TITLE:

Coculture of axotomized rat retinal ganglion neurons with olfactory ensheathing glia, as an in vitro model of adult axonal regeneration.

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KEYWORDS:

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SUMMARY:

We present an in vitro model to assess olfactory ensheathing glia (OEG) neuroregenerative capacity, after neural injury. It is based on a coculture of axotomized adult retinal ganglion neurons (RGN) on OEG monolayers and subsequent study of axonal regeneration, by analyzing RGN axonal and somatodendritic markers.

ABSTRACT:

Olfactory ensheathing glia (OEG) cells are localized all the way from the olfactory mucosa to and into the olfactory nerve layer (ONL) of the olfactory bulb. Throughout adult life, they are key for axonal growing of newly generated olfactory neurons, from the lamina propria to the ONL. Due to their pro-regenerative properties, these cells have been used to foster axonal regeneration in spinal cord or optic nerve injury models.

We present an in vitro model to assay and measure OEG neuroregenerative capacity after neural injury. In this model, reversibly immortalized human OEG (ihOEG) is cultured as a monolayer, retinas are extracted from adult rats and retinal ganglion neurons (RGN) are cocultured onto the OEG monolayer. After 96h, axonal and somatodendritic markers in RGNs are analyzed by immunofluorescence and the number of RGNs with axon and the mean axonal length/neuron are quantified.

This protocol has the advantage over other in vitro assays that rely on embryonic or postnatal neurons, that it evaluates OEG neuroregenerative properties in adult tissue. Also, it is not only useful for assessing the neuroregenerative potential of ihOEG but can be extended to different sources of OEG or other glial cells.

INTRODUCTION:

Adult central nervous system (CNS) neurons have limited regenerative capacity after injury or disease. A common strategy to promote CNS regeneration is transplantation, at the injury site, of cell types that induce axonal or neuronal growth such as stem cells, Schwann cells, astrocytes or olfactory ensheathing glia (OEG) cells[1-5].

OEG derives from the neural crest[6] and locates in the olfactory mucosa and in the olfactory bulb. In the adult, olfactory sensory neurons die regularly as the result of environmental exposure and they are replaced by newly differentiated neurons. OEG surrounds and guides these new olfactory axons to enter the olfactory bulb and to establish new synapses with their targets in the CNS[7]. Due to these physiological attributes, OEG has been used in models of CNS injury such as spinal cord or optic nerve injury and its neuroregenerative and neuroprotective properties become proven[8-11]. Several factors have been identified as responsible of the pro-regenerative characteristics of these cells, including extracellular matrix proteases production or secretion of neurotrophic and axonal growth factors[12-14].

Given the technical limitations to expand primary OEG cells, our group previously established and characterized reversible immortalized human OEG (ihOEG) clonal lines, which provide an unlimited supply of homogeneous OEG. These ihOEG cells derive from primary cultures, prepared from olfactory bulbs obtained in autopsies. They were immortalized by transduction of the telomerase catalytic subunit (TERT) and the oncogene Bmi-1 and modified with the SV40 virus large T antigen[15-18]. Two of these ihOEG cell lines are Ts14, which maintains the regenerative capacity of the original cultures and Ts12, a low regenerative line that is used as a low regeneration control in these experiments[18].

To assess OEG capacity to foster axonal regeneration after neural injury, several in vitro models have been implemented. In these models, OEG is applied to cultures of different neuronal origin and neurite formation and elongation, in response to glial coculture, are assayed. Examples of such neuronal sources are neonatal rat cortical neurons[19], scratch wounds performed on rat

embryonic neurons from cortical tissue[20], rat retinal explants[21], rat hypothalamic or hippocampal postnatal neurons[22,23], postnatal rat dorsal root ganglion neurons[24], postnatal mouse corticospinal tract neurons[25], human NT2 neurons[26] or postnatal cerebral cortical neurons on reactive astrocyte scar-like cultures[27].

