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A novel and rapid method for culturing pure rat spinal cord astrocytes on untreated glass

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Abstract

Astrocytes are the major population of glial cells, and are key players in the development, maintenance, and functioning of the central nervous system (CNS). Their potential as targets of therapeutic intervention following CNS injury makes the elucidation of their cellular and subcellular physiology a primary research goal. Well defined and pure cell culture systems are required to examine astrocytic physiology, biochemical pathways and underlying responses to pathophysiologically altered conditions. Previously published protocols for establishing primary astrocyte cultures are time- and resource-consuming or suffer high contamination from other undesired cell types. Here we describe a new and simple procedure for producing highly pure ($>99\%$) rat primary astrocyte cultures. The method involves a simple mechanical dissociation of harvested spinal cord tissue through a porous membrane and the subsequent plating of the cells on plain, untreated glass coverslips. Astrocytes adhere very well to the untreated glass while other cell types require a substrate such as poly-L-lysine. The method described here is, therefore, ideal for experiments which require highly pure astrocyte cultures.

Keywords: Astrocytes; Culturing; Spinal Cord; Rat; CNS; Non-enzymatic; Immunocytochemistry; Confocal microscopy

1. Introduction

Astrocytes constitute the major class of cells in the central nervous system (CNS). They are no longer considered to be passive structural and trophic supports for neurons, but rather play important and integral roles in the physiological functioning of the CNS in both health and disease (Travis, 1994; Largo et al., 1996). Astrocytes participate in ionic homeostasis by siphoning away potentially excitotoxic factors such as excess extracellular glutamate and K^+, and are involved in CNS communication and information processing through interactions with neurons and other astrocytes (Travis, 1994; Nedergaard, 1994; Walz, 1989). Glia in general, and astrocytes in particular, have also been directly and indirectly implicated in the pathophysiology of various traumatic and neurodegenerative diseases (Mattson et al., 1996; Schipper, 1996). Of particular clinical interest, they have demonstrated properties which may be targeted in novel therapeutic initiatives based on the neuroprotective properties they display following neuronal injury in vitro (Bruno et al., 1997) and in brain and spinal cord injuries (Theriault, 1995; Mukhin et al., 1996; Agrawal et al., 1997).

It is critical to have well characterized pure astrocyte in vitro systems which allow the isolation and study of specific components of astrocyte physiology and bio-

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chemistry by well established electrophysiological, molecular, and immunohistochemical techniques. In general, three broad techniques have been used for the dissociation and establishment of astrocyte cultures: mechanical dissociation through a porous membrane; mechanical shaking and separation of cellular strata; and enzymatic dissociation. Different protocols vary in their details but most use one (Weibel et al., 1985; Aloisi et al., 1988; Hansson and Ronback, 1989; Hertz et al., 1989; Lim and Miller, 1989) or a combination (McCarthy and De Vellis, 1980; Guthrie et al., 1987) of the above basic mechanisms while at the same time exploiting such parameters as age of the animal at dissection (for primary cultures), feeding schedules, media composition, and chemical inhibitors of contaminating cell types, in order to promote astrocyte growth and discourage the survival and/or growth of other cells.

Typically, methods which aim at establishing highly pure astrocyte cultures have been time- and resource-consuming, and technically complex, while simpler methods yielded cultures that, depending on the procedure, are contaminated with fibroblasts, microglia, oligodendrocytes, endothelial cells, ependymal cells and (occasionally) even neurons. For example, protocols employing protease treatments with enzymes such as trypsin require careful monitoring of temperature and pH (Banker and Goslin, 1991) and incubation periods ranging from minutes to hours (Banker and Goslin, 1991). Separation by mechanical shaking is very time consuming, since it requires shaking of the cultures for 12 h (McCarthy and De Vellis, 1980).

Techniques based on membrane mechanical dissociation while quick and relatively easy to perform, produce cultures with extensive contamination from other cell types such as macrophages (Hertz et al., 1989) and fibroblasts (Lim and Miller, 1989). Eliminating these contaminating cell types after the establishment of primary astrocyte cultures are as time- and resource-consuming as the methods developed to avoid them in the first place (see Estin and Vernadakis, 1989; Levison and McCarthy, 1991).

In an attempt to address these issues, we have developed a simple non-enzymatic technique for culturing astrocytes which takes advantage of the ease and minimal time required for culturing offered by mechanical membrane dissociation methods, and which produce astrocyte cultures which are 99% pure.

