Experimental Neurology 292 (2017) 35–45

Contents lists available at ScienceDirect

Experimental Neurology

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

Research Paper

Fibronectin connecting segment-1 peptide inhibits pathogenic leukocyte trafficking and inflammatory demyelination in experimental models of chronic inflammatory demyelinating polyradiculoneuropathy

Chaoling Dong, Kelsey M. Greathouse, Rebecca L. Beacham, Steven P. Palladino, E. Scott Helton, Eroboghene E. Ubogu *

Neuromuscular Immunopathology Research Laboratory, Division of Neuromuscular Disease, Department of Neurology, University of Alabama at Birmingham, Birmingham, AL, United States

ARTICLE INFO

Article history:
Received 9 November 2016
Received in revised form 25 January 2017
Accepted 14 February 2017
Available online 16 February 2017

Keywords:
Blood-nerve barrier
Chronic inflammatory demyelinating polyradiculoneuropathy
Fibronectin connecting segment-1
Integrin
Leukocyte trafficking
Spontaneous autoimmune peripheral polyneuropathy

ABSTRACT

The molecular determinants of pathogenic leukocyte migration across the blood-nerve barrier (BNB) in chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) are unknown. Specific disease modifying therapies for CIDP are also lacking. Fibronectin connecting segment-1 (FNCS1), an alternatively spliced fibronectin variant expressed by microvascular endothelial cells at sites of inflammation *in vitro* and *in situ*, is a counterligand for leukocyte α_4 integrin (also known as CD49d) implicated in pathogenic leukocyte trafficking in multiple sclerosis and inflammatory bowel disease. We sought to determine the role of FNCS1 in CIDP patient leukocyte trafficking across the BNB *in vitro* and in severe chronic demyelinating neuritis *in vivo* using a representative spontaneous murine CIDP model. Peripheral blood mononuclear leukocytes from 7 untreated CIDP patients were independently infused into a cytokine-treated, flow-dependent *in vitro* BNB model system. Time-lapse digital video microscopy was performed to visualize and quantify leukocyte trafficking, comparing FNCS1 peptide blockade to relevant controls. Fifty 24-week old female B7-2 deficient non-obese diabetic mice with spontaneous autoimmune peripheral polyneuropathy (SAPP) were treated daily with 2 mg/kg FNCS1 peptide for 5 days *via* intraperitoneal injection with appropriate controls. Neurobehavioral measures of disease severity, motor nerve electrophysiology assessments and histopathological quantification of inflammation and morphometric assessment of demyelination were performed to determine *in vivo* efficacy. The biological relevance of FNCS1 and CD49d in CIDP was evaluated by immunohistochemical detection in affected patient sural nerve biopsies. 25 μM FNCS1 peptide maximally inhibited CIDP leukocyte trafficking at the human BNB *in vitro*. FNCS1 peptide treatment resulted in significant improvements in disease severity, motor electrophysiological parameters of demyelination and histological measures of inflammatory demyelination. Microvessels demonstrating FNCS1 expression and CD49d+ leukocytes were seen within the endoneurium of patient nerve biopsies. Taken together, these results imply a role for FNCS1 in pathogenic leukocyte trafficking in CIDP, providing a potential target for therapeutic modulation.

1. Introduction

CIDP is a clinically heterogeneous immune-mediated disorder affecting peripheral nerve and nerve roots with maximum severity attained after 8 weeks following symptom onset. CIDP has an estimated annual incidence of 1-2/100,000 and prevalence as high as 9/100,000 (Dalakas and Medscape, 2011; Laughlin et al., 2009). CIDP is often under-recognized and may account for about 14% of chronic disability in adults above the age of 65 (Chia et al., 1996). Its clinical course may be described as relapsing-remitting, steady progressive or step-wise progressive, with albuminocytologic dissociation commonly observed on cerebrospinal fluid analysis. Different clinical variants exist based on the pattern of nerve and nerve root involvement observed on electrodiagnostic studies. Pathologically, CIDP is characterized by infiltration of predominantly monocytes/macrophages and less commonly T lymphocytes into peripheral nerve and nerve root endoneurium with macrophage-mediated demyelination. However, inflammatory infiltrates may be sparse with prominent evidence of demyelination with or without remyelination seen in nerve biopsies. Onion bulb formation, indicative of repetitive demyelination and remyelination may be observed in more chronic cases (Dalakas, 2015; Mathey et al., 2015; Ubogu, 2015). Several diagnostic criteria exist for clinical and research purposes. Consensus statements have been published by consortia of

