Neurochemistry International 63 (2013) 47–53

Contents lists available at SciVerse ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/nci

Microglia are the major source of TNF-α and TGF-β1 in postnatal glial cultures; regulation by cytokines, lipopolysaccharide, and vitronectin

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ARTICLE INFO

Article history:
Received 3 September 2012
Received in revised form 22 March 2013
Accepted 15 April 2013
Available online 22 April 2013

Keywords:
Microglia
Astrocytes
Activation
TNF-α
TGF-β1
Vitronectin

ABSTRACT

Damage to the central nervous system (CNS) leads to increased production of TNF-α and TGF-β1 cytokines that have pro- or anti-inflammatory actions, respectively. To define whether astrocytes or microglia express these cytokines, prior studies have used mixed glial cultures (MGC) to represent astrocytes, thought these results are inevitably complicated by the presence of contaminating microglia within MGC. To clarify the cellular source of these cytokines, here we employed a recently described method of preparing microglia-free astrocyte cultures, in which neural stem cells (NSC) are differentiated into astrocytes. Using ELISA to quantify cytokine production in three types of glial culture: MGC, pure microglia or pure astrocytes, this showed that microglia but not astrocytes, produce TNF-α, and that this expression is increased by LPS, IFN-γ, and to a lesser extent by vitronectin, but decreased by TGF-β1. In contrast, TGF-β1 was produced by microglia and astrocytes, though at 10-fold higher levels by microglia. TGF-β1 expression in microglia was increased by vitronectin and to a lesser extent by TNF-α and LPS, but astrocyte TGF-β1 expression was not regulated by any factor tested. In summary, our data reveal that microglia, not astrocytes are the major source of TNF-α and TGF-β1 in postnatal glial cultures, and that microglial production of these antagonistic cytokines is tightly regulated by cytokines, LPS, and vitronectin.

1. Introduction

Microglia and astrocytes are two abundant glial cell types in the central nervous system (CNS) that play important roles in maintaining tissue homeostasis. Microglia play a critical homeostatic role by continually sensing the environment, searching for tissue damage or invading microorganisms (Carson, 2002). They are instrumental in removing apoptotic cells both during development (Purkinje cells) and following immune activation (T cells) (Magnus et al., 2001; Marin-Teva et al., 2004), and can also influence oligodendrogenesis in a biphasic manner (Butovsky et al., 2006). This duality of microglial function is well illustrated by the observation that microglia phagocytosing bacteria release pro-inflammatory cytokines, whereas removal of apoptotic cells is associated with microglial production of anti-inflammatory factors (Glezer et al., 2007; Magnus et al., 2001). Following stimulation, microglia are rapidly activated into migratory cells that phagocytose invading microorganisms and tissue debris (Hanisch and Kettenmann, 2007). In this way, microglia play an important role in tissue regeneration, both by clearing away debris and by producing factors that promote tissue repair (Kreutzberg, 1996; Raivich et al., 1999). However, when persistently activated, microglia cause excessive and unnecessary tissue damage, such as that observed in the autoimmune attack on myelin in multiple sclerosis (MS) (Gonzalez-Scarano and Baltuch, 1999; Ransohoff, 1999; Trapp et al., 1999). In contrast to microglia, which have a primary immune, phagocytic function, astrocytes play more of a supportive role, such as maintaining the barrier properties of cerebral blood vessels (Ballabh et al., 2004; del Zoppo and Milner, 2006; Huber et al., 2001; Janzer and Raff, 1987), and buffering excess levels of potassium ions and excitotoxic neurotransmitters such as glutamate (Nedergaard et al., 2003; Ransom et al., 2003; Ridet et al., 1997). Following tissue damage, activated astrocytes become hypertrophic, expressing increased levels of GFAP and form a reactive glial scar (Ridet et al., 1997). While current evidence suggests that the glial scar provides a useful function in limiting the extent of tissue damage, it is also apparent that the reactive glial scar impedes regeneration in the CNS, preventing both nerve regeneration (Fawcett, 1997; Fawcett and Asher, 1999) and remyelination (Franklin and ffrench-Constant, 1996).

During the activation process, microglia and astrocytes show marked changes in their expression profile of cytokines. In particular, the pro-inflammatory cytokine TNF-α shows rapid induction

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0197-0186/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.neuint.2013.04.007

following CNS damage, and this cytokine has been implicated in the pathogenesis of several neurological conditions, including MS (Hofman et al., 1989) and stroke (Lambertsen et al., 2005). The anti-inflammatory cytokine TGF-β1 is also quickly upregulated following cerebral ischemia, and is thought to play an important protective role by preventing cell death of ischemic neurons and by countering some of the destructive effects of pro-inflammatory cytokines (Buisson et al., 2003; Krupinski et al., 1996). One important question to be answered is, which of these cytokines is produced by microglia or astrocytes, and how is this regulated by activating stimuli? In this regard, a large number of studies have described microglial production of both TNF-α (Gregersen et al., 2000; Lambertsen et al., 2005; Zujovic et al., 2000) and TGF-β1 (Lehrman et al., 1998; Wang et al., 1995), but the results on astrocytes have yielded equivocal results, with some describing astrocyte expression of TNF-α (Chung and Benveniste, 1990; Freyer et al., 1996), and TGF-β1 (Morganti-Kossmann et al., 1992), but other studies not supporting these findings (Clausen et al., 2008; Gregersen et al., 2000; Lehrman et al., 1998; Morgan et al., 1993). Significantly, many in vitro studies of astrocytes have been performed using the mixed glial culture (MGC) system (Chung and Benveniste, 1990; Freyer et al., 1996; Lee et al., 1993), which contain a majority of astrocytes, but also a significant population of microglia (Crocker et al., 2008; Liu et al., 2006; Saura et al., 2003). Thus, one possibility is that some of the cytokine responses described using the MGC system may have originated in microglia, not astrocytes. To specifically address this issue, we recently established a novel method of preparing microglia-free astrocyte cultures from the postnatal CNS, in which neural stem cells (NSC) are differentiated into astrocytes, and confirmed by multiple methods that this approach yields pure astrocyte cultures entirely devoid of microglia (Crocker et al., 2008). In the current study we used this approach to characterize TNF-α and TGF-β1 production in microglia and astrocytes, and then determine how microglial or astrocyte expression of these cytokines is regulated by other activating stimuli, including cytokines, LPS, and extracellular matrix (ECM) proteins.

