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Research Article

Olfactory ensheathing cells promote neurite sprouting of injured axons in vitro by direct cellular contact and secretion of soluble factors

R. S. Chung*, A. Woodhouse, S. Fung, T. C. Dickson, A. K. West, J. C. Vickers and M. I. Chuah

NeuroRepair Group, School of Medicine, University of Tasmania, Private Bag 58, Hobart, Tasmania 7001 (Australia), e-mail: rschung@utas.edu.au

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Abstract. Olfactory ensheathing cells (OECs) represent an exciting possibility for promoting axonal regeneration within the injured spinal cord. A number of studies have indicated the ability of these cells to promote significant reactive sprouting of injured axons within the injured spinal cord, and in some cases restoration of functional abilities. However, the cellular and/or molecular mechanisms OECs use to achieve this are unclear. To investigate such mechanisms, we report for the first time the ability of OECs to promote post-injury neurite sprouting in an in vitro model of axonal injury. Using this model, we were able to differentiate between the direct and indirect mechanisms underlying the ability of OECs to promote neuronal recovery from injury. We noted that OECs appeared to act as a physical substrate for the growth of post-injury neurite sprouts. We also found that while post-injury sprouting was promoted most when OECs were allowed to directly contact injured neurons, physical separation using tissue culture inserts (1 μm pore size, permeable to diffusible factors but not cells) did not completely block the promoting properties of OECs, suggesting that they also secrete soluble factors which aid post-injury neurite sprouting. Furthermore, this in vitro model allowed direct observation of the cellular interactions between OECs and sprouting neurites using live-cell-imaging techniques. In summary, we found that OECs separately promote neurite sprouting by providing a physical substrate for growth and through the expression of soluble factors. Our findings provide new insight into the ability of OECs to promote axonal regeneration, and also indicate potential targets for manipulation of these cells to enhance their restorative ability.

Key words. Olfactory ensheathing cells; time-lapse imaging; post-injury neurite sprouting; growth factor.

The olfactory system is the only region of the central nervous system to support continuous growth of new neurons throughout a lifetime [1]. Within this system, new olfactory neurons extend axons from the periphery into the central nervous system [2], a process that also occurs following neurogenesis in the aftermath of injury to the olfactory nerve [3]. These new axons are accompanied to the olfactory bulb by a unique type of glial cell, the olfactory ensheathing cell (OEC). Based on studies within the olfactory system suggesting that OECs aid in the guidance and support of growing axons, they represent a novel method for the treatment of spinal cord injury, in which there is a lack of regenerative capacity. Indeed, there are numerous reports in the literature demonstrating that OECs injected directly into the injured spinal cord promote axonal regeneration and in some cases a degree of functional recovery [4, 5], including remyelination of demyelinated axons [6, 7]. Perhaps most remarkable is the restoration of the climbing ability of rats that have undergone complete transection of the spinal cord [8]. How-

* Corresponding author.

ever, studies have yet to clearly identify the mechanisms underlying the ability of OECs to promote recovery from spinal trauma.

In the context of understanding how OECs mediate axonal recovery following spinal cord injury, in vitro studies have demonstrated that these cells produce a number of growth factors including several forms of neuregulins and some neurotrophins [9–11]. Furthermore, OECs are known to express cell surface molecules such as N-CAM, N-cadherin and laminin [12–14], supporting the proposition that OECs provide a physical substrate to support axonal regeneration [15]. Recent in vitro studies of OEC-neuron interactions have found that OECs promote outgrowth of cultured embryonic chick ganglia via direct cellular interactions [16], and that soluble factors secreted by OECs are able to promote PC12 cell differentation and protect PC12 cells from Zn^{2+} insult [17]. However, whether similar mechanisms are utilised by OECs upon interaction with injured neurons is not clear. In the present study, we attempted to address this by differentiating between the relative contribution of direct contact and expression of soluble growth factors from OECs using an in vitro model of neuronal injury.

