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Isolation and Culture of Spinal Cord Astrocytes

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Abstract

Astrocytes are possibly the most numerous cells of the vertebrate central nervous system, yet a detailed characterization of their functions is still missing. One potential reason for the obscurity of astrocytic function is that they represent a diverse population of cells that all share some critical characteristics. In the CNS, astrocytes have been proposed to perform many functions. For example, they are supportive cells that provide guidance to newly formed migrating neurons and axons. They regulate the functions of endothelial cells at the blood brain barrier, provide nutrients, and maintain homeostasis including ionic balance within the CNS. More recently, dissecting the central role of astrocytes in mediating injury responses in the CNS, particularly the spinal cord, has become an area of considerable importance. The ability to culture-enriched populations of astrocytes has facilitated a detailed dissection of their potential roles in the developing and adult, normal, and injured brain and spinal cord. Most importantly, in vitro models have defined molecular signals that may mediate or regulate astrocyte functions and the capacity to modulate these signals may provide new opportunities for therapeutic intervention after spinal cord injury and other neural insults.

Keywords

Adult astrocytes; Astrocyte culture; Culture; GFAP; Glia; Postnatal astrocytes; Purification; Spinal cord; Spinal cord astrocytes; Type 1 Astrocytes

1. Introduction

The vertebrate brain and spinal cord is comprised of two major classes of cells: neurons, the canonical electrically active cells that are the basis of neural networks underlying all aspects of nervous system function, and glia. Classical studies identified glia as the support cells or glue that held nervous tissue together. However, studies in the last three decades have revolutionized our understanding of the cellular complexity and functions of glial cells in the CNS. Glial cells in the CNS can be segregated into three major classes: (a) microglia, which represent the macrophages of the CNS (1), are derived from the hematopoietic system and act as the primary phagocytic cells in removing damaged tissue; (b) oligodendrocytes, which are derived from the neuroepithelium (2, 3) and are the primary myelinating cells of the brain and spinal cord, and (c) astrocytes. A more recently identified glial cell population is the adult oligodendrocyte precursor (OPC) also termed polydendrocytes (4). These cells that have been proposed to constitute as much as 5% of the cells in the adult CNS are thought to be precursors that can replenish cells lost to damage or disease, although recently they have been shown to be electrically active (5, 6). During development, multiple studies have shown that OPCs with similar characteristics to the adult cells are capable of generating both

oligodendrocytes and a subset of astrocytes depending on the conditions under which they are grown (7).

Astrocytes have been proposed to perform many functions in the developing and adult CNS. It seems likely that astrocytes are a heterogeneous population of cells, all of which share some characteristics such as the expression at some point in their development of intermediate filaments comprised of glial fibrillary acidic protein (GFAP) (8–11). Astrocytes are important for the formation and support of the blood–brain barrier via their end feet associated with the endothelium (12–15). They have also recently been shown to be linked through gap junctions that allow the propagation of calcium signals between coupled cells, facilitating glia–glia communication (16–18). Interestingly, this communication is closed in response to injury. A functional role of astrocytes in regulating synaptic activity has also been defined. Fine processes of astrocytes are found to envelope the synapses of neurons and form many networks within the CNS (19, 20). Astrocytes are also important for providing structure to the brain and spinal cord, maintaining efficient synaptic function and metabolism, and responding to pathological insults in the CNS by sequestering areas of injury (21–24).

It seems likely there are multiple classes of astrocytes in the spinal cord. Lineage studies suggest that one class of astrocytes termed Type 1 astrocytes arises from a glial-restricted precursor cell and not from a bipotential oligodendrocyte precursor. Several studies implicate fibroblast growth factor (FGF) signaling in the emergence of these cells in both dorsal and ventral regions of the embryonic spinal cord. By contrast, OPCs that give rise to type 2 astrocytes arise in a specific region of the ventral spinal cord as a result of local signals, including sonic hedgehog, and disperse widely through the spinal cord. Type 2 astrocytes derived from OPCs are found mainly in tracts of myelinated axons and associated with nodes of Ranvier; however, their precise function is unknown (25), although they have been implicated in spinal cord injury responses.