In these models, however, the regeneration assay relies on embryonic or postnatal neurons, which have an intrinsic plasticity that is absent in injured adult neurons. To overcome this drawback, we present a model of adult axonal regeneration in cocultures of OEG lines with adult retinal ganglion neurons (RGNs), based on the one originally developed by Wigley et al.[28-31] and modified and used by our group [12-18, 32, 33]. Briefly, retinal tissue is extracted from adult rats and digested with papain. Retinal cell suspension is then plated on either polylysine-treated coverslips or onto Ts14 and Ts12 monolayers. Cultures are maintained for 96 h before they are fixed and then immunofluorescence for axonal (MAP1B and NF-H proteins)[34] and somatodendritic (MAP2A and B)[35] markers is performed. Axonal regeneration is quantified as percentage of neurons with axon, respect to total population of RGNs and axonal regeneration index is calculated as mean axonal length per neuron. This protocol is not only useful for assessing the neuroregenerative potential of ihOEG but can be extended to different sources of OEG or other glial cells.

PROTOCOL:

NOTE: Animal experimentation was approved by national and institutional bioethics committees.

1. **ihOEG (Ts12 and Ts14) culture.**

   NOTE: This procedure is done under sterile conditions in a tissue culture biosafety cabinet.

   1. Prepare 50 mL ME10 OEG culture medium as provided in Table 1.
   2. Prepare 5 mL of DMEM/F12-FBS, as provided in Table 1, in a 15 mL conical tube.
   3. Temperate both media at 37 ºC in a clean water bath, for 15 min.
   4. Thaw Ts12 and Ts14 cells vials at 37 ºC in a clean water bath.
   5. Resuspend and add cells to the DMEM/F12-FBS culture medium prepared in Step 2.
   6. Centrifuge for 5 min at 300 x g.
   7. Aspire the supernatant.
   8. Add 500 µL of ME10 medium and resuspend the pellet.
   9. Prepare a p60 cell culture dish with 3 mL of ME10 and add the cellular suspension, dropwise.
   10. Move to distribute the cells uniformly across the plate.
   11. Culture cells at 37 ºC in 5% CO2.

   NOTE: After reaching confluence, at least another passage must be done to optimize cells for coculture. 90% confluence is needed before seeding them on coverslips for coculture. A confluent p-60 has a mean cell number of 7×10^5 for Ts14 and 2.5×10^6 for Ts12 cell lines. Ts12 and Ts14 cell lines should be passaged every 2-3 days.

2. Preparation of ihOEG (Ts12 and Ts14) for the assay.

NOTE: This step must be done 24 h before RGN dissection and coculture.

1. Treat 12 mm Ø coverslips with 10 µg/mL poly-L-lysine (PLL) for 1 h.

NOTE: Coverslips can be left O.N.in PLL solution.

2. Wash coverslips with phosphate buffer saline (PBS) 1X, three times.
3. Detach Ts12 and Ts14 ihOEG cells from p60 cell culture dish:
   - 3.1 Add 4 mL of DMEM/F12-FBS culture medium (see Table 1) to a 15 mL conical tube. Temperate at 37 ºC in a clean water bath.
   - 3.2 Remove medium from plates and wash cells with 1 mL PBS-EDTA 1X, once.
   - 3.3 Add 1 mL trypsin-EDTA to the OEG cells and incubate for 3-5 min at 37 ºC, 5% CO2.
   - 3.4 Collect cells with a p1000 pipette and transfer them to medium prepared in step 3.1.
   - 3.5 Centrifuge for 5 min at 200 x g.
   - 3.6 Aspire the supernatant.
   - 3.7 Add 1 mL of ME10 medium and resuspend the pellet.
   - 3.8 Count cell number in a hemocytometer.
4. Seed 80,000 Ts14 cells or 100,000 Ts12 cells onto coverslips in 24 well plates in 500 µL of ME10 medium.
5. Culture cells at 37 ºC in 5% CO2, for 24 h.

3. Retinal tissue dissection.

NOTE: 2-month old male Wistar rats are used as RGN source. Two retinas (one rat) for 20 wells of a 24 well cell dish.

NOTE: Autoclave surgical material before use.

NOTE: Papain dissociation kit is commercially purchased (Table of Materials). Follow provider´s instructions for reconstitution.

NOTE: Reconstitute D,L-2-amino-5-phosphonovaleric acid (APV) in 5 mM stock and prepare aliquots.

1. On the day of the assay, prepare the following media:
   - 1.1 A p60 cell culture dish with 5 mL of cold EBSS (vial 1 of the papain dissociation kit).
   - 1.2 A p60 cell culture dish with:
     - 1.2.1 Reconstituted vial 2 (papain) of the papain dissociation kit plus 50 µL of APV.
     - 1.2.2 250 µL of vial 3 (DNAse) plus 5 µL of APV.
   - 1.3 In a sterile tube mix 2.7 mL vial 1 with 300 µL vial 4 (albumin-ovomucoid protease inhibitor). Add 150 µL vial 3 (DNAse) plus 30 µL APV.
   - 1.4 20 mL of Neurobasal-B27 medium (NB-B27) as provided in Table 1.
2. Sacrifice a rat by asphyxiation with CO2.
3. Remove the head by decapitation with guillotine, place it in a 100 mm Petri dish and spray the head with ethanol 70% before placing it in a laminar flow

hood.

