Experimental Eye Research 169 (2018) 99–110

Contents lists available at ScienceDirect

Experimental Eye Research

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

Research article

Retinal neuroinflammatory induced neuronal degeneration - Role of toll-like receptor-4 and relationship with gliosis

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ARTICLE INFO

Keywords:
Retinal neuroinflammation
Gliosis
Toll-like receptor 4
Lipopolysaccharide
Galectin 3

ABSTRACT

The purpose of this study was to explore retina-intrinsic neuroinflammatory reactions, effects on neuronal survival, relationship with classic gliosis, and possible role of the toll-like receptor 4 (TLR4). To isolate the adult retina from the systemic immune system, a previously described large animal explant culture model was used in which full-thickness porcine retinal sheets can be kept in vitro for extended time periods. Explants were kept for 5 days in vitro (DIV) and were treated with either; lipopolysaccharide (LPS), a Toll-like receptor-4 (TLR4) inhibitor (CLI-095), LPS + CLI-095, or solvent vehicle throughout the culture period after which retinal sections were examined with hematoxylin and eosin staining and extensive immunohistochemistry. In addition, the culture medium of all explants was assayed for a panel of cytokines at 2 and 5DIV. Compared with in vivo controls, vehicle controls (CT) as well as CLI-095 explants displayed moderate reduction of total thickness and number of retinal neurons with upregulation of glial fibrillary acidic protein (GFAP) throughout the Müller cells. In contrast, LPS and LPS + CLI-095 treated counterparts showed extensive overall thinning with widespread neuronal degeneration but only minimal signs of classical Müller cell gliosis (limited upregulation of GFAP and no downregulation of glutamine synthetase (GS). These specimens also displayed a significantly increased expression of galectin-3 and TGF-beta activated kinase 1 (TAK1). Multiplex proteomic analysis of culture medium at 2DIV revealed elevated levels of IL-1β, IL-6, IL-4 and IL-12 in LPS-treated explants compared to CLI-095 and CT counterparts. LPS stimulation of the isolated adult retina results in substantial neuronal cell death despite only minimal signs of gliosis indicating a retina-intrinsic neuroinflammatory response directly related to the degenerative process. This response is characterized by early upregulation of several inflammatory related cytokines with subsequent upregulation of Galectin-3, TLR4 and TAK1. Pharmacological block of TLR4 does not attenuate neuronal loss indicating that LPS induced retinal degeneration is mediated by TLR4 independent neuroinflammatory pathways.

1. Introduction

The central nervous system (CNS) including the brain and retina, is an immune-privileged site, which through several mechanisms resists immunogenic inflammation (Streilein, 2003). However, despite this, neuroinflammation, a CNS-specific set of reactions involving inflammation-like glial reactivity, propagated through the activation of innate immune pathways, has been suggested to significantly contribute to neuronal degeneration and pathological tissue alterations in brain diseases such as stroke and Alzheimer's disease (Streit et al., 2004; Burguillos et al., 2015). Although very early neuroinflammatory responses are believed to be protective, chronic neuroinflammation is considered to create a highly detrimental cellular environment, and current consensus therefore suggests that inhibiting these responses may be an avenue of treatment (Heneka et al., 2015; Streit et al., 2004).

Recently, our group has shown that a well-known brain neuroinflammatory factor, galectin-3, mediates neuronal degeneration and glial activation in the retina during chronic cerebral hypoperfusion (Manouchehrian et al., 2015). Galectin-3 can potentiate neuroinflammatory reactions through several mechanisms, most notably by acting as a ligand for the innate immune receptor toll-like receptor 4 (TLR4), which is expressed in abundance within the CNS (Burguillos et al., 2015; Kumar and Shamsuddin, 2012). The activated TLR4, which in the retina is expressed in photoreceptors, Müller cells, astrocytes and microglia, has been shown to contribute to neuronal degeneration and inflammatory reactions during endotoxin-induced uveitis, through involvement of the peripheral immune system (Tu et al., 2011; Lin et al., 2013; Kohno et al., 2013; Xu and Wang, 2016; Takeda et al., 2002). In

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https://doi.org/10.1016/j.exer.2018.02.002
Received 2 November 2017; Received in revised form 21 January 2018; Accepted 3 February 2018
Available online 07 February 2018
0014-4835/

several non-uveitis related common retinal neurodegenerative diseases, including glaucoma and diabetic retinopathy, inflammatory processes associated with TLR4 activation have been suggested to contribute to the pathological progression, although it is not yet clear whether this process is driven solely by endogenous retinal reactions independent of immune system involvement (Xu and Wang, 2016; Williams et al., 2017).

The aim of this paper was to explore retina-intrinsic neuroinflammatory responses and their relationship with glial reactions and neuronal degeneration. To this end, we used the well known immune activator lipopolysaccharide (LPS) in a previously described minimum-trauma organotypic retinal explant system and assayed neuronal and glial morphology as well as cytokine release (Li et al., 2008; Taylor et al., 2014; Caspi, 2006; Rosenbaum et al., 2011). Furthermore, we also used a potent TLR4 inhibitor within the culture system to establish the role of TLR4 activation in the retinal neuroinflammatory response.

2. Material and methods

2.1. Animals

All proceedings and animal treatment were in accordance with contemporary best practice, and the guidelines and requirements of the government committee on animal experimentation at Lund University, the EU Directive 2010/63/EU for animal experiments, and with the ARVO statement on the use of animals in ophthalmic and vision research. Six-month old adult Yorkshire/Hampshire pigs were euthanized by an overdose of sodium pentobarbital (Apoteket, Umeå, Sweden), after which eyes were harvested. In total, 16 eyes from 8 animals were used, yielding 58 retinal specimens for culture and 6 eyes serving as a normal adult in vivo controls.