2. Materials and methods

2.1. Cell cultures

The protocol used for obtaining spinal cord astrocyte cultures is a combination of several previously published techniques (c.f. Black et al., 1993; Richardson et al., 1996). Newborn (0–12 h) Wistar rat pups were deeply anesthetized by cold standard operating procedure (SOP) and decapitated. The skin and muscles overlying the spinal column were quickly dissected away, and vertebrae from the mid-cervical to mid-lumbar spinal cord were cut by a single incision with a No. 15 scalpel. Enough force was applied so that only the vertebral column was cut without damaging the underlying spinal cord. Dorsal and ventral roots of the cord were cut from inside the vertebral foramen with a scalpel, and the mid-cervical to lumbar portions of the spinal cord were removed and quickly cleaned of meninges in ice-cold Hank's balanced salt solution (BSS; low Ca^{2+} and Mg^{2+}, Gibco BRL). Spinal cord tissue from 13 to 15 animals (whole litters) was pooled in fresh ice cold BSS. Isolated spinal cords were then disaggregated by gently pushing the tissue through a 75 μm pore Nitex mesh (B and SH Thompson and Co. Ltd., Toronto) with a polished glass rod. The disaggregated cell suspension was collected in modified minimum essential medium (MEM) containing Eagles MEM with Ca^{2+} (2 mM glutamine, 5.6 mM glucose final concentration), 10% horse serum (Gibco BRL), and 50 units/ml of penicillin and 50 units/ml of streptomycin (Gibco BRL). The cell suspension was then spun at 900 rpm for 5 min, the supernatant removed, and the pellet resuspended in fresh MEM at 1 ml per dissected cord segment. The resuspended cells were plated onto dry sterile 25 mm circular or square glass coverslips that had been washed in 10 N nitric acid for 6 h followed by subsequent washes of distilled water and 100% ethanol and stored in 100% ethanol. The excess ethanol was flamed off prior to putting one coverslip per culture dish well. To allow for better cell attachment, a 200 μl aliquot of cell suspension per coverslip was initially plated and 1.8 ml MEM was added 3 h after plating to bring the total volume to 2 ml. Alternately, we incubated the initial 200 μl cell suspension for 18 h prior to topping it off ($n = 12$ cultures). Cell cultures were individually housed in 6-well culture plates, and maintained at 37°C in a 5% CO_2/95% air atmosphere. Cultures were fed every second to third day by replacement of 1 ml of the culture medium with fresh medium. For this report we have established cultures using this method on five separate occasions (using five different litters), on average obtaining 11 to 12 6-well plates of astrocyte cultures per litter.

2.2. Immunocytochemistry

The basic immunocytochemical protocol is a modification of Black et al. (1993). Cultures were rinsed in Tris-buffered saline (TBS: 2.4 g $C_4H_{11}NO_3$, 0.8 g NaCl in 1000 ml distilled H_2O), 2×2 min, and then fixed in phosphate buffered 4% paraformaldehyde (10 min, at

Table 1
Summary of results for spinal cord astrocyte cultures grown on uncoated glass and poly-L-lysine coated glass

Substrate	Number of GFAP-positive cells	Identified morphologies	Days in vitro (DIV) to confluencey
Glass	997 out of 1000 ($n = 6$ coverslips)	Stellate pancake elongated	Approximately 14
Poly-L-lysine	66 out of 100 ($n = 10$ coverslips)	Stellate pancake elongated	Approximately 14

4°C). Sequential incubation of the fixed cultures then took place at room temperature in: (1) wash in TBS with 0.1% Triton X-100, 3 × 5 min; (2) wash in TBS, 3 × 5 min; (3) block in 10% normal goat serum (NGS), 10 min; (4) primary antibody incubation: using monoclonal anti-GFAP (Boehringer Mannheim Biochemica, clone G-A-5) at 1:250 dilution in 2% NGS/TBS for 1 h; (5) wash in TBS, 3 × 5 min; (6) block in 10% NGS/TBS, 10 min; (7) secondary antibody incubation with biotinylated anti-mouse IgG (Vector)), at 1:250 dilution in 2% NGS/TBS, 1 h; (8) wash in TBS, 5 × 5 min; (9) incubation with fluorescein Avidin D (Vector) at 1:250 in TBS or Avidin-Biotin complex horse radish peroxidase (ABC–HRP) at 1:1 μl/ml of both reagents in TBS, 40 min; (10) wash in TBS, 5 × 5 min; (11) fluorescein labeled cells were then ready for coverslipping with a glycerol/Mowiol 4–88 (Hoechst) mounting medium, containing 2.5% DABCO to reduce fluorescent bleaching (c.f. Theriault and Tator, 1994). ABC–HRP incubated cultures were then reacted in chromogenic solution (0.05% DAB/0.03% H_2O_2 in TBS) until the optimal reaction product was observed, typically a light brown staining of cells obtained within 5–30 s; (12) wash in TBS, 3 × 10 min. DAB labeled cultures were air dried overnight and coverslipped with permanent mounting medium after three washes in xylene.

