Neurobiology of Disease 99 (2017) 24–35

Contents lists available at ScienceDirect

Neurobiology of Disease

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

Combined chondroitinase and KLF7 expression reduce net retraction of sensory and CST axons from sites of spinal injury

Zimei Wang, Kristen Winsor, Christopher Nienhaus, Evan Hess, Murray G. Blackmore *

Department of Biomedical Sciences, Marquette University, 53201, USA

ARTICLE INFO

Article history:
Received 20 April 2016
Revised 2 December 2016
Accepted 13 December 2016
Available online 14 December 2016

Keywords:
Spinal cord injury
Axon regeneration
Corticospinal
Dorsal root ganglion
Chondroitin sulfate proteoglycan
Chondroitinase
Transcription factor
Adeno-associated virus
Lentivirus
KLF7

ABSTRACT

Axon regeneration in the central nervous system is limited both by inhibitory extracellular cues and by an intrinsically low capacity for axon growth in some CNS populations. Chondroitin sulfate proteoglycans (CSPGs) are well-studied inhibitors of axon growth in the CNS, and degradation of CSPGs by chondroitinase has been shown to improve the extension of injured axons. Alternatively, axon growth can be improved by targeting the neuron-intrinsic growth capacity through forced expression of regeneration-associated transcription factors. For example, a transcriptionally active chimera of Krüppel-like Factor 7 (KLF7) and a VP16 domain improves axon growth when expressed in corticospinal tract neurons. Here we tested the hypothesis that combined expression of chondroitinase and VP16-KLF7 would lead to further improvements in axon growth after spinal injury. Chondroitinase was expressed by viral transduction of cells in the spinal cord, while VP16-KLF7 was virally expressed in sensory neurons of the dorsal root ganglia or corticospinal tract (CST) neurons. After transection of the dorsal columns, both chondroitinase and VP16-KLF7 increased the proximity of severed sensory axons to the injury site. Similarly, after complete crush injuries, VP16-KLF7 expression increased the approach of CST axons to the injury site. In neither paradigm however, did single or combined treatment with chondroitinase or VP16-KLF7 enable regenerative growth distal to the injury. These results substantiate a role for CSPG inhibition and low KLF7 activity in determining the net retraction of axons from sites of spinal injury, while suggesting that additional factors act to limit a full regenerative response.

1. Introduction

Recovery from injury to the central nervous system (CNS) is limited by the inability of severed axons to regenerate and re-establish effective communication. Axon regeneration is constrained in part by cell-intrinsic mechanisms in many axotomized CNS neurons, including a failure to initiate needed pro-regenerative transcriptional programs (Blackmore, 2012; Moore and Goldberg, 2011). One well-studied example is the differential regenerative ability of the peripherally *versus* centrally projecting branches of sensory axons. Successful axon regeneration of the peripheral branch depends on the transcription of regeneration associated genes (RAGs) in the sensory cell body (Smith and Skene, 1997), which in turn depends on transcription factors that are themselves upregulated or activated in response to peripheral axotomy (Ben-Yaakov et al., 2012; Broude et al., 1997; Jankowski et al., 2009; Michaelevski et al., 2010; Tsujino et al., 2000). In contrast, injury to the centrally projecting axon triggers RAG expression that is smaller in magnitude and shorter in duration (Broude et al., 1997; Geeven et al., 2011; Ma and Willis, 2015; Stam et al., 2007). In the same way, many CNS neurons respond to axotomy with modest and/or transient RAG expression (Chaisuksunt et al., 2000). This failure of RAG expression appears particularly acute in the case of corticospinal tract (CST) neurons after spinal axotomy (Mason et al., 2003), and likely contributes to the relatively modest CST response to a wide range of attempted pro-regenerative therapies (Hollis et al., 2009; Lee et al., 2010b; Pearse et al., 2004; Richardson et al., 1984).

These observations have led to the hypothesis that regeneration by central DRG axons and/or CNS axons might be improved by forced expression of RAGs, particularly key transcription factors that may orchestrate regenerative gene expression (Ma and Willis, 2015; van Kesteren et al., 2011). For example, Krüppel-like factor 7 is a pro-regenerative transcription factor that is normally expressed in peripheral neurons and in CNS neurons during embryonic periods of axon growth, but downregulated in the adult CNS (Laub et al., 2001). We have shown previously that forced expression of a transcriptionally active form, VP16-KLF7, improves axon growth in adult CST neurons after spinal injury (Blackmore et al., 2012). The degree of axon regeneration remains incomplete, however, highlighting the need to identify and overcome additional mechanisms that limit CNS axon growth.