* Corresponding author at: Division of Neuromuscular Disease, Department of Neurology, University of Alabama at Birmingham, 1720 7th Avenue South, Sparks Center 200, Birmingham, AL 35294-0017, United States.
E-mail address: eeubogu@uabmc.edu (E.E. Ubogu).

http://dx.doi.org/10.1016/j.expneurol.2017.02.012
0014-4886/

experts to aid clinicians diagnose patients early and institute therapy (Dalakas, 2015; EFNS/PNS, 2010; Mathey et al., 2015)

Current treatments for CIDP include corticosteroids, intravenous immunoglobulin (IVIg) and plasma exchange based on randomized controlled trials, and immunosuppressant drugs such as azathioprine, mycophenolate, cyclosporine and cyclophosphamide based on small case series, case reports or specialist physician experience (Brannagan, 2009). Despite significant advances in understanding the molecular pathogenesis of immune-mediated disorders and the development of disease-specific therapies for disorders such as rheumatoid arthritis, psoriasis, inflammatory bowel disease and multiple sclerosis over the last 20 years, specific disease-modifying therapies for CIDP do not currently exist. CIDP heterogeneity and clinical recognition provide challenges; however lack of patient nerve and nerve root biopsies for extensive exploratory studies, dearth of *in vitro* human BNB models to study pathogenic inflammatory mechanisms and the limitations of representative animal models needed to ascertain pathogenic mechanisms and test potential drugs *in vivo* before clinical trials are planned have adversely hampered translational strategies needed for novel therapeutic development in CIDP (Meyer zu Horste et al., 2007; Ubogu, 2015).

Inflammatory leukocyte migration across microvessels is a sequential, coordinated process (*i.e.* multi-step paradigm) involving specific selectins, chemokines and adhesion molecules on endothelial cells, and selectin counterligands, chemokine receptors, integrins and matrix metalloproteases expressed by leukocytes (Man et al., 2007; Muller, 2011; Simon and Green, 2005; Ubogu, 2015; Yonekawa and Harlan, 2005). Direct evidence of pathogenic leukocyte trafficking at the human BNB *via* the paracellular route has been demonstrated *in vitro* and *in situ* (Dong et al., 2016; Yosef and Ubogu, 2012). Observational studies evaluating CIDP patient nerve biopsies, cerebrospinal fluid, plasma and sera have shown increased expression of specific pro-inflammatory cytokines, chemokines and chemokine receptors, adhesion molecules, matrix metalloproteinases and other inflammatory mediators (Dalakas and Medscape, 2011; Mathey et al., 2015; Ubogu, 2015). The molecular determinants and signaling mechanisms of pathogenic leukocyte trafficking at the BNB in CIDP patients are unknown. Targeting pathogenic leukocyte trafficking at the BNB is a plausible approach to limit inflammatory demyelination and improve patient outcomes, as seen with natalizumab (humanized mouse anti-human α_4 integrin monoclonal antibody) in relapsing-remitting multiple sclerosis (Pucci et al., 2011). Interestingly, there is some conflicting evidence on the efficacy of natalizumab in medically refractory CIDP based on a single case report showing no benefit and a small three-patient case series showing clinical effect (Vallat et al., 2015; Wolf et al., 2010).

Fibronectin, a major extracellular matrix macromolecule, consists of an alternatively spliced type III connecting segment that generates a high affinity binding domain for leukocyte α_4 integrin. This binding domain, called fibronectin connecting segment-1 (FNCS1) has a conserved peptide sequence, leucine-aspartate-valine (LDV), critical for integrin binding (Humphries et al., 1987; Mould et al., 1994). FNCS1 may be involved in the pathogenesis of chronic inflammatory conditions such as rheumatoid arthritis, allergic contact dermatitis and multiple sclerosis (Elices et al., 1994; Man et al., 2009; Martín et al., 2003; Müller-Ladner et al., 1997; Ubogu et al., 2006). FNCS1 expression has been demonstrated on human endoneurial endothelial cells that form the BNB *in vitro*, with increased relative expression of splice variants that contain the LDV sequence and time-dependent protein expression observed following physiological cytokine stimulus (Yosef and Ubogu, 2012; Yosef et al., 2010). Vascular cell adhesion molecule-1 (VCAM-1) is also a counterligand for α_4 integrin implicated in lymphocyte migration during infection and inflammation, as well as lymphocyte bone marrow retention and homing into lymph nodes *via* high endothelial venules during normal immune processes (Boscacci et al., 2010; Faveeuw et al., 2000; Koni et al., 2001; Papayannopoulou and Craddock, 1997; Xu et al., 2003). Competitive antagonism of FNCS1-α_4 integrin binding with small molecular antagonists that target the LDV peptide sequence could result in a specific disease modifying therapy for CIDP without interfering with α_4 integrin-VCAM-1 binding implicated in normal immunity (Haworth et al., 1999; Jackson, 2002; Liu et al., 2015; Man et al., 2009; Ubogu et al., 2006).

