Journal of Neuroimmunology 286 (2015) 25–32

Contents lists available at ScienceDirect

Journal of Neuroimmunology

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

Activation and function of murine primary microglia in the absence of the prion protein

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ARTICLE INFO

Article history:
Received 10 March 2015
Received in revised form 2 July 2015
Accepted 4 July 2015

Keywords:
Prion protein
Microglia
Inflammation
Cytokine
Phagocytosis
Cell migration

ABSTRACT

The prion protein (PrP^C) is predominantly expressed in the nervous and immune systems and is involved in relevant cell signaling. Microglia participate in neuroimmune interactions, and their regulatory mechanisms are critical for both health and disease. Despite recent reports with a microglial cell line, little is known about the relevance of PrP^C in brain microglia. We investigated the role of PrP^C in mouse primary microglia, and found no differences between wild type and *Prnp*-null cells in cell morphology or the expression of a microglial marker. Translocation of NF-κB to the nucleus also did not differ, nor did cytokine production. The levels of iNOS were also similar and, finally, microglia of either genotype showed no differences in either rates of phagocytosis or migration, even following activation. Thus, functional roles of PrP^C in primary microglial cells are — if present — much more subtle than in transformed microglial cell lines.

1. Introduction

The prion protein (PrP^C) is a highly conserved glycosylphosphatidylinositol (GPI)-anchored glycoprotein, located at the surface of the plasma membrane and expressed mainly in the nervous and immune system (Stahl et al., 1987; Prusiner, 1998). Despite a known association with neurodegenerative diseases, its physiological functions are still controversial, although it is believed that PrP^C acts as a scaffold for the assembly of multi-component signaling complexes at the cell surface (Aguzzi et al., 2008; Linden et al., 2008).

Among the immune cells, PrP^C has been implicated in the physiology of lymphocytes, macrophages, dendritic cells, neutrophils and others (Isaacs et al., 2006; Mariante et al., 2012), and a role of PrP^C was reported in the activation of microglia (Brown et al., 1998). The latter are mononuclear phagocytes resident in the brain parenchyma, and constitute about 5 to 20% of the total number of glial cells, depending on the specific region of the brain (Lawson et al., 1990; Perry and Gordon, 1991). They constitute the main representative of the immune system in the central nervous system, and participate in both neuronal homeostasis and in inflammation (Saijo and Glass, 2011).

Microglia are activated in the context of numerous injuries or diseases that affect the CNS, both in human and in corresponding animal models (Perry et al., 2010). However, the functions of these cells in neurodegenerative diseases are not entirely understood. Microglia can have either a protective role, by removing protein aggregates in transmissible spongiform encephalopathies (Brown and Kretzschmar, 1997; Rezaie and Lantos, 2001) and Aβ aggregates in Alzheimer disease (Lee and Landreth, 2010; Sokolowski and Mandell, 2011), or a pathogenic role, through exacerbation of inflammation with the production of cytokines, inflammatory mediators and other neurotoxins that may contribute to neuronal dysfunction and damage (Perry et al., 2010; Saijo and Glass, 2011). Thus, it is expected that the activation of microglia is tightly regulated, and investigation of its control mechanisms should contribute to better understanding of both the modulation of its phenotypes and its roles in pathophysiology.

Microglial PrP^C has received little attention following the early evidence for its role in cellular activation (Brown et al., 1998), and only recently a series of studies of the microglial cell line BV-2 revived this issue. Thus, an increase in the transcription of the PrP^C-coding Prnp gene was reported following stimuli such as treatment with the neurotoxic prion peptide $PrP_{106–126}$ (Bai et al., 2010) or infection with *Mycobacterium bovis* (Ding et al., 2013). Further, the silencing of PrP^C with siRNA indicated that PrP^C affects the response of BV-2 cells to bacterial infection (Ding et al., 2013) and modulates the change of those cells' phenotype from a quiescent to an activated state (Shi et al., 2013).

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; Iba-1, ionized calcium binding adaptor molecule-1; IL, interleukin; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NF-κB, nuclear transcription factor kappa B; NO, nitric oxide; PrP^C, prion protein; TNF-α, tumor necrosis factor alpha.

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http://dx.doi.org/10.1016/j.jneuroim.2015.07.002
0165-5728/

Nonetheless, despite the evidence for the involvement of PrP^C in both the activation and cell responses of this microglial cell line, the relationship between PrP^C and the physiology of microglia *in vivo* or *ex vivo* is unclear. Here, we examined the functional properties of primary cultures of microglia obtained from both wild type and *Prnp*-null mouse brains. The results showed no evidence of participation of PrP^C in microglial activation and functions.

2. Materials and methods

2.1. Reagents

DMEM-F12 culture medium was from Gibco. Fetal bovine serum was from Cultilab. LPS (*Salmonella enterica*) and FITC-labeled zymosan (*Saccharomyces cerevisiae*) were from Sigma and Invitrogen, respectively. Monoclonal anti-NF-κB p65 (20/NF-κB/p65) and anti-PrP^C (SAF83) antibodies were from BD Biosciences and Cayman Chemical, respectively. Polyclonal anti-Iba-1 and anti-iNOS were from Wako and Abcam, respectively, and anti-Erk2 was from Santa Cruz Biotechnology. Secondary antibodies for immunofluorescence (Alexa Fluor 488 and Alexa Fluor 555) and western blots (anti-mouse IgG-HRP) were from Life Technologies and Cell Signaling, respectively. ELISA kits for cytokine assays were from eBioscience.

