Neurobiology of Disease 83 (2015) 75–89

Contents lists available at ScienceDirect

Neurobiology of Disease

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

Genetic targeting of protease activated receptor 2 reduces inflammatory astrogliosis and improves recovery of function after spinal cord injury

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ARTICLE INFO

Article history:
Received 7 April 2015
Revised 1 August 2015
Accepted 19 August 2015
Available online 24 August 2015

Keywords:
Traumatic spinal cord injury
Astrogliosis
Inflammation
Cytokine
GPCR
Serine protease

ABSTRACT

Inflammatory-astrogliosis exacerbates damage in the injured spinal cord and limits repair. Here we identify Protease Activated Receptor 2 (PAR2) as an essential regulator of these events with mice lacking the PAR2 gene showing greater improvements in motor coordination and strength after compression-spinal cord injury (SCI) compared to wild type littermates. Molecular profiling of the injury epicenter, and spinal segments above and below, demonstrated that mice lacking PAR2 had significantly attenuated elevations in key hallmarks of astrogliosis (glial fibrillary acidic protein (GFAP), vimentin and neurocan) and in expression of pro-inflammatory cytokines (interleukin-6 (IL-6), tumor necrosis factor (TNF) and interleukin-1 beta (IL-1β)). SCI in PAR2 −/− mice was also accompanied by improved preservation of protein kinase C gamma (PKCγ)-immunopositive corticospinal axons and reductions in GFAP-immunoreactivity, expression of the pro-apoptotic marker BCL2-interacting mediator of cell death (BIM), and in signal transducer and activator of transcription 3 (STAT3). The potential mechanistic link between PAR2, STAT3 and astrogliosis was further investigated in primary astrocytes to reveal that the SCI-related serine protease, neurosin (kallikrein 6) promotes IL-6 secretion in a PAR2 and STAT3-dependent manner. Data point to a signaling circuit in primary astrocytes in which neurosin signaling at PAR2 promotes IL-6 secretion and canonical STAT3 signaling. IL-6 promotes expression of GFAP, vimentin, additional IL-6 and robust increases in both neurosin and PAR2, thereby driving the PAR2-signaling circuit forward. Given the significant reductions in astrogliosis and inflammation as well as superior neuromotor recovery observed in PAR2 knockout mice after SCI, we suggest that this receptor and its agonists represent new drug targets to foster neuromotor recovery.

1. Introduction

Astrogliosis and inflammation are integrated contributors to the cascade of events occurring after spinal cord trauma. While certain components of this cascade worsen tissue damage and limit wound healing, other facets are essential to creating an environment favorable to repair. SCI-provoked astroglial and inflammatory responses fit on this complex continuum of injury and repair and this complicates targeted therapy. For example, the SCI-associated astroglial scar limits secondary damage by restricting inflammatory cell infiltration and providing mechanical support (Pekny et al., 1999; Okada et al., 2006; Herrmann et al., 2008; Wanner et al., 2013). In addition however, reactive astrocytes along with microglia and infiltrating macrophages secrete pro-inflammatory cytokines that can injure sparred axons and oligodendroglia (Klusman and Schwab, 1997; Kim et al., 2001; Okada et al., 2004; Wang et al., 2005). Moreover, the compact glial scar creates a chemical and physical barrier limiting regeneration of severed axons (Asher et al., 2001; Bradbury et al., 2002; Silver and Miller, 2004; Okada et al., 2006; Sofroniew, 2009). A better understanding of the molecular underpinnings of inflammatory-astrogliosis in SCI may enable selective targeting to foster an environment ultimately conducive to wound healing and compatible with emerging regenerative interventions.

Protease Activated Receptor 2 (PAR2) is a G-protein coupled receptor (GPCR) playing fundamental roles in neural injury, including effects across neurons, astrocytes and neuroinflammatory responses (Luo et al., 2007), although its activities in SCI are essentially unknown. There are four PARs (PARs1–4), each a classic seven transmembrane receptor. PARs are unique, being activated by cleavage within their extracellular domain to reveal a new amino-terminus that binds to the second extracellular loop to elicit intracellular signaling (Ramachandran et al., 2012).

