vulnerable unmyelinated axon segments as they enter the optic nerve head. In this paper we explore whether a transscleral route of injection could be used to introduce OECs into the optic nerve head.

Tissue was obtained from adult female AS rats. Most of the earlier studies of transplanted OECs involved cells cultured from the olfactory bulb (e.g. [9,19]) or, more recently, from the olfactory mucosa [3,11,26]. For clinical application it is more attractive to avoid craniotomy and obtain OECs by endoscopic sampling of tissue from the olfactory mucosa [6,12]. In the present study we have cultured a mixture of OECs and their associated olfactory nerve fibroblasts (ONFs) from samples of primary tissue taken from the adult rat nasal mucosa.

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1 http://www.ion.ucl.ac.uk/research/hbir/spinal_repair_unit.htm.

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Adult AS rats were decapitated under terminal anaesthesia and the nasal septum exposed. Under a dissecting microscope, the olfactory mucosa marked by its unique amber colour was cut free with a fine scalpel, transferred immediately into ice-cold Hanks'-balanced salt solution (HBSS) without calcium and magnesium supplemented with 1% penicillin–streptomycin (Invitrogen, UK), washed twice in HBSS to remove excessive mucus and then incubated in 1 ml of dispase II (2.4 units/ml, in Puck's solution; Roche Diagnostics GmbH, Mannheim, Germany) at 37 °C for 45 min. The enzymatic reaction was stopped by transferring the tissue to HBSS, and the olfactory epithelium and the lamina propria separated using a fine spatula under a dissection microscope. The lamina propria was cut to small pieces (approximately 0.1 mm^2) on a McIlwain tissue chopper (Campden, Loughborough, UK), collected into 2 ml of 0.25% collagenase type I (Sigma–Aldrich Company Ltd., Gillingham, UK) in a Dulbecco's-modified Eagle medium (DMEM)/Ham's F-12 with GlutaMAX™ 1.0 mg/ml insulin, 0.67 mg/ml transferrin and 0.55 mg/ml selenium, 1% penicillin–streptomycin and 10% deactivated fetal calf serum (FCS; all from Invitrogen), and incubated in 37 °C for 5 min. The pieces of the lamina propria were triturated to dissociate the cells using a flame-polished Pasteur pipette and the collagenase reaction was stopped by adding 8 ml of HBSS. After centrifuging at 300 × g for 5 min and discarding the supernatant, the tissue pellet was further triturated into cell suspension in a DMEM/F12 medium. The resulting cells were seeded to a density of 20–25,000 cm^{-2} on 35 mm culture dishes coated with poly-L-lysine (0.1 mg/ml; Sigma–Aldrich) and maintained in

a humidified incubator enriched with 5% CO_2 for 10 days at 37 °C. The culture medium was replaced every 3 days. At day 7 the cells were transfected with a GFP gene harbouring a lentiviral construct which is known to give long-term stable expression [5,15,23] and which labelled about 80% of the cultured cells. After a further 3 days in culture, the cells were rinsed three times with fresh, serum-free medium, and a cell suspension was made at a concentration of 20×10^6 ml^{-1} in DMEM-F12 without FCS. The mixed cell suspension was kept on ice during surgery time. (Unpublished data based on immunostaining of the cultured monolayers with p75 LANGFR and fibronectin, and fluorescent activated cell sorting suggests that the ratio of OECs to ONFs is around 1–10).

In 18 adult female AS rats (200–220 g body weight) under intraperitoneal tribromoethanol anaesthesia (Avertin, Sigma; 20 mg/100 g body weight), the orbit was opened through the superior conjunctival fornix and the extraorbital muscles and glands separated to expose the optic nerve head. At a point 1–2 mm to one side of the optic nerve head an incision through the sclera, choroid and retina was made with a 15° stab knife (F.S.T. No. 10315-12, Reading, UK) to an estimated depth of around 1 mm. This incision was used to insert fine glass pipette with an internal diameter of around 70 μm guided by hand at an oblique angle for a depth of 1–2 mm (marked on the outside of the pipette) in a direction towards the optic nerve head, and 2–3 μl of the unsorted, mixed cell suspension (about 50,000 cells) was injected by pressure via a 50-ml syringe attached to the pipette.

Two (n = 9) to four (n = 9) weeks after operation, the rats were perfused with 50 ml of 0.1 M PBS followed by 500 ml of 4% paraformaldehyde for 30 min. The heads were left in the fixative at 4 °C overnight. Next day the eye and attached optic nerve were removed and immersed in 10%, then 20% aqueous sucrose solution until the tissue sank. Four series of continuous adjacent 20 μm cryostat sections were cut in a plane longitudinal to the optic nerve. Four normal rats were prepared in the same way for the control study.

