Journal of Neuroimmunology 284 (2015) 57–66

Contents lists available at ScienceDirect

Journal of Neuroimmunology

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

Transitory loss of glia and the subsequent modulation in inflammatory cytokines/chemokines regulate paracellular claudin-5 expression in endothelial cells

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ARTICLE INFO

Article history:
Received 29 January 2015
Received in revised form 6 May 2015
Accepted 11 May 2015

Keywords:
Astrocytes
Blood–brain barrier
Chemokine
Cytokine
Claudin-5
Microglia

ABSTRACT

Signaling mechanisms involved in regulating blood–brain barrier (BBB) integrity during central nervous system (CNS) inflammation remain unclear. We show that an imbalance between pro-/anti-inflammatory cytokines/chemokines alters claudin-5 expression. *In vivo*, gliotoxin-induced changes in glial populations and an imbalance between pro-/anti-inflammatory cytokine/chemokine expression occurred as BBB integrity was compromised. The balance was restored as BBB integrity was re-established. *In vitro*, TNF-α, IL-6, and MCP-1 induced paracellular claudin-5 expression loss. TNF-α- and IL-6- effects were mediated through the PI3K pathway and IL-10 attenuated TNF-α's effect. This study shows that pro-/anti-inflammatory modulators play a critical role in BBB integrity during CNS inflammation.

1. Introduction

The blood–brain barrier (BBB) is a key regulator of central nervous system (CNS) homeostasis, promoting neuronal support and limiting lymphocytes, toxins, blood proteins, and microorganisms from entering the brain and spinal tissue. This highly selective and dynamic barrier is composed of endothelial cells that express tight and adherens junction proteins, astrocytes, microglia, and pericytes (Willis et al., 2008; Abbott et al., 2010). Although the BBB is central to CNS homeostasis, the factors responsible for maintaining BBB properties remain unclear. A number of studies have shown that astrocytes play an important role in BBB regulation. *In vitro*, cultured primary brain endothelial cells lose their BBB characteristics (Rubin et al., 1991). These features are restored when endothelial cells are co-cultured with cells of astroglial origin or when incubated with astroglial membrane fractions or astrocyte/glioma-conditioned medium (Tao-Cheng et al., 1987; Abbott et al., 1992; Hurst and Fritz, 1996; Sobue et al., 1999; Prat et al., 2001). *In vivo*, we have previously shown that astrocytes play a critical role in tight junction protein expression and in maintaining BBB integrity (Willis et al., 2004a,b, 2013). Systemic administration of the gliotoxin, 3-chloropropanediol, induced a loss of glial fibrillary acidic protein (GFAP)-immunoreactive astrocytes and CD11b-immunoreactive microglia in the rat inferior colliculus. Vascular endothelial cells lost paracellular expression of tight junction proteins claudin-5, occludin, and zonula occludens-1 (ZO-1), but not adherens junction markers VE-cadherin nor β-catenin (Willis et al., 2004b, 2013). These morphological changes culminated in the loss of BBB integrity to 4–70 kDa dextran tracers (Willis et al., 2004b). As the pathology progressed, a gliosis developed in which GFAP-immunoreactive astrocytes and CD11b-immunoreactive microglia repopulated the lesion, and tight junction proteins returned to paracellular domains with restoration of BBB integrity to dextran tracers. Results from this study suggest a restorative role primarily for astrocytes at the BBB (Willis et al., 2004b). However, the mechanism of signal transduction between glial cells and endothelial cells was not studied.