Materials and methods

Chemicals and antibodies

MEM HEPES, MEM D-valine, collagenase, dialysed fetal calf serum, trypsin, trypsin inhibitor, cytosine-β-D-arabinofuranoside, bovine pituitary extract, L-glutamine, gentamicin, poly-L-lysine and mouse anti-NCAM antibody were supplied from Sigma, (Sydney, Australia). Neurobasal medium, B-27 supplement and penicillin-streptomycin-amphotericin B were purchased from GIBCO (Auckland, New Zealand). Anti-tau antibody was from DAKO (Glostrup, Denmark). Anti-βIII tubulin and rabbit anti-$P75^{NTR}$ antibodies were from Promega (Madison, Wis.) Alexafluor secondary antibodies were from Molecular Probes (Eugene, Ore.). Mouse anti-$P75^{NTR}$ antibody was purchased from Chemicon, (Melbourne, Australia).

OEC cultures

All procedures involving animals were approved by the Animal Experimentation Ethics Committee of the University of Tasmania and were consistent with the Australian Code of Practise for the Care and Use of Animals for Scientific Purposes.

Ensheathing cells were purified and cultured based upon the method of Chuah and Teague [18] with minor modifications to increase the yield of OECs whilst maintaining a high level of purity [19]. The olfactory nerve layer (ONL) and mucosa (OM) were dissected from 3-day-old Hooded Wistar rats euthanised on ice. The ONL was peeled away from the glomerular and deeper layers of the olfactory bulb and the OM was scraped from both sides of the nasal septum after cutting away the surrounding respiratory tissue. The pooled tissues were digested in MEM HEPES modification containing 0.25% trypsin and 0.03% collagenase for 15 min at 37 °C. This incubation was repeated twice with fresh solution, which was then replaced with culture medium composed of MEM D-valine modification supplemented with 10% dialysed fetal calf serum and penicillin-streptomycin-amphotericin B [20]. The digested tissue was triturated, and the cell suspension passed through a sterile nylon gauze (80 µm pore size) followed by centrifugation for 10 min at 500 g. Resuspended cells were plated in culture medium. After 24 h, the culture was treated with 10^{-4} M cytosine-β-D-arabinofuranoside for 48 h to minimise the population of fibroblasts. The highly purified OEC cultures [>95% pure as indicated by immunoreactivity with antibody against the low-affinity nerve growth-factor (NGF) receptor ($P75^{NTR}$)] were expanded by addition of 125 µg/ml bovine pituitary extract for 2 days before application to neurons.

Neuron/astrocyte co-cultures

Co-cultures were prepared as reported previously [21]. Briefly. cortical tissue (including meningeal layers) was removed from embryonic day 19 (sperm positive day = E1) Hooded Wistar rat embryos and incubated in sterile 10 mM HEPES buffer (37 °C). This was followed by trypsin digestion (0.25%), followed by three gentle washes of the cell pellet using fresh HEPES buffer. The cell suspension was then triturated carefully using a 1-ml pipette. Cells were then plated onto glass coverslips (132 mm^2) pre-coated overnight with 0.01% poly-L-lysine, at a cell density of 5×10^5 cells/well. Cultures were maintained at 37 °C in humidified air containing 5% CO_2 for 21 days before injury. The culture medium consisted of Neurobasal medium, supplemented with 0.1% (final concentration; f/c) B-27 supplement, 0.1 mM (f/c) L-glutamine and 200 U/ml gentamicin. Scratch wounds were made on an inverted microscope (Leitz Fluovent, Melbourne, Australia) using a fine goniotomy knife. Immediately following injury, OECs were applied to neuron/astrocyte co-cultures. In some experiments, the same number of OECs were plated into membranous cell inserts (1 µm pore size, PET track-etched membrane; Falcon, Melbourne, Australia) suspended in empty wells containing Neurobasal medium. OECs were plated into the inserts 1 day prior to injury, but were not placed in the presence of neurons until immediately following injury. Following injury, the cell inserts (containing OECs) were suspended 800 µm above coverslips containing scratch-injured co-cultures.