All classes of astrocytes are thought to react to injury via up-regulation of GFAP expression, proliferation, and process expansion. Depending on the timing of injury, these set of reactions, collectively termed astrogliosis, may or may not be detrimental to repair (26–30). Astrocytes form a fibrous scar that has been proposed to impede regeneration (31) and such glial scar formation is thought to be mediated by type 1 astrocytes (32). Many CNS insults are characterized by axonal damage, demyelination, and presence of glial scarring. This glial scarring is a consequence of astrocyte and glial precursor responses and is manifested by up-regulation of a number of intermediate filament proteins including GFAP and vimentin (31, 33, 34). In addition, glial scarring is typically associated with up-regulation of a number of extracellular matrix (ECM) proteins known as chondroitin sulfate proteoglycans (CSPGs), which create a nonpermissive environment for axonal regeneration (35) for example, NG2. The glial scar is thought to create a chemical and physical barrier, preventing axonal repair and remyelination of injured tissue. The precise roles of astrogliosis and the formation of glial scars are poorly understood, but may reflect stimulation by members of the bone morphogenetic protein family (BMPs) (36). It remains uncertain as to whether astrogliosis is detrimental or beneficial to repair. Ablation of proliferating reactive astrocytes in transgenic mice has demonstrated that these cells are important for the spatiotemporal regulation of inflammation after CNS injury (37, 38). An understanding of the glial scar and its influences on cell migration, proliferation, and differentiation will be beneficial to designing techniques to modulate ongoing damage, improve remyelination, and enhance axonal function after spinal cord injury. The ability to identify, isolate, and grow spinal cord astrocytes in culture has provided critical insights into the lineage association, origins, and biological properties of these complex cells. The methods for isolation and growth of spinal cord astrocytes are outlined in this chapter.

2. Materials

1. Dulbecco's modified eagle's medium (DMEM) supplemented with 1% penicillin–streptomycin and 10% fetal bovine serum (FBS).
2. GlutaMax (Gibco/Invitrogen; 200 mM).
3. 50 mg/ml poly-L-lysine (working solution: 25 mg/500 μ l dissolved in distilled water, stir 1 h, and sterile-filtered).
4. 0.1% trypsin: dissolve 100 mg of trypsin into 100 ml CMF-DMEM, stir until dissolved, filter sterilize, aliquot into 1 ml aliquots.
5. Deoxyribonuclease I (1 mg/ml in DMEM).
6. Calcium- and magnesium-free modified eagle's medium (CMF-MEM) (Gibco/Invitrogen).
7. Solution of CMF-MEM containing 0.25% ethylenediamine tetraacetic acid (EDTA) (Gibco/Invitrogen).
8. 60 mm tissue culture-treated petri dishes.
9. Tissue culture flasks, 75 cm², tissue culture treated with filter cap.
10. Two pairs of five forceps, scalpel blade, and holder or razor blade, large scissors, small 3-mm blade Vannas spring scissors; all autoclaved prior to use.
11. Papain (Worthington Biochemical). Dissolve 20 mg of Papan in 5 ml of MEM. Warm the solution for 5 min at 37°C. Filter sterilize the solution and store at 4°C in a 50 ml sterile plastic centrifuge tube for no more than 3 h.
12. 30 μ m mesh nitex funnel.
13. 15 ml conical tubes.
14. MOPS/Saline stock. Dissolve 0.21 g MOPS and 0.8 g NaCl in 100 ml of dd H₂O to make a solution of 10 mM MOPS, 137 mM NaCl.
15. Optiprep stock solution (Accurate Chemical). Combine 0.495 ml of Optiprep and 0.505 ml of MOPS/Saline solution to make 1 ml of stock solution.
16. Optiprep gradient:
From bottom of a 15 ml conical tube:
0–1 ml: 35% = combine 350 μ l Optiprep stock with 650 μ l DMEM with 10% FBS.
1–2 ml: 25% = combine 250 μ l Optiprep stock with 750 μ l DMEM with 10% FBS.
2–3 ml: 20% = combine 200 μ l Optiprep stock with 800 μ l DMEM with 10% FBS.
3–4 ml: 15% = combine 150 μ l Optiprep stock with 850 μ l DMEM with 10% FBS.