2.2. Mouse strains and genotyping

Prion protein knockout mice originally produced in 129/Ola background (Manson et al., 1994), and serially backcrossed into the C57BL/10SnJ strain (Tribouillard-Tanvier et al., 2009), were kindly provided by Bruce Chesebro and Richard Race (Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, USA). Descendants of heterozygous littermates were maintained by serial backcrossing into C57BL/10SnJ, and used to derive wild type and *Prnp*-null mice, here referred to as B10.129Ola. Animals were housed in plastic boxes with food and water *ad libitum*, and were maintained in a 12 h light/dark cycle. Experiments were done with newborn mice (0 to 2 postnatal days) in accordance with current guidelines for the care and use of laboratory animals, as described by the National Institutes of Health and approved by the Committee for the Use of Experimental Animals from the Center of Health Sciences, Federal University of Rio de Janeiro.

All mice used in the present study were genotyped by PCR as described elsewhere (Steele et al., 2006). Briefly, DNA from tails was amplified in a multiplex reaction using the primer pairs 5′-TCATCCCA CGATCAGGAAGATGAG-3′ and 5′-ATGGCGAACCTTGGCTACTGGCTG-3′, which anneal to the start and stop codons of the *Prnp* gene open reading frame and generate a fragment of 750 bp, and 5′-TTGAGCCTGGCGAACA GTTC-3′ and 5′-GATGGATTGCACGCAGGTTC-3′, which anneal to the neomycin resistance gene present in *Prnp*-null mice and generate a product of 550 bp. Cycling conditions were 94 °C for 3 min, 30 cycles at 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. PrP^C content from microglial cell lysates was further verified by western blots (Supplemental methods and Supplemental Fig. 1).

2.3. Primary cultures of microglial cells

Microglial cells were obtained from the brains of newborn mice as described previously (Théry et al., 1991; Fonseca et al., 2012). Briefly, mice were decapitated, their brains were dissected and the meninges were carefully stripped off. Tissues were mechanically dissociated and resuspended in DMEM-F12 medium supplemented with L-glutamine, 1% penicillin/streptomycin, and 10% fetal bovine serum. Dissociated cells were plated in poly-L-ornithine-coated flasks and incubated at 37 °C for 14 days in a humidified chamber with 5% CO_2. Floating microglial cells were collected from culture supernatants, counted, plated as described in the following sections and either stimulated or not with 1 μg/ml LPS for 24–72 h. Four to six mice pups of each genotype were used for each culture. Microglial cultures presented >97% purity as verified by Iba-1 expression.

2.4. Immunocytochemistry

Microglial cells plated in a 24-well plate containing round coverslips (7×10^4 cells per well) were either treated or not with LPS for 24 h, fixed with 4% paraformaldehyde in phosphate buffer 0.1 M (pH 7.4), permeabilized with 0.5% Triton X-100 and blocked with 3% bovine serum albumin. The coverslips were incubated overnight at 4 °C with primary antibodies anti-Iba-1 (1:200), anti-NF-κB (1:50) or anti-iNOS (1:300), followed by a secondary antibody (Alexa Fluor 488 or 555, 1:300) for 90 min at room temperature. Cell nuclei were stained with DAPI (1 μg/ml). The coverslips were mounted and observed in Zeiss Axio Imager.M2 fluorescence microscope equipped with an ApoTome module. The number of iNOS-positive cells was calculated from images acquired with the AxioVision mosaic tool, which generated images consisting of 225 photos (15 × 15 matrix) covering virtually the entire area of each coverslip. At least 12,000 cells of each group were counted and analyzed in each experiment. The fluorescence intensity of iNOS staining was estimated as described elsewhere (Gavet and Pines, 2010) and normalized to the fluorescence of WT untreated microglial cells. We measured the individual fluorescence of at least 300 cells of each group. Image analysis, processing and quantification were done with AxioVision 4.8, Adobe Photoshop CS5 and ImageJ 1.48v softwares.

2.5. Cytokine measurements

The release of cytokines was estimated from cells seeded in 24-well plates (5×10^5 cells per well), and either treated or not with LPS for 24 h as described above. Supernatants were collected, centrifuged at 10,000 *g* for 5 min, and the levels of IL-1β, IL-6, TNF-α, IL-10 and IL-4 were measured by ELISA according to the manufacturer's instructions.

2.6. Phagocytosis assay

Microglial cells were plated (7×10^4 cells per well) and either treated or not with LPS as described above. Zymosan-FITC bioparticles were opsonized with 30% normal mouse serum in DMEM-F12 for 1 h at 37 °C, and added to cell cultures 90 min prior to the end of LPS treatment at a ratio of 10:1 (zymosan:microglial cell). Afterwards, cells were washed three times with cold phosphate buffered saline to remove non-phagocytized particles, fixed with 4% paraformaldehyde and labeled with anti-Iba-1 (1:200) and DAPI as described before. The slides were examined and photographed in a Zeiss Axio Imager.M2 fluorescence microscope. For quantification, cells containing three or

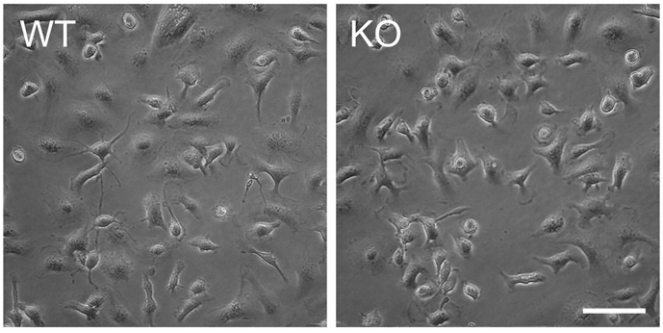

Fig. 1. Primary microglia cells isolated from cultures of cells obtained from brain of wild type (WT) or *Prnp*-null (KO) mice, and spontaneously adhered to glass coverslips. Bar = 40 μm.