Fluorescent immunocytochemistry of primary cultures

At the appropriate time, cells were fixed with 4% paraformaldehyde for 20 min. Following blocking of non-specific adsorption using DAKO protein block solution, coverslips were incubated with a combination of either rabbit anti-$P75^{NTR}$ (1:100) and mouse anti-βIII-tubulin (1:10,000), or mouse anti-$P75^{NTR}$ (1:100) and rabbit anti-tau (1:5000) antibodies diluted in 0.1% PBS, 0.03% TritonX-100. Similar to other laboratories [22, 23], we routinely use $P75^{NTR}$ as a marker of OECs [19] and we have never observed any $P75^{NTR}$ immunoreactivity in neurons or astrocytes within tissue culture conditions (results not shown). In previous studies, we have used anti-tau and anti-βIII-tubulin antibodies (neuronal cytoskeletal markers) to visualize post-injury neurite sprouts [24, 25]. Anti-tau binds with the microtubule-associated tau protein [26], and we have previously found that this antibody only identifies neurons, not astrocytes or OECs (results not shown). Anti-βIII-tubulin binds with the major building block of microtubules, and is neuron specific [27]. Coverslips were then incubated with two secondary antibodies (horse anti-mouse IgG conjugated to alexafluor 488 and goat anti-rabbit IgG conjugated to alexafluor 594, 1:1000 dilution), applied in 0.1% PBS.

Quantitative analysis of post-injury neurite sprouting

For analysis of post-injury neurite sprouting in neuron/astrocyte co-cultures, five digital images along an injury site were captured from each coverslip at a magnification of ×40 (Olympus BX-60, Melbourne, Australia). All experimental groups consisted of at least three coverslips, and experiments were repeated in triplicate (three different neuronal cultures). t test analysis of results was performed using SigmaStat (Jandel Scientific Software Corporation, San Rafael, Calif.).

Live-cell imaging of OECs within the in vitro injury environment

We observed the cellular interactions between OECs and sprouting neurites using live-cell-imaging techniques. A Leica inverted microscope (Leica DMIRB, Melbourne, Australia) with differential interference contrast optics (Nomarski) was used. Briefly, glass coverslips bearing the neuron cultures were transferred from tissue culture wells to glass Petri dishes and incubated in imaging buffer consisting of 124 mM NaCl, 5 mM KCl, 0.2 mM $CaCl_2$, 1 mM $MgCl_2$, 30 mM dextrose, 25 mM HEPES, pH 7.3 [28] for 2 h prior to imaging. Immediately prior to imaging, cultures were injured as described previously, and OECs added. Imaging was then performed on the inverted microscope with the stage heated to 37 °C, typically over a 3- to 6-h period using a low-intensity light setting for the brightfield imaging. Time-lapse imaging movies and single digital images were captured using a Magnafire (Optronics) digital camera and OpenLab software (Improvision). After the imaging period, cultures were processed for immunocytochemistry as described above.

Scanning electron microscopy of cultured OECs

Scanning electron microscopy was performed as described previously [13]. Briefly, OEC cultures were fixed for 1 h in 2.5% glutaraldehyde and 0.5% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Following washing with buffer, cultures were post-fixed for 1 h with 1% osmium tetraoxide. Following another buffer wash, they were dehydrated in an ascending series of ethanol washes, at which point they were critical point dried by Freon. Surface coating of specimens with a layer of gold was performed using a sputter coater (Edwards S150B, London, UK), and examined with a JEM-35CF scanning electron microscope.

Results

OECs promote neurite sprouting following scratch injury in mature neuron/astrocyte co-cultures

At 24 h post-injury (PI) in neuron/astrocyte co-cultures that did not receive OECs, there were very few neuritic processes entering into the injury tract, as identified by tau immunoreactivity (fig. 1A, B). However, at 24 h PI in cultures which had received OECs immediately after injury, there were a number of neurites observed entering the site of injury, as identified by immunocytochemistry