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Available online on ScienceDirect (www.sciencedirect.com).

http://dx.doi.org/10.1016/j.nbd.2015.08.021
0969-9961/

PARs enable cells to respond, or to over respond, to rapid changes in the proteolytic microenvironment occurring with CNS trauma, inflammation and blood brain barrier breakdown.

The role of PAR2 in neuropathogenesis is complex, depressing synaptic activity in hippocampal slices (Gan et al., 2011) and exacerbating neurotoxicity *in vitro* (Yoon et al., 2013) and in experimental autoimmune encephalomyelitis (Noorbakhsh et al., 2006). However, PAR2 serves a neuroprotective role in acute ischemic injury (Jin et al., 2005) and in human immunodeficiency virus-elicited neuroinflammation (Noorbakhsh et al., 2005). PAR2 activation also prevents ceramide-induced astrocyte apoptosis (Wang et al., 2007). The role of PAR2 in inflammation is also context specific, with anti-inflammatory actions in airways (Kouzaki et al., 2009) and intestine (Vergnolle et al., 2004), but pro-inflammatory effects in adjuvant induced arthritis (Ferrell et al., 2003), and allergic dermatitis (Kawagoe et al., 2002).

Given the unique position of PAR2 to mediate effects across multiple cell types in SCI, we sought to clarify its role and potential mechanism of action by determining the impact of PAR2 gene deletion on functional recovery and cellular and molecular signs of pathogenesis in murine experimental contusion–compression SCI. Complementary *in vivo* and *in vitro* approaches demonstrate that PAR2 can be targeted genetically to improve motor outcomes in SCI in part by limiting the canonical interleukin 6 (IL-6)-signal transducer and activator of transcription 3 (STAT3)-dependent signaling axis and the deleterious effects of inflammatory-astrogliosis.

2. Materials and methods

2.1. Experimental contusion–compression SCI

To investigate the role of PAR2 on neurobehavioral recovery after spinal cord trauma, we generated experimental SCI in twelve-week old (19–23 g) adult female PAR2 +/+ or PAR2 −/− (B6.Cg-F2rl1-^{tm1Nwb}) mice by application of a modified aneurysm clip (FEJOTA^{TM} mouse clip, 8 g closing force) as previously described (Joshi and Fehlings, 2002b; Yu and Fehlings, 2011; Radulovic et al., 2013). This model generates a severe injury with high clinical relevance related in part to the generation of an initial contusion as well as a persistent dorsal and ventral compression that results in a robust neuroinflammatory response, astrogliosis and axon degeneration (Joshi and Fehlings, 2002a, 2002b; Yu and Fehlings, 2011). PAR2 −/− mice were obtained from Jackson Laboratories (Bar Harbor, ME), backcrossed to C57BL/6 mice for more than 35 generations, and genotyped as described (Burda et al., 2013; Yoon et al., 2013). Uninjured PAR2 +/+ littermates served as controls in each case. The main endpoints examined were 3, 7 or 30 days after SCI (dpi), corresponding to acute and more chronic periods post-lesion.

Prior to spinal cord compression injury, mice were deeply anesthetized with Xylazine (0.125 mg/kg, Akom, Inc., Decatur, IL) and Ketaset (1 mg/kg, Fort Dodge Animal Health, Fort Dodge, IA). A laminectomy was performed and injury induced at the level of L1/L3 by extradural application of the FEJOTA clip for a period of 1 min. After surgery, the skin was repaired using AUTOCLIP (9 mm, BD Biosciences, San Jose, CA). Before and after surgery, 1 ml of saline was administered subcutaneously to replace lost blood volume. Pain was minimized by administration of Buprenorphine (0.05 mg/kg, Hospira, Lake Forest, IL) subcutaneously every 12 h for 96 h post-surgery. Food and water were available *ad libitum* and cage temperatures maintained at 37 C. To avoid infection, Baytril (10 mg/kg, Bayer Health Care, Shawnee Mission, KS) was administered intraperitoneally in a prophylactic fashion for the first 72 h post-surgery. Bladders were manually voided twice daily until the endpoint of each experiment. Any mice with signs of infection or which were moribund were immediately euthanized and excluded from the study. For all studies, mice were randomized with respect to genotype prior to surgery and investigators participating in surgery and evaluation of subsequent neurobehavioral or histological outcomes blinded to genotype throughout the duration of the experiment. Animals were housed in individual cages. All animal experiments were carried out with careful attention to animal comfort and in strict adherence to NIH Guidelines for animal care and safety. The Mayo Clinic Institutional Animal Care and Use Committee approved these studies.

