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IMPROVED AXONAL REGENERATION AFTER SPINAL CORD INJURY IN MICE WITH CONDITIONAL DELETION OF EPHRIN B2 UNDER THE GFAP PROMOTER

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Abstract—Spinal cord injury (SCI) initiates a cascade of processes that ultimately form a nonpermissive environment for axonal regeneration. Emerging evidence suggests that regenerative failure may be due in part to inhibitory factors expressed by reactive spinal cord glial cells and meningeal fibroblasts, such as the Eph receptor protein-tyrosine kinases and their corresponding ligands (ephrins). Here we sought to assess the role of ephrin B2, an inhibitory axonal guidance molecule, as an inhibitor of the recovery process following SCI. To determine the extent of ephrin B2 involvement in axonal regenerative failure, a SCI model was performed on a conditional ephrin B2 knockout mouse strain (ephrin B2$^{-/-}$), in which the ephrin B2 gene was deleted under the GFAP promoter . The expression of ephrin B2 was significantly decreased in astrocytes of injured and uninjured ephrin B2$^{-/-}$ mice compared to wild-type mice. Notably, in the ephrin B2$^{-/-}$ mice, the deletion of ephrin B2 reduced astrogliosis, and accelerated motor function recovery after SCI. Anterograde axonal tracing on a hemisection model of SCI further showed that ephrin B2$^{-/-}$ mice exhibited increased regeneration of injured corticospinal axons and a reduced glial scar, when compared to littermate controls exposed to similar injury. These results were confirmed by an *in vitro* neurite outgrowth assay and ephrin B2 functional blockage, which showed that ephrin B2 expressed on astrocytes inhibited axonal growth. Combined these findings suggest that ephrin B2 ligands expressed by reactive astrocytes impede the recovery process following SCI.

Key words: spinal cord injury, axonal guidance molecules, ephrin B2, axonal regeneration, astrocytic gliosis.

INTRODUCTION

Traumatic insult to the adult mammalian central nervous system (CNS), such as spinal cord injury (SCI), initiates a cascade of events that ultimately lead to regenerative failure. It is widely recognized that various components of the post-traumatic spinal cord (SC) milieu are inhibitory to axonal re-growth, and that this inhibitory microenvironment is a major contributor to poor functional recovery following SCI. A number of myelin-derived inhibitors including, the reticulon family member RTN4a/Nogo-A (Bandtlow and Schwab, 2000; GrandPre et al., 2000) myelin-associated glycoprotein (McKerracher et al., 1994; DeBellard et al., 1996; Schafer et al., 1996; Filbin, 2003) oligodendrocyte myelin glycoprotein (Wang et al., 2002), and chondroitin sulfate proteoglycans (CSPG) (Bradbury et al., 2002) have been intensively studied as potential mediators of the inhibitory microenvironment. Laboratory experiments and treatment strategies based on these identified inhibitory factors have met with some success, however, the mechanisms that initiate the cascade of post-SCI pathological events remain undefined.

Many studies have shown that ephrin/Eph signaling plays a role in the regulation of axon guidance through contact repulsion, inducing neuronal growth cone collapse in the developing brain and SC (Flanagan and Vanderhaeghen, 1998; Wilkinson, 2001). Members of this family are up-regulated following CNS injury (Miranda et al., 1999; Willson et al., 2003) and in a previous study, ephrin B3 was shown to inhibit axonal regeneration (Duffy et al., 2012). Another member of the family, ephrin B2 has been suggested to be a possible mediator of astrogliosis and scar formation (Fabes et al., 2006; Goldshmit et al., 2006; Curinga and Smith,

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Abbreviations: ANOVA, analysis of variance; BDA, biotinylated dextran amine; BMS, Basso mouse scale; CNS, central nervous system; CPG, central pattern generator; CSPG, chondroitin sulfate proteoglycans; CST, corticospinal tract; GFAP, glial-fibrillary-acidic-protein; PCR, polymerase chain reaction; RPTPσ, receptor protein tyrosine phosphatase sigma; SC, spinal cord; SCI, spinal cord injury; siRNA, small interfering RNA.