2.2. Tissue culturing

The neuroretinas were removed from the surrounding ocular tissues using a standard method previously described (Taylor et al., 2014). To summarize, the eyes immersed in ice cold CO_2 –independent medium (Invitrogen, Paisley, UK) immediately after harvest. The anterior segment was excised, and the neuroretinas were removed by gentle dissection with microforceps, cutting at the optic nerve head. The central portion of each neuroretina was sectioned into 5-6 pieces, measuring approximately 7 × 7 mm. The 58 retinal pieces were explanted onto Millicell- PCF 0.4 μm culture plate inserts (Millipore, Billerica, MA, USA), with the inner limiting membrane facing the culture membrane. Specimens were cultured in 1.5 ml DMEM/F12 (Invitrogen), with 10% fetal calf serum (Sigma-Aldrich, St Louis, MO, USA) and antibiotics (2 mM L-glutamine, 100 U/ml penicillin and 100 ng/ml streptomycin (Sigma Aldrich)), with the following treatments, throughout the culture period: 1 μg/ml LPS, 2 μg/ml CLI-095, 1 μg/ml LPS + 2 μg/ml CLI-095, and a corresponding solvent control (see Table 1). 1 mg CLI-095 was dissolved in 1 ml dimethyl sulfoxide (DMSO) according to the manufacturers instructions, and then diluted in the culture medium for a final concentration of 2 μg/ml. Each eye yielded 5-6 explants, which were evenly distributed among the treatment groups. The concentrations of LPS and CLI-095 was selected based on previously reported effective concentrations for CNS primary cells and organ cultures (Wu et al., 2015; Forgione and Tropepe. 2012; Burguillos et al., 2015). The cultures were maintained in an incubator at 37 °C at 95% humidity and 5% CO2. The medium was exchanged every second day. Explants were fixed after 5 days in vitro (DIV). Culture medium was collected on days 2 and 5 and was flash frozen for a multiplex proteomic cytokine assay (described below).

Table 1
Overview of all experimental groups. LPS = Lipopolysaccharide; DMSO = dimethyl sulfoxide.

Group name	Culture modality	Number
Solvent control	0.2% DMSO	12
CLI	2 μg/ml CLI-095	12
LPS	1 μg/ml LPS	18
LPS + CLI	1 μg/ml LPS + 2 μg/ml CLI-095	16

2.3. Histology

Histological examinations were performed as previously described by Taylor et al., 2014). To summarize, after culturing, the explants were fixed in 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.2) for 1 h at room temperature. The anterior segments were removed as described above from the normal adult in vivo control eyes, after which they were fixed using the same paraformaldehyde concentration for 4 h in room temperature. For cryoprotection, the explants were then incubated in 0.1M Sörensens medium with increasing concentrations of sucrose up to 25%. They were then embedded in egg albumin/gelatin medium for cryosectioning at -20 °C, with a section thickness of 12 μm. For light microscopy, every 10th slide was stained with hematoxylin and eosin (HTX). For immunohistochemical labeling, adjoining slides with sections originating from the center of the explants, and sections including the area centralis in the normal control, were chosen. The specimens were rinsed thrice with PBS containing 0.1% Triton- X, and then blocked with PBS containing 0.1% Triton-X and 1% bovine serum albumin (BSA) for 20 min at room temperature. The slides were incubated overnight at 4 °C with the respective primary antibody (Table 2). For double-labelings, both primary antibodies were added at this stage. The specimens were then rinsed in PBS-Triton-X (0.1%), and incubated for 45 min with a secondary fluorescein isothiocyanate (FITC) or Rhodamine Red-conjugated antibody (Table 2). For double-labelings, both secondary antibodies were added at this stage. After three rinses in PBS Triton-X (0.1%), the specimens were mounted in hard-set Vectashield mounting medium with 4′,6-diamidino-2-phenylindole (DAPI; Vector laboratories Inc. CA, USA). Negative control experiments were performed as above, replacing the primary antibody with PBS containing 0,1% Triton-X and 1% BSA. Normal porcine adult retina was used as a positive control. Normal control tissue and cultured specimens were processed in the same batch for each immunohistochemical labeling.

2.4. Multiplex cytokine assay

Culture medium was collected at 2 and 5 DIV, and cytokine profiles (IL-1 β, IL-4, IL-6, IL-8, IL-10, IL-12p40, IFNα, IFNγ and TNFα) were analyzed with the ProcartaPlex Porcine Cytokine and Chemokine Panel I (EPX090-60829-901, Thermo Fisher Scientific, Waltham, MA, USA) on a Bio-plex 200 System (Bio-Rad Laboratories Inc, Hercules, CA, USA) according to the manufacturer's instructions. Briefly, the culture medium samples were incubated with the magnetic beads coupled to specific antibodies, washed, followed by addition of a biotinylated detection antibody, washed again, and lastly incubated with streptavidin-phycoerythrin. The run included a blank as well as controls of known concentrations for each cytokine. All samples were processed and analyzed in the same batch.

2.5. Microscopy and image analysis

The histological sections and immunohistochemically labeled specimens were examined using an using an optical and epifluorescence microscope (Axio Imager M2, Carl Zeiss Microscopy GmbH, Jena, Germany) equipped with a digital camera system (AxioCam MRm, Carl Zeiss) and a digital acquisition system (ZEN, 2012 blue edition, Carl Zeiss). Photographs were taken centrally as well as from the end of each retinal section, using a 20× objective. Images were viewed and