3. Methods

3.1. Preparation of Astrocyte Cultures from Early Postnatal Rodent Spinal

1. Prepare poly-L-lysine (PLL)-coated flasks by adding 5–7 ml PLL (50 mg/ml) to a T75 flask and incubate 1 h in a 37°C incubator. Before use, wash 3 $\times$ using sterile water (see Note 1).

2. Ensure sterile conditions by using aseptic technique and frequent use of ethanol wipes and replacement of gloves. Flame sterilize the instruments and container tops as needed.
3. Place a 60-mm petri dish containing CMF-MEM on a sterile ice pack in the tissue culture hood.
4. Place a litter (at least ten pups) of P2 rat pups in a CO₂ chamber for anesthesia. Wait until they have ceased respiration.
5. Clean the outer surface of the animals with ethanol and dry. Decapitate using large scissors and a quick motion.
6. Place the torso on its ventral surface to expose the dorsal aspect and cut along the midline. Remove the skin and expose the spinal column by removing the fat pad and trapezius muscle associated with this area.
7. Using the Vannas scissors, cut down the length of the spinal column bilaterally removing vertebra. Begin at the most rostral region and move down the length of the cord, caudally. End near the bottom of the spinal cord marked by the hip region.
8. Run the forceps along the length of the cord so as to remove all dorsal root ganglia. Using forceps, remove the entire spinal cord and place in a 60-mm petri dish containing CMF-MEM. Repeat for all the animals (see Note 2).
9. After completion of the initial dissection of the spinal cord, remove the meninges covering the outer surface of the spinal cord under a dissecting microscope. Successful removal of the meninges is important to avoid subsequent contamination of the astrocytes cultures with fibroblasts. Add the cleaned spinal cord tissue to a new 60-mm dish containing a drop of CMF-MEM. Chop the tissue into a slurry using a scalpel or razor blade to allow enzymatic digestion to be effective. Add 1 ml of 0.25% EDTA in CMF-MEM to the plate, then add 1 ml of 0.1% trypsin.
10. Incubate the tissue at 37°C in a CO₂ balanced incubator for 20 min until the chemical digestion is complete. In the mean-time, flame a plugged Pasteur pipette to remove the sharp edges that will damage cells on trituration, and ensure that the media is warmed to 37°C.
11. After the 20 min incubation, add 5 ml DMEM with 10% FBS to stop the trypsin enzymatic action. Add 350 µl of DNase to the plate and triturate the cell suspension using the fire-polished pipette, being careful not to generate air bubbles, as this will cause cell lysis and a subsequent dramatic drop in cell yield (see Note 3). Triturate the entire solution approximately 10×.

¹It is important to thoroughly wash the PLL-coated flasks/plates. If cell viability is low after plating, it is often a reflection of an insufficient washing following PLL coating. Increased washes usually solve this problem. If plating onto coverslips, ensure they have been thoroughly washed before use, since traces of residual cutting oils will kill neural cells.

²In order to facilitate the removal of the meninges from the spinal cord, it is important to keep the tissue as intact as possible. When dissecting the spinal cord, hold the animal with dorsal side up between your thumb and forefinger in order to immobilize the animal. Using small Vannas scissors, remove the spinal column by cutting down each side laterally. Remove vertebrae as you move rostral to caudal and keep sight of the spinal cord by removing debris and blood. Keep the spinal cord within the confines of the spinal column except for the upper most portions. Using forceps, disconnect the dorsal root ganglia before trying to excise from the spinal column since these will tear the meninges and damage the cord tissue.

³If cell viability is low at the time of plating, it is likely due to one of two steps. First, most likely the operator allowed air bubbles to enter the pipette during trituration. This must be done with great care to avoid excess mechanical sheer forces. Reducing the speed of trituration and leaving a small amount of fluid in the tube at all times will solve this problem. Second, the tissue is being over trypsinized. The solution to this is to reduce the digestion interval until viability improves.