2. Experimental procedures

2.1. Cell culture

The use of animals in this protocol was approved by the Committee on Animal Protocols, Department of Animal Resources, The Scripps Research Institute. Mixed glial cultures (MGC) were prepared from postnatal (day 0–2) C57BL6 mouse pups, as previously described (Milner and Campbell, 2002, 2003). Cultures were maintained in poly-D-lysine coated T75 flasks in DMEM (Sigma–Aldrich, St. Louis, MO) containing 10% fetal bovine serum (FBS) (Sigma–Aldrich) for 7–10 days, before being mechanically shaken to yield microglia, which were plated into 6 well plates (Nunc, Naperville, IL), previously coated with poly-D-lysine, fibronectin, laminin or vitronectin. Plates were coated by incubating with a solution containing either 5 μg/ml poly-D-lysine or 10 μg/ml of the different ECM proteins (all from Sigma) for 2 h at 37 °C. The purity of these microglial cultures was >99% as determined by Mac-1 positivity in flow cytometry. The remaining adherent cells, consisting predominantly of astrocytes, were also plated into poly-D-lysine or ECM coated 6 well plates.

Primary cultures of neurospheres were obtained from postnatal (P0–P2) C57BL6 mouse brains as described previously (Jacques et al., 1998; Milner, 2007). Briefly, spheres of neural precursors were grown in uncoated Nunc non-adherent T25 tissue culture flasks in DMEM/Hams F12 (Sigma) (50:50) supplemented with 1% B27, 20 μg/ml epidermal growth factor (EGF) and 20 μg/ml fibroblast growth factor-2 (FGF2) (all obtained from Invitrogen). Neurospheres were subsequently passaged every 5–7 days into fresh flasks. Neurospheres were differentiated into astrocytes by culturing in poly-D-lysine or ECM coated 6-well plates in DMEM containing 10% FBS, and grown to confluence. The astrocyte purity of these cultures was >99% as determined by GFAP positivity by immunocytochemistry. Flow cytometry and immunocytochemistry demonstrated the total absence of Mac-1 positive cells in these cultures, as previously described (Crocker et al., 2008). When all three types of cell culture reached confluence, growth media was removed and replaced with serum free media containing the cytokines IFN-γ (10 U/ml), IL-1β (10 ng/ml), TNF-α (10 ng/ml), or TGF-β1 (2 ng/ml), all from R&D Systems, Minneapolis, MN, or LPS (1 μg/ml, Sigma). All cytokines were murine except for TGF-β1, which was human After 2 days culture, the supernatants were harvested and levels of TNF-α or TGF-β1 analyzed by ELISA.

2.2. Immunocytochemistry

Mixed glial cultures and NSC-derived astrocytes were cultured on poly-D-lysine coated glass coverslips in DMEM containing 10% FBS. All incubations were performed at room temperature with washes in between. Cells were blocked in 5% normal goat serum (NGS) in PBS for 30 min then live-labeled with a Mac-1 monoclonal antibody (M1/70 clone from BD Pharmingen , La Jolla, CA) for 1 h, then incubated with anti-rat Alexafluor 488 (Invitrogen) for 30 min, fixed in acid/alcohol (95:5) at −20 °C for 30 min, then incubated with anti-GFAP-Cy3 (Sigma) for 1 h, before being mounted in aquamount (Polysciences, Warrington, PA). The co-localization studies were performed the same way except that cultures were first fixed in acetone/methanol (50:50) at −20 °C for 30 min. The TNF-α goat polyclonal antibody was obtained from R&D Systems. The antibodies against S100β and glutamine synthetase (GS) were obtained from Sigma.

2.3. ELISA analysis of glial cytokine production

Concentrations of TNF-α and TGF-β1 in microglial and astrocyte conditioned media were quantified by standard ELISA techniques using the Duoset ELISA system (R&D) according to the manufacturer's instructions. Results are expressed as concentrations of cytokine (pg/ml), and represent the mean ± SEM of 4 experiments, with each sample examined in duplicate within each experiment. Statistical significance was assessed by Student's t test in which a value of $p < 0.05$ was defined as statistically significant.

3. Results

3.1. Microglial TNF-α production is regulated by cytokines and LPS

To determine whether microglia or astrocytes express TNF-α and TGF-β1, we prepared three different types of postnatal glial culture (Fig. 1). Mixed glial cultures (MGC) represent a mixture of predominantly GFAP-positive astrocytes with a smaller though significant population of Mac-1-positive microglia (Crocker et al., 2008; Liu et al., 2006; Saura et al., 2003). Pure cultures of microglia were prepared by the well-established method of mechanical shaking MGC to harvest loosely-attached microglia (Milner and Campbell, 2003) and contain only Mac-1-positive cells, with no GFAP-positive cells within. Pure astrocyte cultures were obtained by differentiating neural stem cells (NSC) into astrocytes, as recently described (Crocker et al., 2008). The advantage of this method over the traditional MGC system is that the resulting astrocyte cultures are totally microglia-free, and as illustrated in Fig. 1B, contain only GFAP-positive astrocytes with absolutely no Mac-1-posi-