Sections were examined in the confocal microscope. The transplanted cells were identified by the green fluorescent protein introduced via the lentiviral construct. One series of sections was immunostained for neurofilament 200 (1:500, rabbit polyclonal, Serotec, UK) and a second for fibronectin (Dako, Cytomation, Denmark), both visualised with red fluorescent secondary antibody (Alexa Fluor goat anti-Rabbit red, 1:400; Molecular Probes, Eugene, OR). Samples sections were immunostained for 1:3000 mouse monoclonal P0 antibody (gift of JJ Archelos, Max Planck Institute, Munich). In a reference series of sections the overall cellular pattern was visualised by Sytox Orange Nucleic Acid (Molecular Probes, Eugene, OR) staining at a final concentration of 0.1 μl in 0.1 M PBS for 5 min at room temperature in the dark.

Animal procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986, and adequate measures were taken to minimise pain and discomfort.

The transplanted cells were clearly identified by the green fluorescent label introduced via the lentiviral vector. In one rat the transplant failed to survive. In two rats the tip of the injection pipette was located deep in the vitreous and the transplanted cells formed a tight layer adherent to the back of the lens. In all the remaining 15, which are the subject of this paper, the injection track of the transplants was around 200 μm in diameter, penetrated at an angle through the entire thickness of all retinal layers from the scleral to the vitreal surfaces, and was densely packed with cells. From this initial deposit, the transplanted cells migrated into a number of different retinal layers and into the optic nerve head. The morphology and axon relationships of the transplanted cells differed according to their location. We were able to discern no differences in any of these features between the retinae at 2 weeks or at 4 weeks after transplantation, and there was no obvious decrease in numbers of labelled cells over this period.

Within the stratum opticum the cells extend for around 3 mm in a centripetal direction (Figs. 1–3Figs. 1A and 2A). In this region the transplanted cells are embedded in a dense fibronectin-positive extracellular matrix (Figs. 1–3 Fig. 1B). They consist of

(1) fibronectin-negative cells (green fluorescence), which are the presumed OECs. The cells are smooth-surfaced and bipolar, with a central ovoid cell body around 5 μm across, flattening to 10–15 μm in the long axis, and giving rise on either side to rather uniform, axon-associated processes extending for up to as much as 100 μm and intimately associated with the retinal ganglion cell axons.
(2) Olfactory nerve fibroblasts (ONFs) are identified by the surrounding red extracellular fibronectin fluorescence or yellow as a result of overlap with the green fluorescence of the cellular GFP label (arrows in Figs. 1–3 Fig. 1B). The ONFs are more rectangular in shape than the OECs, with less distinct outlines and fewer processes visible at the light microscopic level. The ONFs generally lie closer to the vitreal surface of the stratum opticum, and accumulate towards the leading edge of the centripetal migration towards the optic nerve head. Compared with the monolayer of cells in the cultures, the close interweaving of the OECs and ONFs in the transplanted tissues makes it difficult to estimate the ratio of the two cell types.

A major proportion of the transplanted cells migrated into the optic nerve head to a depth of up to around 0.5 mm where they form dense accumulations occupying almost the full width of the optic nerve (Figs. 1–3 Fig. 1C-F), where they are intimately associated with the RGC axons (see below).

From the scleral aspect of the injection track another stream of elongated bipolar cells spreads into an expanded subretinal space through which cells can also reach the pial surface of the optic nerve (arrowhead in Figs. 1–3 Fig. 1C). Within the retina, a small number of highly flattened single cells with elongated processes become insinuated as a monolayer in the narrow outer plexiform layer (small arrows in Figs. 1–3 Fig. 2A).

In the normal retina, and in the adjacent parts of the transplanted retina unaffected by the transplants, positive staining for the neurofilament 200 (NF200) is restricted to the layer of retinal ganglion cell (RGC) axons in the stratum opticum. In the transplanted retinae, there is an increased density of NF200+ fibres associated with the OECs along the injection track, in the stratum opticum, as well as in the subretinal space, and in the optic nerve head. These fibres appear yellow (e.g. at high power in Figs. 1–3 Fig. 1E) due to overlap of the green fluorescence of the OECs and the red fluorescence of the NF200 immunostaining. At higher magnification the ensheathing cell processes can be resolved as double green fluorescent tracks enclosing a single, red fluorescent, unbranched RGC axon (or a single compact bundle of finer axons which cannot be resolved at the light microscope level) between them (arrows Figs. 1–3 Fig. 3C and D). The absence of P0 immunostaining shows that these ensheathments do not result in myelination, at least up to the 4-week point studied.

A separate population of small, highly characteristic green fluorescent (fibronectin-negative) OECs migrate into the inner third of the IPL (ip in Figs. 1–3 Figs. 1A and 2A and B) where favourable sections show that they tend to be arranged in two tiers parallel with the surface of the IPL. The cells in the IPL are small, ovoid cells, around 5 μm across, with a tuft of branched processes, up to around 20-μm long, usually arising from the pole facing towards the stratum opticum (Figs. 1–3 Fig. 2D). The ovoid cells are scattered singly, with virtually no overlap of either the cell bodies or their processes.

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