2. Methods

2.1. CIDP patient leukocytes and sural nerve biopsies

Peripheral blood mononuclear leukocytes (PBMLs) were obtained from whole heparinized blood donated by seven untreated adult patients with clinical, electrophysiological and supportive cerebrospinal fluid or histopathological evidence of CIDP based on the Inflammatory Neuropathy Cause and Treatment criteria. PBMLs were isolated using density gradient centrifugation and cryopreserved in liquid nitrogen using a controlled rate freezing process. This was performed due to donor unpredictability and to ensure experiments were performed concurrently to limit experimental variation. Leukocyte viability was >99% following reconstitution in warmed medium at 37 °C using the 0.4% trypan blue exclusion test. Duration of storage had no significant effect on PBML viability or composition provided experiments were performed within 6 months of preservation, as previously published (Yosef and Ubogu, 2012). Archived frozen sural nerve biopsies from four untreated adult CIDP patients and two normal controls stored in optimum cutting temperature compound at −80 °C were obtained from the Shin J. Oh Muscle and Nerve Histopathology Laboratory, Department of Neurology, University of Alabama at Birmingham. The study was approved by the Institutional Review Board, with an exemption obtained to use archived pathological specimens for research. Written informed consent was obtained from each CIDP patient blood donor.

2.2. Flow-dependent human in vitro blood-nerve barrier trafficking assay

Untreated CIDP patient PBML trafficking across the human BNB *in vitro* was studied in real time using a flow-dependent leukocyte trafficking assay. A parallel plate flow chamber was attached to basal and cytokine-treated confluent primary human endoneurial endothelial cells (that form the BNB) cultured on rat tail collagen-coated CellBIND® Petri dishes, coupled to time-lapse video microscopy as previously described (Greathouse et al., 2016; Ubogu, 2013; Yosef and Ubogu, 2012). CIDP patient PBMLs were incubated with FNCS1 peptide EILDVPST (GenScript, Piscataway, NJ; 0–100 μM), a scrambled control peptide DELPQLVTL (designated as FNCS1C, GenScript, 25 μM: negative control), human IVIg (Carimune® nanofiltered, CSL Behring AG, Bern, Switzerland, 5 mg/mL; current CIDP treatment: positive control) and 6-alpha methylprednisolone (6αMP; Sigma-Aldrich, St. Louis, MO; 200 μg/mL: current CIDP treatment: positive control) for 10 min at 37 °C before performing the leukocyte-BNB trafficking assay with the drug in solution. Each experiment lasted 30 min. FNCS1 peptide was compared to current CIDP treatments, 6αMP and human IVIg, to determine equivalence or superiority in modulating leukocyte trafficking at the BNB *in vitro*. FNCS1 peptide is being evaluated as a competitive antagonist occupying the critical α_4 integrin binding site on PBMLs. This is hypothesized to prevent leukocyte adhesion to endothelial cell-expressed FNCS1 during the trafficking cascade (Man et al., 2009). Leukocytes from healthy controls or patients with other neurological disorders were not used in this study as the aim was to determine comparative drug efficacy in CIDP patients, with each patient serving as his/her own control.

Videos were generated by merging digital photomicrographs generated by an Axiocam MRc 5 digital camera (Carl Zeiss Microscopy, Jena, Germany) using the National Institutes of Health Image J software (field of view 870 μm long × 650 μm wide) or directly using an Eclipse Ci-S Upright epifluorescent microscope with a D5-Qi2 camera (Nikon Instruments Inc., Melville, NY) coupled to the Nikon NIS-Elements AR software program (field of view 1420 μm long × 950 μm wide).