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Research report

Establishment and characterization of primary astrocyte culture from adult mouse brain

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ABSTRACT

As a major class of glial cells, astrocytes have been indicated to play multi-roles in physiological and pathological brain. Astrocyte cultures derived from postnatal mouse brains have been extensively used to characterize their biological properties. However, the inability to culture adult mouse primary astrocytes has long stymied studies of function in adult brain. Here, we developed a protocol to successfully establish highly enriched astrocyte cultures from the brains of adult mouse. Cortical tissues were collected to prepare cell suspension by enzymatic digestion and mechanical dissociation, and then plated onto vessels pre-coated with gelatin and matrigel and cultured in DMEM medium containing 20% fetal bovine serum (FBS). Forskolin (FSK) and glial-derived neurotrophic factor (GDNF) were use to promote astrocyte proliferation and survival respectively. These adult astrocyte cultures were identified by immunocytochemical, immunobloting and PCR analysis. Furthermore, biological and functional analysis indicated that they possess the biochemical and physiological properties of astrocytes, suggestive of a useful cell model for astroglial studies in adult brain.

1. Introduction

Neurons and glia are two major cellular constituents of the nervous system. For a long time, glial cells have been thought to play primarily passive support roles for neurons. However, increasing evidence indicates that glial cells are actually important and active players in multiple aspects of neural development, function, and disease (Barres, 2008; Robel and Sontheimer, 2016; Zuchero and Barres, 2015). Astrocytes represent an important fraction of glial cells in the central nerve system (CNS), broadly distributed in the brain and spinal cord. Astrocytes play multiple key roles in maintaining homeostasis of the CNS in physiological and pathological conditions. For instance, it is well accepted that astrocytes fulfil their house-keeping functions to establish a viable microenvironment for neurons, including providing nutrient and structural support, regulating extracellular concentrations of ions and neurotransmitters, and maintaining the blood-brain barrier (BBB) (Barres, 2008). Astrocytes are also reported to shape the structural plasticity of synapses and control synaptic activity by fast release of gliotransmitters(Dallerac and Rouach, 2016; De Pitta et al., 2016; Zhou and Liu, 2016). Additionally, astrocytes are involved in the neuropathologenesis by response to various stimuli, such as traumatic injury, stroke and degenerative disease (Sofroniew, 2009; Sofroniew, 2014). Despite these highlighted advances, our understanding of astrocyte development and function still remain rudimentary.

In vitro primary cell culture provides an ideal model for the studies of astrocytes in CNS. Classically, postnatal astrocyte cultures derived from the rodent brain have been extensively used to characterize their biological functions and neuron-astrocyte interactions (Booher and Sensenbrenner, 1972; Skytt et al., 2010). However, progress in understanding of the biology and pathology of astrocytes in adult CNS has long been hindered by lack of effective primary astrocyte culture obtained from adult brain. In contrast to the traditional view that astrocytes are a homogeneous group of cells in the CNS, growing evidence shows their significant heterogeneity in many aspects of astrocytes in a spatio-temporal manner, including developmental origin, morphology, gene expression profile, physiological properties, function (Hu et al., 2016; Zhang and Barres, 2010). The astrocytic heterogeneity results from a number of factors, such as the differences of age and species. For example, newborn astrocytes are plastic and labile to stimuli (Sun et al., 2014; Yoshiura et al., 2005). However, adult astrocytes contain well established synaptic connections and make more reliable response (Souza et al., 2013; Sun et al., 2013).

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Therefore, it is essential to establish and characterize a model of adult astrocyte culture for studies in ageing brain. Mouse is regarded as one of the most popular and best laboratory animal for medical research. In spite of the model of primary astrocyte culture derived from adult rat or monkey (Guillemin et al., 1997; Souza et al., 2013), the methodology of adult mouse astrocyte culture is still not obtained. Indeed, it had been reported that the phenotype, differentiation and function of astrocytes depend on species (Ahlemeyer et al., 2013; Puschmann et al., 2010). Thus, it would be of great interest to develop primary cultures of adult mouse astrocytes, providing an ideal tool for understanding of physiological and pathological functions of astrocytes.

In present study, we successfully developed a new protocol for culturing astrocytes derived from adult mouse brain tissue. Adult astrocytes were isolated from mice aged 2–3 months and plated onto gelatin and matrigel-coated vessels. Cells were cultured in a defined medium, DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 20% FBS, 10 μM FSK and 10 ng/mL GDNF, which enables the survival and proliferation of adult astrocytes. Purified astrocytes were obtained by shaking to remove contaminated microglia and oligodendrocyte precursor cells (OPCs) that loosely attached on the cell monolayer. Immunocytochemical and immunobloting analysis was applied to identify these cultured adult astrocytes. Using this adult astrocytes preparation, furthermore, we begin to unravel some of their fundamental functional properties. Taken together, these primary astrocytes derived from adult mouse brain may provide cellular models to uncover roles of astrocytes in normal neurodevelopment and pathogenesis of neurological injuries or diseases.