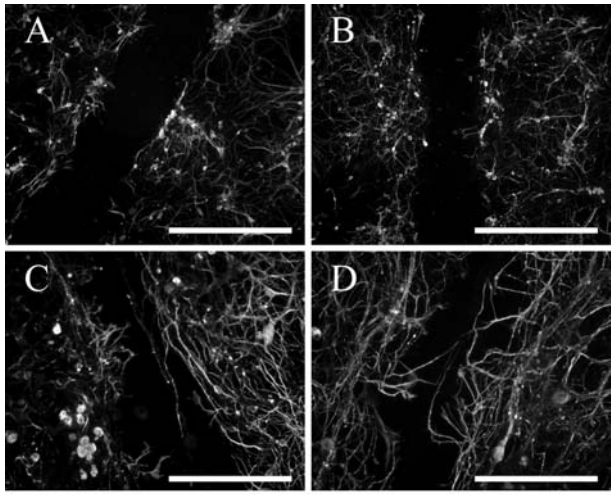

Figure 1. Scratch wound injuries were performed on neuron/astrocyte co-cultures, and post-injury neurite sprouting assessed at 24 h post-injury by immunocytochemistry against the axon-specific microtubule-associated tau protein. In cultures that received no OECs, there were very few, short neurite sprouts observed entering into the injury tract (*A*, *B*). However, in cultures that received OECs immediately after injury, a number of neurites were observed entering the injury tract (*C*, *D*). Scale bars, 250 µm (*A*–*C*) and 125 µm (*D*).

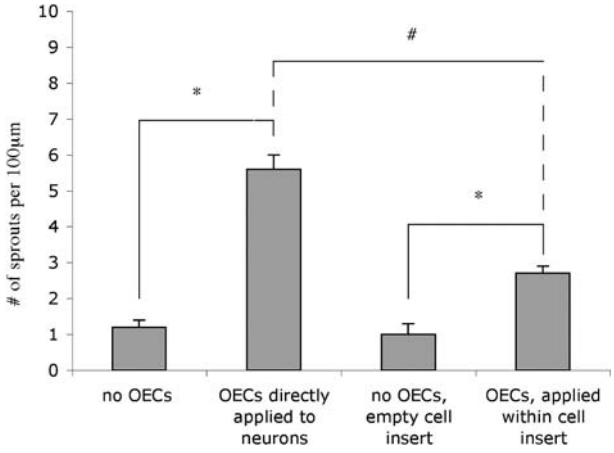

Figure 2. To investigate the effect of soluble growth factors produced by OECs upon neurite sprouting, OECs were applied to injured cultures either directly, or within membranous culture inserts, preventing direct contact between OECs and neurons. At 24 h PI to co-culture, cultures with OECs directly applied had significantly more sprouting neurites compared to those without. Similarly, cultures with OECs within the cell inserts had significantly more neurites compared to those with empty inserts. *$p < 0.01$ compared to appropriate no OEC control, paired t test. Furthermore, significantly more neurites were observed when OECs were directly applied to neurons than when OECs were separated by the membranous insert (#$p < 0.01$, paired t test).

against the microtubule-associated protein tau (fig. 1C, D). Quantitative analysis indicated that there were significantly more neurite sprouts following OEC treatment (5.6 ± 0.4 sprouts per 100 µm of injury tract) compared to untreated cultures (1.2 ± 0.2 sprouts per 100 µm of injury tract; $p < 0.01$, Student's t test; fig. 2). Similar results were obtained when immunocytochemistry was performed for βIII-tubulin (results not shown)

Intriguingly, we noted a very close association between sprouting neurites and OECs found within the injury tract. Sprouting neurites were often observed to grow initially towards the centre of the scratch site and then make a right-angle turn and grow parallel to the original injury (see fig. 1C, D). These neurites were found to be coursing directly on top of an underlying OEC (fig. 3A, B). This suggests that in accordance with previous reports in the literature, OECs express cell adhesion molecules that are involved in promoting the direct interactions between themselves and neurites [for a recent review, see ref. 29].