* Corresponding author at: Department of Biomedical Sciences, Marquette University, Milwaukee, WI 53201, USA.
E-mail address: murray.blackmore@marquette.edu (M.G. Blackmore).
Available online on ScienceDirect (www.sciencedirect.com).

http://dx.doi.org/10.1016/j.nbd.2016.12.010
0969-9961/

Besides neuron-intrinsic limits, regeneration by CNS axons is also constrained by inhibitory extracellular cues. Prominent among these are chondroitin sulfate proteoglycans (CSPGs), which are produced at high levels by oligodendrocytes and reactive astrocytes near sites of CNS injury (Asher et al., 2002; Jones et al., 2003; Jones et al., 2002; Silver and Miller, 2004). CSPGs consist of a protein core adorned with sulphated glycosoaminoglycan (GAG) side chains (Bandtlow and Zimmermann, 2000), and inhibit axon extension in a variety of *in vitro* assays (Snow et al., 1990; Tom et al., 2004; Usher et al., 2010). Chondroitinase, a bacterial enzyme that cleaves GAG side chains, has been shown repeatedly to reduce CSPG's inhibitory properties *in vitro* (Niederost et al., 1999; Snow et al., 1990; Yamada et al., 1997), and to promote axon growth *in vivo* after spinal injury (Bartus et al., 2012; Bradbury et al., 2002; Cheng et al., 2015; Iseda et al., 2008). Thus chondroitinase-mediated degradation of CSPGs has emerged as an important component of combinatorial treatments to promote axon regeneration (Hunanyan et al., 2013; Kanno et al., 2014; Lee et al., 2013; Steinmetz et al., 2005; Tom et al., 2009; Tropea et al., 2003).

Here we tested the hypothesis that combined expression of VP16-KLF7 and application of chondroitinase can improve axon growth in the injured spinal cord. Based on previously developed and validated vectors, we created lentivirus to drive expression of chondroitinase optimized for mammalian expression and thermal stability (Muir et al., 2010; Nazari-Robati et al., 2013; Zhao et al., 2011). VP16-KLF7 was delivered by adeno-associated virus to sensory neurons in dorsal root ganglia, or in separate experiments, to cortical neurons. Spinally projecting axons were injured by transection or crush injury, and axon regeneration was assessed in the presence or absence of lentiviral chondroitinase. In DRG neurons, VP16-KLF7 expression increased the proximity of injured axons to the site of transection injury, but in contrast to previous findings in CST axons responding to a similar partial injury, did not evoke robust growth through spared tissue. Similarly, in CST neurons responding to complete spinal crush, forced expression of VP16-KLF7 increased proximity to the injury site but not growth distal to the injury. Chondroitinase treatment also increased the approach of injured DRG axons to the injury center but not extension beyond the injury. In neither DRG nor CST neurons did chondroitinase significantly potentiate the effects of VP16-KLF7 expression. These results demonstrate regulation of net retraction from injury sites by KLF7 transcription and CSPGs, while highlighting the existence of additional constraints to full axon growth.

2. Methods

2.1. Cloning of KLF7 and lenti-chondroitinase

DNA encoding *P. vulgaris* chondroitinase ABC (accession number AAB43331) with a 5′ signal sequence from matrix metalloprotease two (MMP2, accession NM008610) and codon optimized for mammalian expression was synthesized by Genscript. The construct included N282K, N338Q, N345Q, and S517A mutations, previously shown to block inappropriate N-glycosylation (Muir et al., 2010; Zhao et al., 2011), as well as Q140A, shown previously to increase thermostability (Nazari-Robati et al., 2013). Chondroitinase was cloned into Lenti-MP2 vector, supplied by the University of Miami Viral Vector Core, which produced Lenti-Chase. AAV8-VP16-KLF7-2A-mCherry, AAV-EBFP-2A-mCherry, and AAV8-EGFP were cloned and produced by the Univ. of Miami Viral Core as described previously in (Blackmore et al., 2012).

