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Characterization of an improved procedure for the removal of microglia from confluent monolayers of primary astrocytes

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Abstract

Cultures of astrocytes can be readily established and are widely used to study the biological functions of these glial cells in isolation. Unfortunately, contamination by microglia can confound results from such studies. Herein, a simple and highly effective modification of a common procedure to remove microglia from astrocyte cultures is described. After becoming confluent, astrocytes were exposed to a mitotic inhibitor for 5–6 days then treated with 50–75 mM L-leucine methyl ester (LME) for 60–90 min. Unlike previous protocols that employed lower LME concentrations on subconfluent cultures or during passage of astrocytes, this protocol effectively depleted microglia from high-density astrocyte monolayers. This was evidenced by the selective depletion of microglial-specific markers. Purified monolayers appeared morphologically normal 24 h after LME treatment and expressed nitric oxide synthase-2 (NOS-2) and cyclooxygenase-2 (COX-2) proteins upon stimulation with LPS plus IFNγ, albeit to a lower level than unpurified monolayers. This difference could be attributed to removal of contaminating microglia from monolayers and not to astrocyte dysfunction, since LME treatment did not alter global protein synthesis and a reactive phenotype could be induced in the purified monolayers. Thus, this modified protocol selectively depletes microglia from high-density primary astrocyte monolayers without compromising their functional integrity.

Keywords: Cell culture; Astrocytes; Microglia; L-Leucine methyl ester; Inducible nitric oxide synthase (iNOS; NOS-2); Nitric oxide (NO); Cyclooxygenase-2 (COX-2); Reactive astrocytosis; Lipopolysaccharide (LPS)

1. Introduction

Astrocytes are the predominant cell type in the central nervous system (CNS). They function to maintain normal brain physiology, including survival and guidance of migrating neurons during development, formation and preservation of the blood–brain barrier, and maintenance of neuronal homeostasis and plasticity (reviewed in Araque et al., 2001; Doetsch, 2003; Fields and Stevens-Graham, 2002; Janzer, 1993; Montgomery, 1994; Parri and Crunelli, 2003). In addition, astrocytes become reactive following injury to the CNS and serve important modulatory roles during CNS inflammation (Dong and Benveniste, 2001; Malhotra et al., 1990; Smith and Lassmann, 2002). In this regard, the expression of a number of genes can be upregulated in astrocytes in response to proinflammatory stimuli (John et al., 2005). Among these are nitric oxide synthase-2 (NOS-2) and cyclooxygenase-2 (COX-2), which have been implicated in the pathogenesis of CNS inflammation in several instances (Murphy, 2000; O'Banion, 1999).

The elucidation of astrocyte function has benefited from the ability to study these glial cells under defined conditions in vitro. Although astrocyte cultures are relatively easy to establish, these cultures can be contaminated by microglia, which have been shown to modify astrocyte responses under certain circumstances (Brown et al., 1996; Ciccarelli et al., 2000; Xiong et al., 1999). Furthermore, because proinflammatory stimuli can affect the expression of similar genes in microglia and astrocytes, interpretation of results using astrocyte cultures can be confounded by the presence of contaminating microglia. This could be especially problematic when sensitive biochemical measures are employed. Control of microglial growth is particularly difficult in astrocyte

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cultures because astrocytes are a primary source of colony stimulating factor-1 (Hao et al., 1990; Thery et al., 1992), which is a potent and selective growth factor for microglia (Giulian and Ingeman, 1988). Thus, microglia will proliferate extensively in cultures of astrocytes if not controlled.

Anti-mitotic agents have been employed to inhibit microglial growth in primary astrocyte cultures (Hewett, 1999; Solenov et al., 2004; Swanson et al., 1997; Tedeschi et al., 1986; Uliasz and Hewett, 2000). However, such an approach can be applied only on high-density monolayers after the astrocytes have entered a non-proliferative state induced by cell–cell contact. This allows a period of microglial cell growth prior to mitotic inhibition; thus, such cultures can still have a considerable number of microglia present. L-Leucine methyl ester (LME), a lysosomotropic agent originally used to selectively destroy macrophages (Thiele et al., 1983), has also been employed to deplete microglia from neural cultures including astrocytes (Giulian et al., 1993; Guillemin et al., 1997) and oligodendrocytes (Hewett et al., 1999). Typically, such protocols have employed 1–10 mM LME for this purpose (Bowman et al., 2003; Simmons and Murphy, 1992). However, this approach is most effective when performed on cells at low density or during cell passage. Even then, astrocyte cultures cannot be considered to be free from microglia (Giulian and Baker, 1986).