Astrocytes perform a wide range of functions, including neuronal metabolic support, regulation of the extracellular space ion concentration, neurotransmitter uptake and release, modulation of synaptic transmission, vasomodulation, and repair of the CNS scarring process (Abbott et al., 2006). The myeloid-derived microglia are the resident monocytes of the CNS. Hypertrophy and proliferation of microglial cells are morphological features of the CNS innate immune response to stimuli such as inflammation and endotoxins during neurodegenerative disease (McMahon et al., 2005; Inoue, 2006). Under inflammatory conditions, both activated astrocytes and hypertropic microglia secrete

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http://dx.doi.org/10.1016/j.jneuroim.2015.05.008
0165-5728/

a variety of inflammatory mediators including interleukin (IL)-1β, IL-6, tumor necrosis factor-α (TNF-α), and interferon-gamma (IFN-γ) (DeLeo et al., 2004; Coull et al., 2005; McMahon et al., 2005; Abbott et al., 2006). There is a growing body of evidence that inflammatory mediators play a role in BBB breakdown (Capaldo and Nusrat, 2009; Pan et al., 2011; Erikson et al., 2012). Patients with multiple sclerosis (MS) show elevated levels of cytokines and chemokines in the CNS and a loss of BBB integrity (Plumb et al., 2002; Erikson et al., 2012). However, the effect of pro- and anti-inflammatory mediators regulating the integrity of the mature BBB is not fully understood.

We reasoned that modulation of astrocyte and microglia populations would initiate an inflammatory response with marked changes in pro- and anti-inflammatory mediator expression, and that these mediators directly regulate markers of BBB integrity. To test this hypothesis, we employed both an *in vivo* and *in vitro* experimental design. *In vivo*, we used the well-defined 3-chloropropanediol lesion model (Willis et al., 2004b). *In vitro*, we employed use of the endothelial cell line, bEnd.3, to determine the effect of select cytokines/chemokines directly on vascular endothelial cells. We show that a chemically-induced change in glial populations and an imbalance in pro-/anti-inflammatory cytokine/chemokine expression in the rat inferior colliculus occur when the integrity of the BBB is compromised. *In vitro*, TNF-α, IL-6, and MCP-1, but not IL-10, induced a loss of paracellular claudin-5 expression. Pre-treatment with IL-10 attenuated TNF-α's effects on claudin-5 expression. In addition, TNF-α-, and IL-6-, but not MCP-1-, induced effects are in part mediated through the PI3K pathway. Therapeutics based on anti-inflammatory cytokine activity, or that restore the pro-/anti-inflammatory balance may slow progression of, or even reverse, neurodegenerative disease pathology, such as seen with MS, by preserving BBB integrity.

2. Methods

2.1. Animals and dosing

Male Fischer F344 rats (180–220 g; Harlan, Indianapolis, IN, USA) were maintained on a 12:12 h day/night light cycle. Food and water was provided *ad libitum*. Rats were given a single i.p. dose of 140 mg/kg 3-chloropropanediol ((S)-(+)-3-chloro-1,2-propanediol, S-α-chlorohydrin); (Sigma-Aldrich Inc., St. Louis, MO, USA) in sterile saline (1 ml/kg) under light isoflurane anesthesia. The rats were allowed to recover and were then sacrificed up to 14 days after dosing. Vehicle injected animals were used for control inferior colliculus tissue studies. Four to six animals were used in each group. All animal procedures were carried out in accordance with National Institutes of Health guidelines and were approved by the University of New England Institutional Animal Care and Use Committee. All efforts were made to minimize the number of animals used and their suffering.

2.2. Tissue preparation

For confocal microscopy and MILLIPLEX immunoassays, animals were killed by over-dose of isoflurane anesthetic followed by decapitation. For confocal microscopy whole brains were rapidly removed and the hind brain collected. For MILLIPLEX immunoassays, the inferior colliculus was dissected out. All samples were snap-frozen in dry ice-cooled isopentane at −40 °C and stored at −80 °C until required.