Live-cell imaging of OECs within the in vitro injury environment

Live-cell imaging was performed to investigate the cellular dynamics between OECs and sprouting neurites within the scratch injury tract. At 3 h post-plating, OECs within the scratch injury tract were often elongated along the middle of the injury site, with a number of neuritic processes observed growing from the margins of the injury tract towards the elongated OEC (fig. 4). Occasionally, some neurons were observed to move into the injury tract (fig. 5A), and in some cases to actually travel along an OEC (fig. 4A–D, supplementary movie 1, available for download at http://www.neurorepair.utas.edu.au/supplemental.html). Post-imaging immunocytochemistry indicated that these small moving cells were tau-immunoreactive neurons (fig. 5E). Important to note is that in cultures that did not receive OECs, no cells were observed within the middle of the injury tract (fig. 5F) although some neurons were found marginally within the border of the injury tract (fig. 5F). At higher magnification, we found that some OECs extended numerous, small protrusions around the entire cell body (fig. 6A, B), which were more readily distinguishable during time-lapse imaging where they were observed to be constantly extended and retracted (supplementary movie 2, available

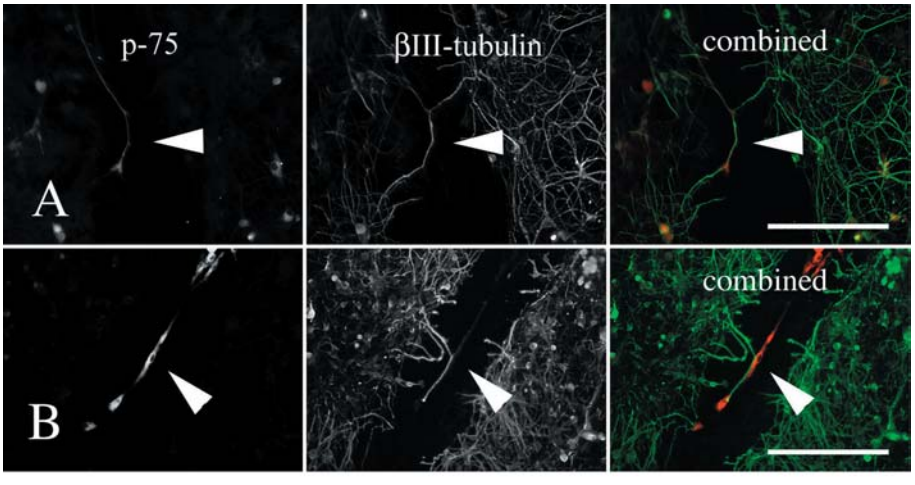

Figure 3. Post-injury neurite sprouts (anti-βIII-tubulin, green) were often found in close association with underlying OECs (anti-$P75^{NTR}$, red) as they grew across the injury tract (two separate examples, *A*, *B*). Arrowheads indicate OECs. Scale bars, 125 µm.

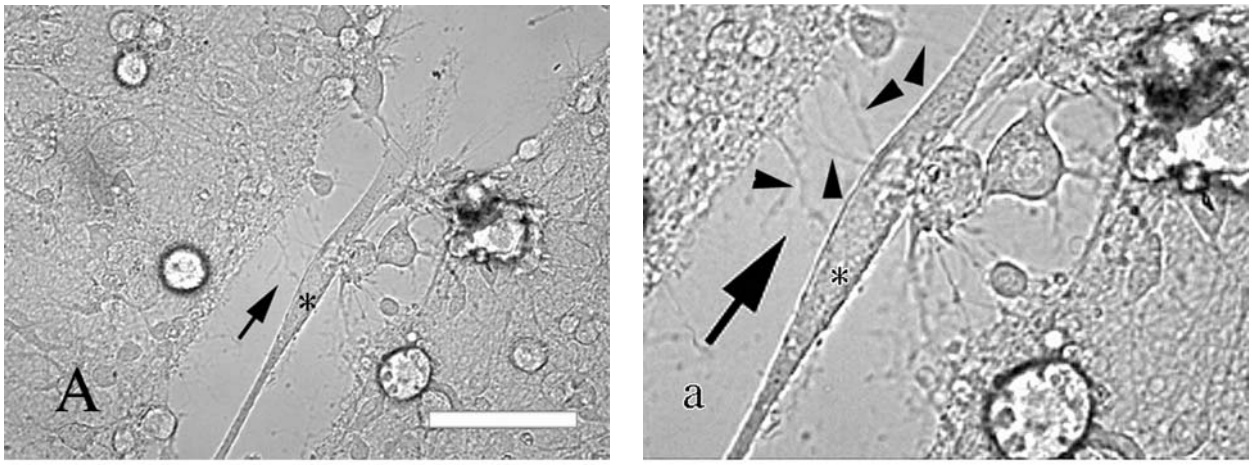

Figure 4. At 3 h PI (*A*), OECs within the scratch injury tract had elongated along the middle of the injury site (OEC indicated by asterisk), and a number of neuritic processes (indicated by arrow) were observed growing from the margins of the injury tract towards the elongated OEC. *a* is a twofold magnification of *A*, with neuritic processes growing towards and making contact with the OEC clearly distinguishable (small arrowheads). Scale bar, 100 µm.