4. Cut the rat´s whiskers with scissors so they do not interfere with the eye manipulation.
5. Grip the optic nerve with forceps to pull out the eyeball enough to be able to make an incision across the eye with a scalpel.
6. Remove the lens and vitreous humor and pull out the retina (orange-like tissue), while the remaining layers of the eye stay inside (including the pigment epithelial layer).
7. Place the retina in the p60 cell culture dish prepared in Step 3.1.1.
8. Transfer the retina to the p60 cell culture dish prepared in Step 3.1.2 and cut it with the scalpel in small pieces of an approximate size < 1 mm.
9. Transfer to a 15 mL plastic tube.
10. Incubate the tissue for 30 min, in a humidified incubator at 37 ºC under 5% CO2, with agitation every 10 min.
11. Dissociate cell clumps by pipetting up and down with a glass Pasteur pipette.
12. Centrifuge the cell suspension at 200 x g for 5 min.
13. Discard supernatant and to inactivate papain, resuspend the cell pellet in the solution prepared in step 3.1.3. (NOTE: 1.5 mL for 2 eyes).
14. Pipet carefully this cell suspension onto 5 mLµ of reconstituted vial 4.
15. Centrifuge at 200 x g for 5 min.
16. While centrifuging, completely remove the ME-10 medium from the OEG 24 well cell plate (previously prepared in Step 2 of the protocol - Preparation of ihOEG (Ts12 and Ts14) for the assay -) and replace it with 500 µL of NB-B27 medium, per well.
17. Discard supernatant and resuspend the cells in 2 mL of NB-B27 medium.
18. Plate 100 µL of retinal cell suspension, per well of the m24 plate, onto PLL-treated or OEG monolayers-coverslips.
19. Maintain cultures at 37 ºC with 5% CO2 for 96 h in NB-B27 medium.

4. Immunostaining

1. After 96 h, fix cells for 10 min by adding the same volume of 4% paraformaldehyde (PFA) in PBS1X to the culture medium (600 µL) (PFA final concentration 2%).
2. Remove media and PFA from 24 multiwell plate and add again 500 µL of 4% paraformaldehyde (PFA) in PBS1X. Incubate for 10 min.
3. Discard fixer and wash 3 times with PBS1X for 5 min.
4. Block with 0,1% Triton X-100/1% FBS in PBS (PBS-TS) for 30-40 min.
5. Prepare primary antibodies in PBS-TS buffer as follows:
    5.1 SMI31 (against MAP1B and NF-H proteins) monoclonal antibody (1:500).
    5.2 514 (recognizes MAP2A and B proteins) rabbit polyclonal antiserum (1:400).
6. Add primary antibodies to cocultures and incubate overnight at 4 ºC.
7. Next day, discard antibodies and wash coverslips with PBS1X, 3 times, for 5 min.
8. Prepare secondary antibodies in PBS-TS buffer as follows:
    8.1 For SMI-31, anti-mouse Alexa Fluor 488 (1:500).

8.2 For 514, anti-rabbit Alexa-594 (1:500).

9. Incubate cells with the corresponding fluorescent secondary antibodies for 1h, at RT, in the dark.
10. Wash coverslips with PBS1X, 3 times, for 5 min, in the dark.
11. Finally, mount coverslips with mounting medium (see Table of Materials) and keep at 4ºC.

NOTE: Whenever necessary, fluorescent nuclei staining with DAPI (4,6-diamidino-2-phenylindole) may be performed. Before mounting, incubate cells for 10 min in the dark with DAPI (10 µg/mL in PBS1X). Wash coverslips 3 times with PBS1X and finally, mount coverslips with mounting medium.

5. Axonal regeneration quantification

NOTE: Samples are quantified under the 40x objective of an epifluorescence microscope. A minimum of 30 pictures should be taken on random fields, with at least 200 neurons, to be quantified for each treatment. Each experiment should be repeated a minimum of three times.