2.3. In vivo confocal microscopy

Cryostat sections (30 μm) containing inferior colliculus (+0.4 to +0.6 mm from the interaural line) (Paxinos and Wilson, 1998) were mounted on gelatin-coated glass slides and stored at −80 °C until used for staining. Tissue sections were air-dried and fixed in 100% ethanol for 10 min. Following fixation, sections were washed in phosphate buffered saline (PBS) (pH 7.2), then in buffer (1% bovine serum albumin (BSA)/0.2% Tween-20 in PBS), and incubated in normal goat serum (2 mg/ml in buffer; Dako A/S, Glostrup, Denmark) for 30 min. Indirect immunofluorescence was performed using GFAP (0.8 μg/ml; Sigma-Aldrich), and CD11b (0.5 μg/ml; AbD Serotec, Raleigh, NC, USA). Primary antibodies were diluted in buffer and incubated on sections for 2 h. Following incubation, sections were washed in buffer and incubated in purified goat anti-mouse IgG secondary antibodies conjugated to Alexa-Fluor-488 (4 μg/ml; Life Technologies, Carlsbad, CA, USA) for 1 h in the dark. Finally, sections were washed in buffer, then in PBS, and mounted in ProLong Gold antifade with DAPI (Life Technologies) under coverslips. All incubations were performed at room temperature (RT).

Sections were examined using a Leica TCS SP5 laser scanning confocal microscope with an argon–krypton laser and three channel scan head (Leica, Buffalo Grove, IL, USA). Sequential scans were created through the 30 μm sections and maximum projection images were obtained. These were exported and viewed using Paint Shop Pro 7.0 (Jasc Software, Inc. Eden Prairie, MN, USA) and uniformly adjusted to optimize brightness and contrast.

2.4. MILLIPLEX immunoassay analysis of cytokines/chemokines in vivo

The inferior colliculus was homogenized using a handheld Teflon homogenizer in ice-cold CelLytic buffer (Sigma-Aldrich) containing protease inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitor cocktails 2&3 (Sigma-Aldrich). The homogenate was cleared by centrifugation (10,000 *g*, 10 min, 4 °C). The protein concentration of each sample was determined using the bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, IL, USA) and used for immunoassay analysis. A commercially available MILLIPLEX MAP Rat Cytokine/Chemokine Magnetic Bead Immunoassay (Millipore, Billerica, MA, USA) was used to measure the tissue TNF-α, IL-6, IL-13, MCP-1, IL-4, and IL-10, levels in the rat inferior colliculus. All procedures followed the manufacturer's protocol. Briefly, assay buffer was added to a 96-well plate and incubated for 10 min at RT. Assay buffer was removed and replaced with rat inferior colliculus tissue homogenate sample or CelLytic buffer (without inhibitors) for background. A standard curve was prepared using supplied standards and control samples. Finally, well-mixed and sonicated magnetic beads were added to each well. The plate was incubated at 4 °C on a plate shaker overnight. The plate was then washed with wash buffer (Millipore), detection antibodies added, and then incubated for 1 h at RT while shaking. Without removing the detection antibodies, Streptavidin-Phycoerythrin was added and the plate incubated for 30 min at RT while shaking. Finally, the plate was washed, sheath fluid (Millipore) added, and the overall median fluorescent intensity in each well was determined using MAGPIX hardware. The median fluorescent intensity of each analyte was determined through the logistic curve-fitting method. Analytes were normalized to total protein concentration.

2.5. Mouse bEnd.3 endothelial cell-culture

bEnd.3 endothelial cells are BALB/c mouse brain endothelial cells transformed by a retrovirus vector that expresses polyomavirus middle T antigen (CRL-2299 American Type Culture Collection, Manassas, VA, USA). Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, D6046)/10% fetal bovine serum (FBS) (Sigma-Aldrich) in a humidified cell culture incubator at 37 °C and 5% CO_2/95% room air following supplier's instructions. For experimental use, cells were trypsinized and plated at a density of 0.5–1.0 × 10^4 cells/cm^2 (Omidi et al., 2003) onto 12-well plates with collagen I-coated glass coverslips, to be viewed by immunofluorescence microscopy. Cells were grown until confluent and medium was monitored and changed every three days and again 24 h before cell treatment.