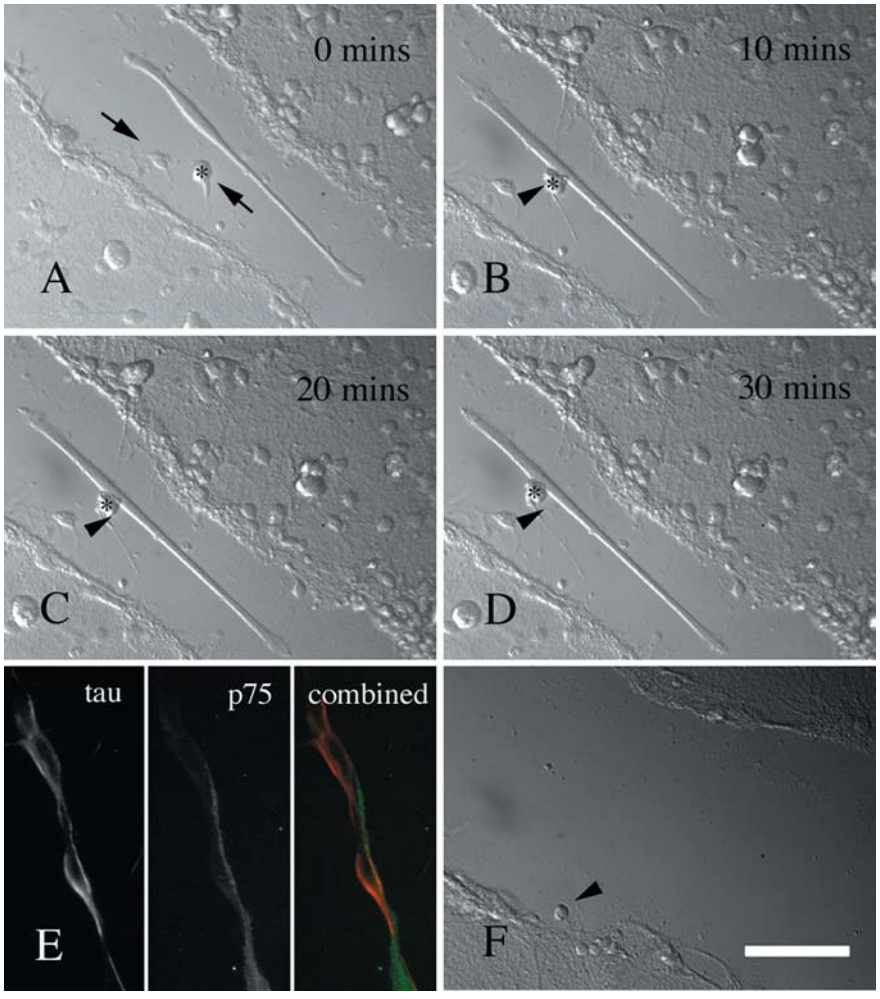

Figure 5. At 3 h post-plating, neurons were observed to have moved into the injury tract (indicated by arrows) from the bordering margins (*A*), and in some cases to have travelled along an OEC (*A–D*) (arrowhead indicates starting point of asterisk-labelled neuron). Immunocytochemistry confirmed that these small cells observed to move along underlying OECs (anti-$p75^{NTR}$, green) were tau-immunoreactive neurons (red) (*E*). In neuron cultures that did not receive OECs, no cells were observed within the middle of the injury tract (*F*), although some neurons were found marginally within the borders of the injury site (indicated by arrowhead). Scale bars, 100 µm.