2.3. Analysis of cultures

Cultures that had 200 μl of cell suspension incubated for a 3-h period prior to adding the remaining 1.8 ml of medium were analyzed at 7, 11, 14, 21, and 30 days in vitro (DIV). We analyzed $n = 6$ cultures per incubation period per time point. Cultures in which the 200 μl aliquot of cell suspension sat for 18 h prior to being topped off with the remaining 1.8 ml of culture media, were analyzed at 14 and 21 DIV. Cell growth patterns, morphology, and numbers of GFAP-expressing cells were analyzed and compared to control cultures grown on 70–100 kD poly-L-lysine coated glass coverslips and tissue culture treated polystyrene 6-well culture dishes (Corning). Immunocytochemistry runs omitting the primary antisera provided very clean controls, with no visible background fluorescence or DAB reaction product. To quantitate the fraction of GFAP-positive cells in cultures grown on glass and on poly-L-lysine coated coverslips, three random fields were chosen from six slides and approximately 50 cells were counted from each field ($n \cong 1000$ cells). Cultures were viewed under phase and fluorescence microscopy using a BioRad 600 confocal microscope and a Leitz DM/RB fluorescence microscope.

3. Results

A comparison of the cultures grown on untreated glass coverslips versus those grown on poly-L-lysine or tissue culture treated polystyrene culture dishes yielded no differences in cell growth patterns, morphology, or confluency (Table 1). The typical morphologies and growth patterns of GFAP-positive astrocytes grown on untreated glass are shown in Fig. 1. The cell density of these cultures was typically much higher, but this field was chosen so that the morphologies could be clearly seen. Astrocytes on all three substrates showed a random pattern of growth with no regionally dependent morphology patterns, e.g. no particular cellular morphologies were confined to specific regions of the coverslip, such as the outer rim or the center. All three surfaces exhibited similar cellular morphologies (e.g. stellate, pancake, or elongated) to the point that it was impossible to distinguish by this criterion which substrate the cultures were grown on. A minor difference detected at earlier time points, up to about a week, was that a fraction of the astrocytes grown on glass took

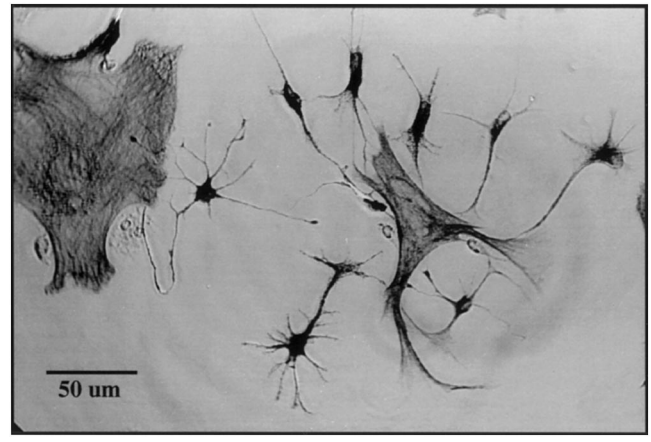

Fig. 1. GFAP-positive astrocytes cultured on plain, untreated glass coverslips. There were no visible regional differences in morphology, growth patterns, or confluency between cultures established on untreated glass versus those grown on substrate (poly-L-lysine or tissue culture treated polystyrene dishes).

