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Neurite outgrowth promoting effects of enriched and mixed OEC/ONF cultures

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Abstract

Olfactory ensheathing cell (OEC) transplants stimulate axon regeneration and partial functional recovery after spinal cord injury. However, it remains unclear whether enriched OEC or mixed transplants of OEC and olfactory nerve fibroblasts (ONF) are optimal for stimulating axon regrowth. The neurite outgrowth stimulating effects of enriched OEC, ONF, and mixed OEC/ONF cultures on neonatal cerebral cortical neurons were compared using co-cultures. We show that (1) OEC are more neurite outgrowth promoting than ONF, and (2) ONF do not enhance the neurite outgrowth stimulating effects of OEC in mixed OEC/ONF cultures. Hence, our data indicate that there is no preference for the use of enriched OEC or mixed OEC/ONF cultures with respect to stimulation of neurite growth in vitro.

Keywords: Olfactory ensheathing glia; Axon; Rat; Cell adhesion; Purification; Co-culture

During the last decade, transplantation of olfactory ensheathing cells (OEC) has been frequently used to stimulate axon regeneration and functional recovery after experimental spinal cord injury (SCI). The OEC reside in the olfactory system, which is a part of both the peripheral nervous system (PNS) and central nervous system (CNS) and retains a capacity to stimulate olfactory axon regeneration throughout the mammalian life time [12]. Together with the OEC, the olfactory nerve fibroblasts (ONF) are thought to play a major role in this regeneration capacity [9]. When transplanted into the injured spinal cords of adult rats, both enriched OEC and mixed OEC/ONF suspensions have stimulated axon regrowth and partial recovery of body functions [8,14]. Nevertheless, it remains unclear if enriched OEC or mixed OEC/ONF cultures are better at stimulating axon regrowth [1]. In vitro studies have shown that both enriched OEC and OEC/ONF have neurite outgrowth stimulating capacities which are similar or stronger than those of other cell types, such as hippocampal glia [5,6], epineurial fibroblasts, Schwann cells, and cerebral cortical astrocytes [15]. However, the properties of enriched OEC and mixed OEC/ONF have, so far, never been directly compared.

Previously, we showed that mixed OEC/ONF cultures containing about 10% OEC were more effective in stimulating outgrowth of the longest neurite of co-cultured cerebral cortical neurons as compared to cerebral cortical astrocytes [3]. No difference was made between neurites of neurons on either OEC or ONF cells and, thus, the neurite outgrowth stimulating effect was an overall effect of both OEC and ONF together. In the present study, we hypothesized that (1) OEC are more effective in stimulating neurite outgrowth of CNS neurons in vitro as compared to ONF and (2) that ONF increase the neurite outgrowth stimulating effect of OEC in mixed OEC/ONF cultures. These hypotheses were tested using co-cultures of enriched or mixed OEC/ONF with early postnatal cortical neurons. We show that (1) OEC are more potent than ONF in stimulating neurite outgrowth, and (2) ONF do not increase the

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neurite outgrowth promoting effect of OEC in mixed OEC/ONF cultures.

All procedures in this study were approved by the Ethical Committee on Animal Experiments of the Maastricht University (The Netherlands, DEC 2002-95) and were conducted according to the recommendations of the European Commission. Nine-week-old male Lewis rats (inbred; animal facilities Maastricht University) were used for the isolation of OEC and ONF. Neonatal cerebral cortical cultures, including neurons, were obtained from postnatal day 1 male Lewis rat pups.

The isolation of primary cultures of OEC/ONF from adult rats has been described previously [3,13]. The isolated OEC/ONF cells were plated onto poly-L-lysine (PLL; Sigma Chemical Co., St Louis, MO, USA; >300,000 MW, 1% in purified water) coated Petri dishes at a density of 200,000 cells/2 ml/Petri dish. The culture medium was DMEM/NUT mix F12 with glutamax-I (Gibco/Invitrogen, Breda, The Netherlands), supplemented with 10% inactivated fetal calf serum (Bodinco, Alkmaar, The Netherlands) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). The OEC/ONF cultures were grown for 14 days in an incubator (37 °C; 5% CO_2). The culture medium was refreshed after the first 4–5 days and every second day thereafter. This protocol generates mixed OEC/ONF cultures with about 10% p75-low-affinity-nerve growth factor-receptor (NGFr)-positive OEC at 14 days in vitro [3]. The rest of the cells belong to the (NGFr)-negative ONF cell population and contain mainly fibroblasts and astrocytes [3].