1. Quantify the percentage of neurons with axon (SMI31 positive neurite) relative to total population of RGNs (identified with MAP2A/B 514 positive immunostaining of neuronal body and dendrites).
2. Quantify the axonal regeneration index or mean axonal length (µm/neuron). This parameter is defined as the sum of the lengths (in µm) of all identified axons, divided by the total number of counted neurons, whether they presented an axon or not. Axonal length is determined using the plugin NeuronJ of the image software ImageJ (NIH-USA).
3. Calculate mean, standard deviation, and statistical significance using appropriate software.

REPRESENTATIVE RESULTS:

In this protocol we present an in vitro model to assay OEG neuroregenerative capacity after neuronal injury. As shown in Figure 1, the OEG source is a reversible immortalized human OEG clonal cell line -Ts14 and Ts12-, which derives from primary cultures, prepared from olfactory bulbs obtained in autopsies[15, 17, 18]. Retinal tissue is extracted from adult rats, digested and retinal ganglion neurons (RGN) suspension is plated on either PLL-treated coverslips or onto ihOEG monolayers, Ts14 or Ts12. Cultures are maintained for 96 h before they are fixed. Axonal and somatodendritic markers are analyzed by immunofluorescence and axonal regeneration is quantified.

Figure 1. Diagram of rat retinal ganglion neurons with olfactory ensheathing glia cells coculture, as a model of adult axonal regeneration. Immortalized human OEG (ihOEG) clonal cell lines -Ts12 and Ts14- derive from primary cultures from olfactory bulbs. Retinal ganglion neurons from adult rats are plated on either PLL-treated coverslips (negative control) or onto Ts14 or Ts12 monolayers. Cultures are maintained for 96 h before they are fixed and axonal and somatodendritic markers are analyzed by immunofluorescence. Percentage of neurons with axon

and mean axonal length/neuron are quantified to assay RGN axonal regeneration.

Ts14 OEG identity is assessed by immunostaining with markers described to be expressed in ensheathing glia (Figure 2), such as S100 β (2A) and vimentin (2B); GFAP expression was also analyzed to discard astrocyte contamination (2C). As shown, Ts14 expressed S100 β and vimentin but not GFAP.

Figure 2. Identity of ihOEG cell line Ts14. Immunofluorescence images of Ts14 in culture, labeled with anti-S100 β (panel A, green) and vimentin (panel B, red). GFAP expression (panel C, red) was also analyzed to discard astrocyte contamination. Nuclei are stained with DAPI (blue).

In the axonal regeneration assay, Ts14 regenerative capacity is compared to Ts12 in RGN-OEG cocultures, using PLL substrate as a negative control (Figure 3). Both the percentage of cells with axons as well as the average length of the regenerated axons were significantly higher in neurons cocultured on Ts14 monolayers, compared to neurons plated on either Ts12 cells or PLL (3D, E). Representative images show a lack of capacity of RGN to regenerate their axons over PLL or Ts12 cells (3A, B), while Ts14 stimulates the outgrowth of axons in RGN (3C).

Figure 3. Assay for axonal regeneration in cocultures of OEG lines with adult retinal ganglion neurons (RGNs). (A-C) Immunofluorescence images showing somatodendritic labelling with 514 antibody, which recognizes microtubule-associated protein MAP2A and B, in red, and with axon-specific SMI31 antibody in green, against MAP1B and NF-H proteins. Green arrows indicate RGN axons (SMI31-positive: green) and yellow arrows indicate neuronal bodies and dendrites (514 positive: red and yellow). **(D, E)** Graphs show mean and standard deviation of the percentage of neurons exhibiting axons and the axonal regeneration index, a parameter reflecting the mean axonal length (μM) of axons per neuron. A minimum of 30 pictures (40x) were taken on random fields and quantified for each cell sample. Experiments were performed in triplicate, from three different rats (N=3), retinal tissue pooled from both eyes, with duplicates for each experimental condition (each glia population tested). Asterisks indicate the statistical significance: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, NS: non significance (ANOVA and post hoc Tukey test comparisons between parameters quantified for Ts14 vs Ts12, Ts14 vs PLL and Ts12 vs PLL).