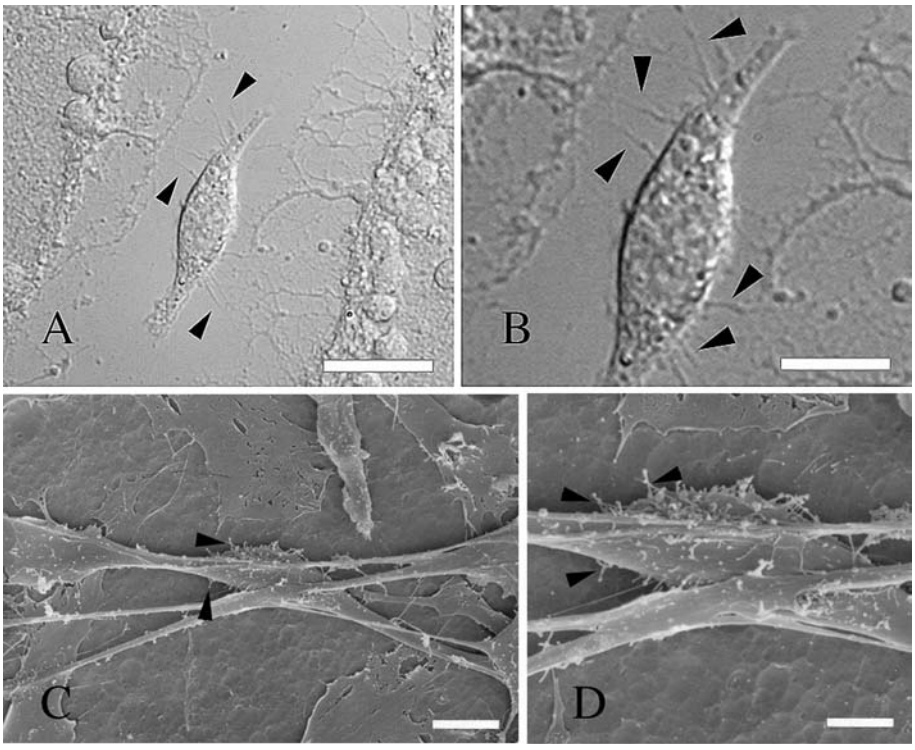

Figure 6. In some instances, OECs within the scratch injury tract were noted to extend numerous, small protrusions around the entire cell body (*A*) (indicated by arrowheads). These protrusions were more distinguishable at higher magnifications (*B* is a ×2 magnification of *A*) and during time-lapse imaging (see supplementary movie 2). Scanning electron microscopy confirmed the presence of these fine projections, which appeared to end in a bulbous tip (indicated by arrowheads), extending from the surface of some OECs (*C*, *D*; *D* is a ×2.5 magnification of *C*). Scale bars, 50 µm (*A*), 75 µm (*B*), 10 µm (*C*) and 25 µm (*D*).

for down-load at http://www.neurorepair.utas.edu.au/supplemental.html). Scanning electron microscopy confirmed the presence of these small projections extending from the surface of some OECs (fig. 6C, D). At this magnitude of resolution, the protrusions were observed as thin processes that ended in bulbous tips (fig. 6D).

OECs produce growth factors that promote post-injury neurite sprouting

To investigate the contribution of soluble growth factor expression by OECs to the ability of these cells to promote post-injury neurite sprouting, OECs were applied to injured co-cultures within membranous culture inserts, preventing direct contact between OECs and neurons. At 24 h PI to co-culture, cultures with OECs within the inserts had significantly more neurite sprouts compared to those with empty inserts (2.7 ± 0.2 vs 1.0 ± 0.3 sprouts per 100 µm, respectively; fig. 2). However, co-cultures that received direct injections of OECs produced significantly more neurite sprouts than co-cultures in which OECs were separated by the membranous inserts (5.6 ± 0.4 vs 2.7 ± 0.2 sprouts per 100 µm, respectively; fig. 2).

Supplementary results

All supplementary movies are in QuickTime format, and are available for download as a compressed file from the following internet address: http://www.neurorepair.utas.edu.au/supplemental.html

Supplementary movie 1

Sometimes neurons were observed to migrate into the injury tract and make physical contact with OECs. We observed that these neurons actually travelled along underlying OECs. A total of 40 frames were captured, at 30-s intervals.