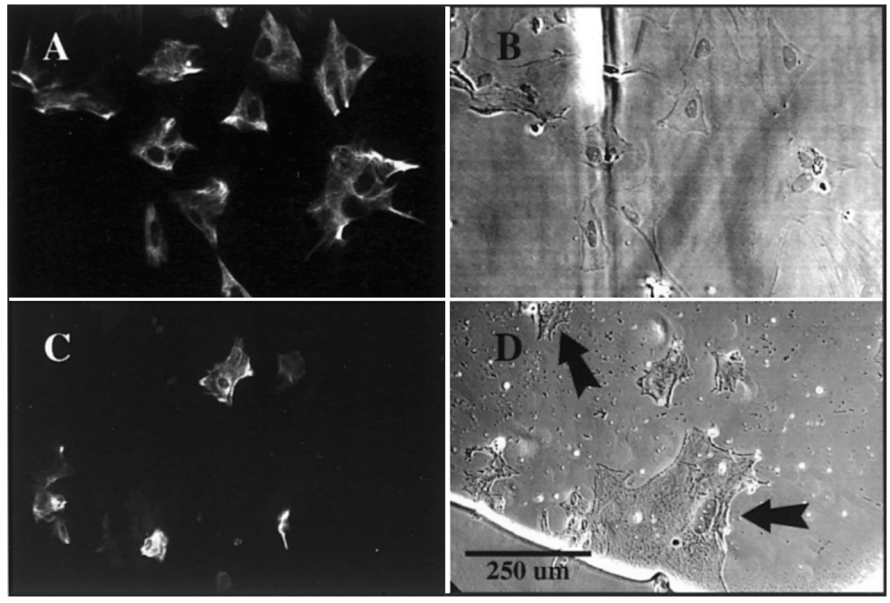

Fig. 2. Fluorescein-labeled GFAP positive astrocytes (A and C) with the corresponding light microscopy phase pictures (B and D). Notice the lack of GFAP-negative cells in the fluorescence (A) and phase (B) micrographs of cultures grown on untreated glass coverslips. In contrast, in (C) fluorescein-labeled GFAP-positive cells grown on poly-L-lysine coated glass coverslips do not correspond to all cell types seen in (D). The presence of GFAP-negative fibroblast-like cells in (D) are indicated with arrows.

somewhat longer to develop the characteristic astrocytic morphology such as elongated processes or flat cell bodies. At these early stages (0–7 DIV) the cell bodies were somewhat more spherical and non-process-bearing. However, by 7 DIV the cultures were indistinguishable regardless of what substrate they were grown on, and by 14 DIV all cultures had reached confluency.

In order to allow better cell attachment to the coverslips, regardless of the surface used, we typically allowed a 3-h incubation of the 200 μl of cell suspension prior to bringing the final volume of the culture wells up to a total 2 ml of medium. In the series of experiments ($n = 12$ cultures) where we incubated the initial cell suspension for 18 h, we found that at 1 DIV the ratio of cell density was at least 5:1 vs. those that were left for 3 h. Therefore, much better cell attachment occurred initially for the longer incubation period, although by 7 DIV both cultures appeared the same. We note that there was no observable dehydration of the 200 μl cell suspension when left for prolonged periods of time in the incubator.

The cultures grown on untreated glass coverslips were found to contain more than 99% GFAP-positive cells, while those established using the same protocol but grown on poly-L-lysine had only 66% GFAP-positive cells (see Table 1). Morphologically identified fibroblast-like cells and microglia were present on the cultures grown on poly-L-lysine and on polystyrene culture dishes, while they were virtually absent on the untreated glass cultures. Typical fields of GFAP-positive cells under fluorescence and the corresponding phase contrast images for cultures grown on untreated glass and poly-L-lysine treated glass are shown in Fig. 2.

4. Discussion

The method described above allows for the rapid establishment of highly pure astrocyte cultures, with minimal resources and comparative ease. By culturing astrocytes from postnatal day 0 rat pups via mechanical dissociation and plating them on untreated glass coverslips, we produced cultures that were virtually pure in GFAP-positive cells. The lack of differences in phenotype and cell density on untreated glass, poly-L-lysine, and tissue culture treated polystyrene dishes, indicates that the substrate itself may not significantly affect the expression of astrocyte phenotypes in vitro. In addition, other cell types such as fibroblasts and microglia, may have a harder time adhering to the uncoated glass. In the series of experiments where we incubated the 200 μl aliquots of cell suspension for 18 h prior to bringing the final volume of medium in the wells to 2 ml, we found that these cultures initially had many more cells attached than the 3-h cultures at 1 DIV. This approach may prove useful for acute experiments which require cultures from 0 DIV to about 7 DIV, for example, those experiments specifically designed to explore properties of astrocytes during early development.

The preparation of highly pure astrocyte cultures ensures that any observed experimental effects can be

attributed to the cell type of interest, without having to go back and confirm its phenotype via immunocytochemistry. In addition, the use of glass coverslips greatly facilitates experiments which require high resolution images of cellular and subcellular in vitro systems, e.g. fluorescent $[Ca^{2+}]_i$ imaging experiments and immunohistochemical descriptions. By exploiting the ability of astrocytes to selectively adhere to and proliferate on uncoated glass, this method combines the best of both worlds: astrocyte cultures which are remarkably easy to establish yet are highly pure.

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