12. Remove the nondissociated tissue by filtering cells through a sterile 30 μ m mesh nitex funnel into a clean 15 ml conical tube. If the solution is very viscous, add more DMEM with 10% FBS and replace the filter if it becomes blocked.
13. Centrifuge at 1,500 rpm for 5 min in a bench top centrifuge. Decant the liquid from the tube. Resuspend the cells in 10 ml of DMEM with 10% FBS. Add cells to a PLL-coated flask, place in sterile incubator at 37°C and 5% CO₂, and allow 24 h for cell adherence.
14. Twenty four hours later, shake the flask at 200 rpm at 37°C overnight. The next day, remove the nonadherent oligodendrocytes, neurons, and microglial cells, wash the plate gently with DMEM containing 10% FBS. Add back 10 ml DMEM with 10% FBS. Allow the cells to recover and proliferate for 1 week prior to plating for the final experiment.

3.2. Preparation of Astrocytes Cultures from the Adult Rodent Spinal

Compared to neonatal rodent spinal cord cultures, the yield and purity of adult spinal cord cultures is generally lower. This is a reflection of the difficulties of dissociating viable cells from intact adult tissue. Spinal cord injury or ongoing neural disease generally increases the ease of dissociation and subsequent yield of viable cells. Because of the increased tissue debris that is generated during dissociation of adult tissue, it is important to isolate viable cells using differential centrifugation through a step gradient (see below).

1. Perfuse a terminally anesthetized adult rat through the ascending aorta with 200 ml of cold saline. Decapitate and remove the spinal cord from the column using a dorsal approach and place in cold DMEM with 10% FBS.
2. Remove the meninges and chop the tissue finely using a scalpel or razor blade to generate a tissue slurry. Transfer the slurry to a 50-ml conical tube containing 5 ml of 4 mg/ml Papain in DMEM. Incubate in a 37°C incubator with gentle shaking to ensure that the spinal cord tissue remains suspended for a minimum of 2 h. (see Note 4).
3. Centrifuge at 2,000 rpm for 3 min. Aspirate the medium and add 5 ml of DMEM with 10% FBS. Triturate with a fire-polished Pasteur pipette at least 10 $\times$, taking care not to introduce air bubbles into the medium (see Note 3). Add a further DMEM with 10% FBS to make up to a final volume of 15 ml.
4. Filter the solution through a 30- μ m nitex mesh filter into a 15-ml conical tube. Centrifuge at 1,500 rpm for 5 min. Aspirate off the medium, resuspend the cells in DMEM with 10% FBS up to 6 ml.
5. Carefully add the 6-ml cell suspension to the top of a gradient of Optiprep in a 15-ml centrifuge tube. The Optiprep gradient is made in four 1-ml steps, 35%, 25%, 20%, and 15%. Optiprep stock in DMEM with 10% FBS supplement. Centrifuge for 15 min at 2,200 rpm at room temperature.
6. Discard the debris from the densest band and above. This band will appear within the 15% layer (see Fig. 1). Collect the cells and solution from the center, approximately 1 ml above the pellet (usually the 25% and a portion of the 20% layer) and transfer to another 15-ml tube. Fill the tube with DMEM containing 10% FBS. Centrifuge for 5 min at 2,000 rpm, remove the supernatant, and resuspend the cells in DMEM with 10% FBS.

⁴The exact time of incubation varies depending on the size of the spinal cord and the fineness of the mincing process, but 2 h is the minimum.

7. Plate the cells in a T75 flask that has been previously coated with poly-L-lysine, and incubate overnight in an incubator at 37°C and 5% CO₂ to allow the cells to adhere.
8. Next day, place the flasks in a 37-C shaker and shake overnight at 200 rpm to remove loosely adherent neurons and glia.
9. After the overnight shake, wash the adherent cells in the flask extensively with fresh DMEM with 10% FBS and feed the residual cells (astrocytes) with fresh media.
10. Allow the adherent astrocytes to recover and expand over the course of 10–14 days, changing the media every 3–4 days before beginning each experiment.