DISCUSSION:

OEG transplantation at CNS injury sites is considered a promising therapy for CNS injury due to its constitutive pro-neuroregenerative properties[7-9]. However, depending on the tissue source - olfactory mucosa (OM-OEG) versus olfactory bulb (OB-OEG) - or age of donor, considerable variation exists in such capacity[26, 31, 33, 36]. Therefore, it is of importance to have an easy and reproducible in vitro model to assay the neuroregenerative capacity of a given OEG sample, before initiating in vivo studies. In the protocol described in this work, adult rat axotomized RGN are cocultured onto a monolayer of the OEG to assay. Subsequent analysis of RGN axonal and somatodendritic markers by immunofluorescence is performed to assess RGN axonal regeneration.

An initial difficulty of the assay is the source of OEG. In this work we use reversible immortalized

human OEG (ihOEG) clonal lines, previously established and characterized by our group[15-18], which provide an unlimited supply of homogeneous OEG. Two of these ihOEG cell lines are Ts14, which maintains the regenerative capacity of the original cultures and Ts12, a low regenerative line that is used as a low regeneration control in these experiments[18] Nevertheless, although technical limitations exist to expand human primary OEG cells, they can also be obtained from nasal endoscopic biopsies – OM- or, in case of OB-OEG, from cadaver donors.

Preparation of monolayer OEG cultures is a crucial procedure, as too many cells could cause the coculture to detach from the plate. Therefore, previously to OEG preparation for the assay, it is recommended that the user determines the optimal number of cells to be plated, depending on their size and division rate.

Another critical issue is retinal tissue dissociation, after retina dissection. It is necessary to break up the tissue fragments, following incubation in the dissociation mix. If done too vigorously cells will be destroyed, but tissue fragments will be left intact if done too weakly. In order to obtain a homogeneous cell suspension, we suggest filling and emptying a Pasteur pipette for 10-15 times, with a tip of intermediate diameter, while avoid bubbling. Pasteur pipettes with wide tips can be narrowed by using a Bunsen burner.

To assess the capacity of different glial populations to foster adult neurons axonal regeneration, we have determined that 96h is the time interval that best suits our aim because: 1) it is the longest time to maintain the culture alive without disturbing the OEG monolayer; 2) it is the time needed for neurons to grow axons long enough to reveal differences between the regenerative capacities of different OEG populations or other non-regenerative cells, i.e. fibroblasts[12-18,32,33]. It would certainly be interesting to determine the time course of the regeneration process, as it could provide information about the differential regenerative properties of the different glial populations, at shorter times of the co-culture. In our hands, for regenerative glia, the time course between 72-96 h is quite similar for all the cell lines, although axons are shorter at 72 h (unpublished data). Also, 96 h of co-culture, permits to study OEG-dependent mechanisms of adult axonal regeneration[12,14].

During axonal regeneration quantification, it is important to take a minimum of thirty pictures at 400 augments (40x objective), at different random areas of the coverslip, but following the complete axons of the photographed neurons. Therefore, the experimenter must take serial pictures in the chosen areas, to measure the real axonal lengths.

Other in vitro approaches have also been developed to evaluate OEG regenerative functions. In these models, OEG is applied to cultures of different neuronal origin and, in response to glial coculture, neurite formation and elongation are assayed[19-27]. However, the regeneration assay relies on embryonic or postnatal neurons, which have an intrinsic plasticity absent from injured adult neurons. Our model of adult axonal regeneration in cocultures of OEG lines with adult retinal ganglion neurons (RGNs) overcomes this drawback. In addition, we are dissecting adult retinas, and because we cut optic nerve and axons retract in the process of dissection, we obtain neuronal bodies clean of myelin, to perform the coculture. This is the difference with other parts of the adult CNS, where myelin can hinder very much the dissection to obtain clean neurons for the coculture.

Based on the one originally developed by Wigley et al[28-31], we highlight the following improvements in our protocol. First, the use of neurobasal medium supplemented with B27 as OEG-RGN coculture medium, which allows growth of neuronal cells and positively affects the

reproducibility of the experiment. Second, we characterize and quantify axonal regeneration by using a specific marker of the axonal compartment; and third, we use an additional direct parameter, the mean axonal length/neuron, that assesses the axonal growth regenerative potential of OEG.

In summary, we consider this is a simple, reproducible, time saving and medium-cost assay, not only useful for assessing the neuroregenerative potential of ihOEG, but also because it can be extended to different sources of OEG or other glial cells. Moreover, it could be used as a valuable proof of concept of the neuroregenerative potential of an OEG or glial sample, before translation to in vivo or clinical studies.

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DISCLOSURES:

The authors have nothing to disclose.

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