2.2. Expression of PAR2 in experimental SCI

To address the potential significance of PAR2 to pathogenesis during the acute and more chronic periods after SCI, we determined the relative abundance of RNA expression for PAR2, and its CNS endogenous agonist, neurosin in RNA isolated from the uninjured spinal cord, or in the 3 mm of spinal cord at the level of the injury epicenter, as well as in the 3 mm above or below the site of injury site, at 7 or 30 dpi (n = 4 per time point). Neurosin is also commonly referred to as kallikrein 6 (Klk6) as well as Zyme, Protease M, or myelencephalon specific protease (MSP) (Scarisbrick and Blaber, 2012). Given the relatively low amount of RNA obtained from sites of injury, particularly at the injury epicenter and below at 30 dpi, samples from individual mice at a given endpoint and injury level were pooled prior to RNA isolation (Kendziorski et al., 2005; Radulovic et al., 2013). RNA was isolated using RNA STAT-60 (Tel-Test, Friendswood, TX) and stored at −70 C until the time of analysis. Amplification of the housekeeping gene 18S in the same RNA samples was used to control for loading. Real-time PCR amplification in each case was accomplished using primers obtained from Integrated DNA Technologies (Coralville, IA), or Applied Biosystems (Grand Island, NY), as detailed in Table 1, on an iCycler iQ5 system (BioRad, Hercules, CA).

2.3. Neurobehavioral outcome measures

All mice received training in the open field, ladder walk and incline plane test prior to surgery. A baseline measurement for each mouse in each assay was collected prior to surgery (0 dpi). The open field was used to assess seven categories of locomotor recovery using the Basso Mouse Scale (BMS), the day after surgery, and weekly thereafter until 30 dpi (Basso et al., 2006). Briefly, mice were placed in a plexiglass enclosed open field and 2 observers evaluated ankle movement, plantar placement, stepping, coordination, paw position, trunk stability and tail position generating a maximum BMS score of 9 and a subscore of 0 to 11.

The ladder walk was used to evaluate sensorimotor coordination between the hind limbs and forelimbs (Cummings et al., 2007). Mice were videotaped crossing a horizontal ladder fitted with an angled mirror to view/record footfalls prior to surgery and on days 8, 15, 22 and 31 after injury. 4 mm rungs of the ladder were spaced between 7.5 to 16 mm apart creating “easy” or “hard” levels of difficulty that included the

Table 1
Real time PCR assays used to quantify molecular changes in gene transcription in response to experimental contusion–compression SCI. Primers were obtained from Integrated DNA Technologies (IDT), or Applied Biosystems (AB), as indicated.

Gene	Accession number	Primer sequence forward/reverse
BIM	NM_207680	Probe Assay ID: Mm.PT.56a.8950841.g (IDT)
GFAP	NM_010277.2	GCAGATGAAGCCACCC TGG/GAGGTCTGGCTTGGCCAC (IDT)
IL-6	NM_031168.1	Probe Assay ID: Mm00446190_m1 (AB)
IL-10	NM_010548.2	Probe Assay ID: Mm00439614_m1 (AB)
IL-1β	NM_008361.3	Probe Assay ID: Mm.PT.51.17212823 (IDT)
Neurocan	NM_007789.3	Probe Assay ID: Mm.PT.56a.10993411 (AB)
Neurosin (Klk6)	NM_011177.2	CCTACCCTGGCAAGAT CAC/GGATCCATCTGATATGAGTGC
PAR2	NM_010170.4	GCATTGAACATCACCACCTG /GGATAGCCCTCTGCCTTTTC (IDT)
Rn18s	NR_003278.3	Probe Assay ID: Mm03928990_g1 (AB)
TGF-β1	NM_011577.1	Probe Assay ID: Mm01178820_m1 (IDT)
TNF	NM_013693.2	Probe Assay ID: Mm00443258_m1 (AB)
VIM	NM_011701.4	Probe Assay ID: Mm.PT.53a.8720419 (IDT)