The 14-day OEC/ONF cultures were enriched using magnetic cell sorting (MACS) (Miltenyi Biotec, Bergisch Gladbach, Germany) as described previously for Schwann cell enrichment [16]. OEC/ONF cultures were washed with phosphate buffered saline (PBS) and then trypsinized by a 5 min incubation in 0.1% trypsin/0.05 mM EDTA (Fluka BioChemika, Buchs, Switzerland) at 37 °C. After stopping the trypsinization with an excess of culture medium, the cell yield was determined by counting trypan blue (0.4%, Sigma, Uithoorn, The Netherlands) excluding cells in a Fuchs-Rosental counting chamber. Next, the cells were centrifuged at 1000× *g* for 10 min and the pellet was resuspended and incubated in 1 ml MACS buffer (2 mM EDTA and 0.5% BSA in Mg^{2+}/Ca^{2+}-free PBS) containing 2 μl of anti-NGFr monoclonal antibodies (undiluted, Chemicon Europe, Hampshire, UK) for 10 min at 4 °C. Another 500 μl MACS buffer was added after the 10 min incubation and the cells were centrifuged (1000× *g*; 5 min; 4 °C). The pellet was washed using 1.5 ml MACS buffer, centrifuged, and the supernatant discarded. The cells were resuspended and incubated in 80 μl MACS buffer and 20 μl of the microbead-linked goat anti-mouse IgG1 (1:5; Miltenyi Biotec, Germany) for 15 min at 4 °C. Thereafter, 1.4 ml MACS buffer was added and the cells centrifuged once again. The pellet was resuspended in 500 μl MACS buffer and passed through a MS^+ magnetic column (Miltenyi Biotec, Germany). Multiple MACS buffer rinsing steps were used to remove unbound cells from the column. These cells constituted the NGFr-negative fraction. After removal from the magnet, the column was flushed with 2 ml MACS buffer, thereby collecting the p^{75}-l-NGFr-positive fraction. The negative fraction contained about 10 times more cells than the positive fraction, which is in line with the 10% NGFr/S100β-immunoreactive OEC contribution to the mixed OEC/ONF cultures at 14 days in vitro [3]. The negative and positive fractions were then centrifuged (1000 × *g*; 10 min; 4 °C) and resuspended in culture medium. In parallel, mixed OEC/ONF cultures from the same cell pool as the OEC/ONF cultures from which the negative and positive fractions were obtained were trypsinized (0.1% trypsin/0.05 mM EDTA; 5 min; 37 °C), centrifuged (1000 × *g*; 10 min; 4 °C) and resuspended in culture medium. The mixed OEC/ONF, the enriched OEC (positive fraction), and the enriched ONF (negative fraction) were plated on poly-L-lysine (PLL; Sigma Chemical Co., St Louis, MO, USA; >300,000 MW; 1% in enriched water) coated Petri dishes at a density of 150,000 cells per Petri dish.

One day after plating, neonatal cerebral cortical cell cultures, including neurons, were prepared for co-culture. These neonatal cerebral cortical cell cultures were selected because of their effectiveness in previous in vitro neurite outgrowth experiments [3,4]. The neonatal cerebral cortical cultures were obtained from postnatal day 1 Lewis rat pups by a method described earlier [3]. The culture medium was removed from the enriched OEC, enriched ONF, and mixed OEC/ONF cultures and replaced with a 2 ml cell suspension containing 300,000 cerebral cortical cells. These co-cultures were maintained for 48 h in culture medium. Hereafter, the co-cultures were fixed by a 15-min-incubation in 4% paraformaldehyde in PBS (Merck, Germany) and processed for immunocytochemistry. The above mentioned co-culture procedure to study neurite outgrowth in vitro was selected for its effectiveness in previous studies [3,4].

The following primary antibodies were used: mouse anti-microtubule-associated protein 2 (anti-MAP2; 1:200; Sigma), mouse anti-neurofilament (clone-RT97; 1:100; Hybridoma Bank, Iowa City, IA, USA), and rabbit anti-S100β (1:1000; Swant, Bellinzona, Switzerland). The following secondary antibodies were used: Cy3-conjugated donkey anti-mouse (1:800; Jackson) and Alexa488-conjugated goat anti-rabbit (1:100; Molecular Probes). Both primary and secondary antibodies were diluted in 0.3% Triton X-100 in Tris-buffered saline (TBS-T). Primary antibody incubations were conducted overnight at room temperature. Secondary antibody incubations were for 1.5 h at room temperature. Before all antibody incubations, washing steps consisted of 10 min TBS-T, 10 min TBS (Tris-buffered saline), and 10 min TBS-T. All other washing steps consisted of three times 10 min TBS incubation. For visualization of nuclei, a 30-min incubation with Hoechst 33342 (1:500; Sigma) was used. All co-cultures were double stained for S100β and MAP2/RT97. The purity of the enriched OEC and ONF cultures at the time of co-culture was >95% on the basis of presence and absence of S100β-positive cells, respectively.

Quantitative analysis of neurite outgrowth supported by the different glial cultures was performed on images of the double stained preparations (Olympus AX-70 microscope with epi-fluorescent illumination; ×20 objective; ×10 projection lens; narrowband MNIBA-type FITC filter to detect the signal for Alexa 488; MNG filter to detect the signal for Cy3; U-MNIBA filter to detect the signal for Hoechst; Sony Power HAD 3CCD Color Video Camera). All pictures were quantified with the anal-