3.3. Analysis of Cell Purity

To assess the composition of the cultures, they should be labeled with antibodies to GFAP to determine the proportion of GFAP+astrocytes (see Notes 5–⁷). In general, this is accomplished after a period of recovery and expansion. The cells are removed from the flask by a combination of cooling and enzymatic digestion and replated onto Poly-L-lysine-coated coverslips for immunofluorescent analyzes using cell type-specific antibodies. A major concern when using differential adhesion to prepare enriched cultures of neonatal or adult astrocytes is the presence of contaminating cells from other lineages. Extensive shaking removes the majority of nonadherent cells including neurons, oligodendrocytes, and microglial cells. For most studies, enrichment in the range of 85–90% GFAP + cells is sufficient (see Notes 8 and ⁹); however, if greater purity is required, contaminating cells can be removed by complement-mediated cell lysis. For example, to eliminate contaminating fibroblasts and oligodendrocytes, the following approach is effective.

1. Six milliliters each of hybridoma supernatant from monoclonal antibodies A2B5 (1:5), O4 (1:5), and Thy1.1(1:100) are added to the flask and incubated at 37°C for 30 min to allow for antibody binding to cells of the oligodendrocyte (A2B5 and O4) and fibroblast (Thy1.1) lineages.
2. Guinea pig complement is added to the flask to a final dilution of a 1:10 in the continued presence of the antibody and incubated for 30 min at 37°C. This will result in targeted cell lysis of those cells that bound the antibody.
3. The reaction is stopped by rinsing the cells with fresh DMEM with 10% FBS and fresh media is added. Allow cells to recover for 1–2 days prior to experiments. The

⁵It is important to ensure the purity of astrocytes prior to experimental procedures in order to provide validity to your experiments. Astrocyte purity can sometimes be achieved by shaking alone. Significant variation exists regarding the outcome of shaking. If shaking is too rigorous, it results in widespread cell death, but too weak shaking results in unacceptable contamination. Cellular contamination must be assessed empirically using immunocytochemistry and followed by antibody-mediated complement lysis to further purify if needed. Primary Astrocyte cultures often harbor residual microglia cells and OPCs. If used soon after purification, this is minimized; however, if the astrocyte cultures are allowed to remain for extended culture periods, the level of contamination can rise to unacceptable levels, depending on the design of the studies.

⁷Cell purity is assessed after cells reach confluency and plated on PLL-coated coverslips. While it is relatively easy to eliminate contaminating fibroblasts, neurons, and oligodendrocytes through shaking and repeated rounds of complement-mediated cell lysis, it is more challenging to eliminate all microglia. Extensive shaking eliminates most but not all microglia, and over extended culture periods, microglial contamination will increase. The most effective studies are conducted rapidly after removing contaminating cells.

⁸Astrocytes are particularly responsive to environmental cues. Most studies require the removal of serum to obtain clear molecular insights and slow withdrawal of serum and replacement with serum-free supplement (e.g., 1% N2 (Invitrogen)) is important. This environmental response is also important to consider when designing in vitro studies.

⁹As in other regions of the CNS, spinal cord astrocytes are a heterogeneous population. Mixed cultures contain several morphologically distinct populations of GFAP + cells including classical type 1 and 2 cells (Fig. 2). Clonal studies have demonstrated several distinct classes of cells (39) suggesting multiple astrocyte lineages. Recent studies have begun to define the molecular control of spinal cord astrocyte development in vivo (40, 41) and further culture studies with defined cell populations are likely to provide a more complete understanding of the role of astrocytes in spinal cord development as well as spinal cord injury, demyelination (36), and neurodegenerative diseases such as Amyotrophic Lateral Sclerosis.

effectiveness of the cell elimination is assayed by labeling with cell type-specific antibodies. If needed, this protocol can be repeated every 1–2 days until no contaminating cells are detected.

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Fig. 1. Representation of the Optiprep gradient. The material layering above the 15% should be discarded as should the pellet. The fractions between the 20% and the 35% layer represent the enriched viable cell fractions and should be kept and resuspended.

Fig. 2.

Spinal cord astrocytes are morphologically and biochemically heterogeneous. Astrocytes generated from postnatal day 2 rat spinal cords later assessed to be of 95% purity by GFAP immunohistochemistry. Cultures initially contained a majority of process bearing type-2 astrocytes (**a**); with extended period in culture (14 days), the cultures become increasingly populated with flat fibroblastic type 1 astrocytes (**b**). This transition likely reflects a proliferative advantage of type 1-astrocyte precursors under the conditions in which the cells were grown rather than a phenotypic transformation.