Supplementary movie 2

We found that some OECs lying within the injury tract constantly extended and retracted numerous small projections around the entirety of the cell body. These small projections appeared to interact with neurite sprouts emanating from the borders of the injury site. Imaging was performed over a 20-min period, with frames captured every 30 s.

Discussion

While OECs have been demonstrated to be capable of promoting axonal regeneration within the injured spinal cord, the cellular and/or molecular mechanisms OECs use to achieve this are unclear. To investigate such mechanisms, we report for the first time the ability of OECs to promote post-injury neurite sprouting in an in vitro model of axonal injury. Using this model, we found that

OECs promote neurite sprouting mediated through the expression of cell adhesion molecules and soluble factors. Furthermore, live-cell imaging revealed that OECs within the injury tract environment exhibit remarkably dynamic morphological plasticity, and actively interact with neurite sprouts. Our findings provide new insight into the ability of OECs to promote axonal regeneration, and also indicate potential targets for manipulation of these cells to enhance their restorative ability.

When OECs were applied directly to injured neuronal/astrocyte co-cultures, we observed a dramatic increase in post-injury neurite sprouting. Intriguingly, we also noted a close association between sprouting neurites and OECs, suggesting that OECs actively provide a permissive substrate to promote post-injury neurite sprouting. This is in accordance with previous work demonstrating that OECs express cell adhesion molecules, both in vivo and in tissue culture conditions [for a review, see ref. 29]. Cell adhesion molecules expressed by OECs have previously been suggested to be involved in direct physical interactions between axons and OECS. For example, Sonigra and colleagues [30] interrupted cell adhesion molecule dynamics with a cell-permeant calcium chelator (BAPTA/AM), resulting in the loss of association between neurons and OECs in an in vitro co-culture system. Using live-cell-imaging techniques we were able to directly observe the cellular interactions between OECs and neurons within the in vitro injury environment. In some cases we observed neurons growing along elongated OECs within the injury tract. Such interactions have been observed previously, but in a different culture system [31], which utilized newly plated intact neurons rather than mature injured neurons (21 days in vitro) which we used in this study. These live-cell-imaging data provide further evidence that OECs provide a physical substrate to sprouting neurites following axonal injury. Furthermore, our observations suggest that the relationship between OECs and neurites sprouts is an active dynamic one, rather than simply providing a physical scaffold for regenerating neurites.

Intriguingly, we also noted during time-lapse imaging that some OECs extended numerous slender protrusions around the entire cell body, which were constantly being extended and retracted. While we are uncertain what function these processes have (to our best knowledge this has not been described in the literature previously), they possibly act like the filopodia of the neuronal growth cone, as a chemical sensor that aids in guiding the OEC the remarkable distances it travels from the periphery to the olfactory bulb as it accompanies new olfactory axons [2].

There are suggestions in the literature that OECs not only provide a physical substrate for axonal regeneration, but also produce soluble growth factors to aid in neuronal recovery. Indeed, in vitro studies have demonstrated that these cells produce a number of growth factors including several forms of neuregulins and some neurotrophins [9–11]. We investigated this possibility by separating OECs from neurons using membranous culture inserts, and found that OECs increase the number of sprouting neurites following injury, although to a lesser degree than OECs applied directly. This suggests that both direct physical interaction between OECs and neurons and the release of soluble factors are important in mediating improved neurite sprouting following injury. An alternative possibility is that the soluble growth factors produced by OECs are only able to act within a short distance. Also possible is that the membranous culture inserts absorb some of the soluble factors secreted by the OECs, hence reducing their effect.

In summary, we demonstrated that OECs promote post-injury neurite sprouting in vitro through direct interactions between OECs and neurons and soluble factors released by OECs. These are likely to be crucial mechanisms underlying the therapeutic success of OEC implantations into the injured spinal cord. Our findings provide new insight into the ability of OECs to promote axonal regeneration, and also indicate potential targets for manipulation of these cells to enhance their restorative ability.

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