In this report, we describe a new method for eradicating microglia from high-density primary astrocytes. In this protocol, treatment of confluent astrocyte monolayers with a mitotic inhibitor followed by a brief exposure to high concentrations of LME (50–75 mM) generates highly purified astrocytes without the need for cell passage.

2. Materials and methods

2.1. Primary cultures

Primary astrocyte cultures were derived from 1 to 3 day postnatal CD1 mice (Charles River Laboratories, Wilmington, MA) as described previously (Trackey et al., 2001). Following an aseptic dissection, cerebral cortices were dissociated and cells plated at a density of 1–1.5 hemispheres/24-well plate (Falcon Primaria, BD Biosciences, Lincoln Park, NJ) or 1.2–1.6 hemispheres/6-well plate (Falcon Primaria, BD Biosciences, Lincoln Park, NJ). Plating medium consisted of media stock (MS) containing 10% bovine growth serum (Hyclone, Logan, UT), 10% iron-supplemented calf serum (CS; Hyclone, Logan, UT), 10 ng/ml epidermal growth factor (Invitrogen, Carlsbad, CA), 2 mM L-glutamine (Mediatech; Herndon, VA), 50 IU/ml penicillin and 50 μg/ml streptomycin (Mediatech; Herndon, VA). MS was comprised of modified Eagle's medium (Earle's salt; Mediatech, Herndon, VA) supplemented with glucose (J.T. Baker, Phillipsburg, NJ) and sodium bicarbonate (FisherChemicals, Fair Lawn, NJ) to a final concentration of 25.7 and 28.2 mM, respectively. Unless stated otherwise, upon reaching confluence, astrocyte monolayers were treated with 8 μM cytosine β-D-arabinofuranoside (Ara-C; Sigma–Aldrich, St. Louis, MO) for 5–6 days to eliminate dividing cells (i.e., microglia). This treatment is not cytotoxic to quiescent contact-inhibited astrocytes. Cultures were subsequently maintained in growth medium consisting of MS containing 10% CS, 2 mM L-glutamine, 50 IU/ml penicillin and 50 μg/ml streptomycin. Such cultures are termed primary astrocytes because they are never subjected to cell passage. Cells were grown, maintained and stimulated at 37 °C in a humidified atmosphere of 6% CO_2. All studies were performed on monolayers between 14 and 31 days in vitro (DIV).

2.2. LME treatment

LME (Sigma–Aldrich, St. Louis, MO) was dissolved in MS and added to cultures at concentrations of either 5 mM for 8 h or 25–75 mM for 60–90 min. All LME solutions were adjusted to pH ∼7.4 and filter sterilized prior to addition to cells. Between 45 and 90 min after addition of high concentration LME, cultures were visually inspected to ensure maximal microglial lysis with minimal astrocytic toxicity. Thereafter, monolayers were washed thoroughly with growth medium and allowed to recover for 1 day in growth medium prior to experimentation.

2.3. Monolayer stimulation

To induce NOS-2 protein expression, monolayers were exposed to 2 μg/ml lipopolysaccharide (LPS; DIFCO, Kansas City, MO) plus 3 ng/ml recombinant mouse interferon-γ (IFNγ; R&D Systems, Minneapolis, MN) for 24–35 h. Both LPS and IFNγ are required to induce NOS-2 in mouse primary astrocyte cultures (Hewett et al., 1993; Vidwans et al., 2001). Stimulation medium contained DMEM (Gibco BRL, Gaithersburg MD) supplemented with 5% CS, 2 mM L-glutamine, 50 IU/ml penicillin and 50 μg/ml streptomycin. NOS-2 protein was assessed by immunoblot or immunofluorescence analysis. Nitric oxide (NO) was assessed by nitrite accumulation in cell supernatants. To induce COX-2 protein expression, astrocyte monolayers were treated with 0.5 mM dibutyryladenosine 3′,5′-cyclic monophosphate (dbcAMP, Sigma–Aldrich, St. Louis, MO) in MS supplemented with 2 mM L-glutamine. COX-2 protein expression was assessed 6 h later by immunoblot analysis. Monolayers were exposed to 5 mM dbcAMP in stimulation medium to induce reactive astrocytosis. The reactive phenotype was assessed 48 h later by expression of glial fibrillary acidic protein (GFAP) depicting a transition from a cobblestone to elongated morphology.

2.4. Measurement of nitrite accumulation

Production of nitric oxide (NO) was assessed indirectly by measurement of nitrite, an oxidative breakdown prod-