2.2. Plasmid transfection of 293T cells and enzyme assay

293T cells (ATCC) were plated at 90% confluency in 6 well plates (Cellstar) and transfected with Lenti-Chase or EGFP control plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions with 2.4 μg DNA and 3 μl Lipofectamine per well. After two days of culture at 37 °C in a 5% CO2 incubator in 2 ml Optimem media (Gibco), media was collected for chondroitinase activity measurement. The Proteoglycan Detection Kit (Amsbio com) quantifies sulfated glycoaminoglycans using 1,9-dimethylmethylene (DMMB) dye, which shifts it's absorption spectrum upon GAG binding. To generate a standard curve of chondroitinase activity, 0.5 μg of CSPGs (Millipore) were incubated with chondroitinase enzyme (Amsbio) at 0, 12.5, 25, 50, and 100 mU/ml for 2 h at 37 °C, exposed briefly to DMMB, and then 525 nM absorbance as quantified by microplate reader (Molecular Devices). To quantify chondroitinase activity generated by transfected cells, 0.5 μg of CSPGs were incubated with a 1:1000 dilution of conditioned media from 293T cells transfected or transduced with chondroitinase constructs or mCherry control. All test and standard curve controls were run in duplicate.

2.3. DRG cell culture, transfection, and neurite outgrowth

24-well plates were prepared for cell culture by overnight incubation with PDL (100 μg/ml, Sigma) followed by extensive rinsing with water. Wells were then incubated overnight at 37 °C with laminin alone (10 μg/ml, Sigma) or with a mixture of laminin and controlled amounts of CSPGs (0.5 to 5 μg/ml; Millipore). Dorsal root ganglia were dissected from adult mice and dissociated by incubation with Collagenase Type 1 (0.5 mg/ml, Invitrogen), Dispase (10 mg/ml, Invitrogen) and DNAse I (2.5 μg/ml, Sigma) at 37 °C for 40 min, followed by trituration with a fire-polished pipette in 1 ml Hibernate E (Gibco). Cells were rinsed in 5 ml Hibernate E, pelleted by centrifugation at 20G for 10 min, and resuspended in DMEM: F12 media (Gibco) supplemented with SM1 (StemCell Technologies), Pen Strep (Gibco), and Glutamax (Thermofisher) for cell counting. Cells were transfected with VP16KLF7-2A-mCherry or EBFP-2A-mCherry control by electroporation in a Nucleofector II interfaced with a Lonza 96-well Shuttle. 20,000 cells were placed in each transfection well with 20 μl P3 buffer (Lonza), mixed with 1.6 μg DNA, and current was delivered by program DR-114. Following transfection, 80 μl of DRG culture media was immediately added. DRG neurons were cultured in the prepared 24-well plates at a density of 4000 cells per well.

After 48 h, cells were fixed in 4% paraformaldehyde (Electron Microscopy Sciences) for 30 min, rinsed in PBS, transferred to a blocking solution 20% goat serum (Invitrogen), 0.2% Triton X-100 (G-Biosciences) for 30 min, incubated overnight at 4 °C with neuronal specific tubulin rabbit polyclonal antibody (1:500, Sigma T2200), followed by secondary Goat-anti-Rabbit AlexaFluor®546 conjugated antibody (1:500, Invitrogen) and 300 nM DAPI nuclear stain (Sigma) for 2 h at room temperature. To quantify neurite outgrowth, neurons were visualized using an inverted Olympus IX81 fluorescence microscope interfaced with a Coolsnap ES2 digital camera (Photometrics). Transfected neurons were identified by mCherry fluorescence, and the longest neurite from each transfected cells was manually traced using NIS Elements Software.

2.4. Viral delivery to DRG and cortical neurons

AAV8-EBFP-2A-mCherry or AAV8-VP16KLF7-2A-mCherry were delivered to sensory neurons by lumbar puncture as described in (Vulchanova et al., 2010(Wang et al., 2015)). Briefly, a 30 G needle attached by PE10 tubing to a 10 μl Hamilton syringe was inserted at the midline at the level of the iliac crest, into the spinal column, until a characteristic tail flick indicated entry into intrathecal space. 1.5 μl of viral particles (5×10^{13} p/ml) were injected and the needle kept in place for 30 s prior to removal. The procedure was performed on two consecutive days prior to spinal injury.

Cortical neurons were transduced as described in (Blackmore et al., 2012; Wang et al., 2015). Briefly, adult mice (>8 wks, 20–22 g) were anesthetized by Ketamine/Xylazine (100/10 mg/kg, IP), mounted in a stereotaxic frame, and targeted regions of cortex exposed by scraping away skull with a scalpel blade. A pulled glass micropipette attached