2. Methods

2.1. Animals

Wild-type male and female postnatal (0–4 days of age) or adult C57/BL6J mice (2–3 months of age) were used for astrocytes preparation. All these mice were housed under a 12 h light/dark cycle and had ad libitum access to food and water. All experimental procedures and protocols strictly conformed to the recommended National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Animal Experimentation Ethics Committee of Second Military Medical University.

2.2. Reagents

DMEM, FBS, and other materials for cells culture were purchased from Gibco. Gelatin, lipopolysaccharides (LPS), FSK, BrdU (5-bromo-2–deoxyuridine) and Hoechst 33342 were from Sigma-Aldrich. Matrigel and GDNF were from BD Biosciences and eBioscience, respectively. The companies where the primary antibodies used for immunofluorescence were obtained are listed in Table S1. The corresponding secondary antibodies conjugated with Alexa Fluor 488, 555 or 647 dye was all from Jackson ImmunoResearch Laboratories.

2.3. Cell isolation and culture

Highly enriched primary astrocytes were prepared from the cerebral cortex of male and female mice at the age of 2–3 months. After mice were killed by CO_2 overdose, cerebral cortexes were aseptically dissected and meninges were carefully removed. Several superficial washings were performed with phosphate buffered saline (PBS) containing 100U/mL penicillin and 100 μg/mL streptomycin to limit contaminations. Superficial blood vessels were carefully extracted using dissection pliers. The tissues were minced finely and enzymatically (0.25% trypsin, 37 °C, 15 min) then mechanically dissociated to produce single cells.

After filtered through a 100 μm pore mesh, the cell suspension was centrifuged at 800*g* for 4 min and resuspended in DMEM containing 20% FBS, 100U/mL penicillin and 100 μg/mL streptomycin. The cells were plated in tissue flasks pre-coated with gelatin and matrigel at a density of 3–5 × 10^5 cells/cm^2, then cultured at 37 °C in a 95% air/5% CO_2 incubator. In the medium, 10 μM FSK and 10 ng/mL GDNF were supplemented to promote cell proliferation and survival, respectively. The medium change occurred once every two days.

When cells grew to confluence (10–14 d), flasks were shaken on a rotary shaker at 260 rpm for 18–20 h at 37 °C to remove the loosely attached contaminated microglia and OPCs. The attached enriched astrocytes were subsequently detached using trypsin-EDTA and then subjected to different treatments.

2.4. Immunofluorescence

For immunocytochemistry, cells cultured on coverslips were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. Fixed cells were permeabilized and blocked with 0.2% Triton X-100 and 3% BSA in 1XPBS for 1 h, followed by overnight incubations at 4 °C with the primary antibodies listed in Supplementary Table 1. Alexa Fluor 488, 555 or 647-conjugated corresponding secondary antibodies were used for indirect fluorescence. Nuclei were counterstained with Hoechst 33342 (Hst). Images were acquired with a Nikon E600FN microscope or a Leica confocal microscopy and were analyzed using Image-Pro Plus software.

2.5. Western blot analysis

Cells were harvested and lysed with cold SDS gel sample buffer. The protein samples were electrophoresed on a 10% SDS PAGE gel, and transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat milk and then were incubated with the indicated primary antibodies against Aldoc, GFAP, Vimentin and β-actin overnight at 4 °C. After incubated with horseradish peroxidase-conjugated secondary antibodies (Sigma), immunoreactive bands were visualized by chemiluminescence reagents (ECL, Amersham). Immunoblot results were analyzed using Image-Pro Plus 6.0 software.

2.6. Real-time PCR

Total RNA was extracted from primary astrocytes with Trizol reagent (Invitrogen) in which contaminating DNA was depleted with RNase-free DNase (Thermo Scientific Fermentas). According to the manufacture's instruction, first-strand cDNA was synthesized using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific Fermentas). As previously described (Su et al. 2013), quantitative PCR was performed to check the indicated genes using a MyiQ™ (Bio-Rad) with SYBR Green Realtime PCR Master Mix (TOYOBO Biotech). Gene expression was expressed as the mRNA level, which was normalized to that of a standard housekeeping gene (GAPDH). Relative levels of target mRNA expression were calculated using the $2_T^{-\triangle\triangle C}$ method. There were at least three independent experiments performed for every set of PCR analysis. The primers used in this study were provided in Supplementary Table 2.

2.7. BrdU labeling and cell proliferation assays

Proliferating cells in culture were labeled by incubation with 10 mM 5-bromo-2-deoxyuridine for 16–18 h. BrdU incorporation was detected by fluorescent staining using an antibody against BrdU. Briefly, paraformaldehyde-fixed cells were treated with 2 M HCl for 30 min at 37 °C, rinsed in 0.1 M boric acid for 10 min, and incubated with blocking solution (0.2% Triton X-100 and 3% BSA in 1XPBS) for 1 h, which was followed by sequential incubations with blocking solutions containing primary and secondary antibodies. In addition to the BrdU incorporation assay, proliferating cells were also detected with an anti-Ki67 or anti-PCNA antibody.