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2008), through its receptor EphB2, expressed by meningeal fibroblasts in the injured adult SC (Bundesen et al., 2003). Ephrin B2 has also been shown to bind to EphA4, expressed on the corticospinal tract (CST) axonal stump (Fabes et al., 2006). EphA4 has been implicated in the response to injury, with expression in astrocytes and neurons (Goldshmit et al., 2004; Fabes et al., 2006; Herrmann et al., 2010). Several lines of evidence, including Eph-A4 knockout mice, peptide antagonist, or soluble recombinant blocker, suggest that the lack of EphA4 enhanced axonal regeneration of the corticospinal tract and improved functional recovery following traumatic SCI (Goldshmit et al., 2004, 2011; Fabes et al., 2007).

Although ephrin B2 has been proposed to play an inhibitory role in axonal regeneration after SCI, no studies have so far directly tested the effect of deletion of ephrin B2. In this study, a conditional ephrin B2 knockout mouse strain, ephrin $B2^{-/-}$, was established with a Cre–LoxP system, in which Cre recombinase targeted toward the ephrin B2 gene was inserted under the GFAP promoter. Because ephrin B2 is expressed by astrocytes, this conditional ephrin B2 knockout mouse line provided a novel tool to test the effect of astrocytic derived ephrin B2 on axonal regeneration. We found that deletion of ephrin B2 in astrocytes enhanced axonal regeneration after SCI. The study also provides additional evidence suggesting that the effect of ephrin B2 deletion is likely a result of suppressing the glial scar formation.

EXPERIMENTAL PROCEDURES

Establishment of ephrin B2 conditional knockout mice

We used two transgenic lines to establish the astrocytic conditional ephrin B2 knockout mice (ephrin $B2^{-/-}$): GFAP-Cre mice [FVB-Tg (GFAP-cre) 25Mes/J] bearing Cre recombinase enzyme gene under control of GFAP promoter, which were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) (Zhuo et al., 2001), and ephrin B2-Lox mice, in which exon 1 of the endogenous ephrin B2 gene was flanked by LoxP sites, which were obtained from the California Institute of Technology (Gerety and Anderson, 2002). All animal housing and procedures were performed in compliance with guidelines established by the University Committee of Animal Resources at the University of Rochester. For genotyping purposes, genomic DNA was isolated from the tails of 4- to 6-week-old mice and used for polymerase chain reaction (PCR). PCR genotyping for the conditional ephrin B2 allele (floxed allele) was performed with a 5′primer specific for the 5′loxP site insertion, 5′-AAGTTATAAGCTTCAACGCGTCC-3′ (TF3), and a 3′primer in the genomic region downstream of exon 1, 5′-GAGCCCCAGGTTCTAGAATAACTTCG-3′ (RF1) (product size of 320 bp). The wild-type ephrin B2 locus was detected with a 5′primer that includes sequence flanking the inserted 5′loxP site, and a 3′primer downstream of the first exon, 5′-GCTGC CCGCGGCCGGTCCCAACG-3′ (BrgF1) and 5′-CCGTTAGTG GCAACGTCCTCCGTCCTCG-3′ (HL-I-R2h) (product size of 500 bp). The GFAP-Cre transgene was detected by allele-specific primers, with the oIMR1900, 5′-ACTCCT TCATAAA GCCCT-3′ and the oIMR1901, 5′-ATCACTCGTTGCATCG ACCG-3′ (product size of 190 bp). Ephrin $B2^{-/-}$ mice were identified by the presence of loxP-allele-specific and GFAP-Cre-specific PCR products. The mice with preservation of ephrin B2 gene (ephrin $B2^{+/+}$) in the same litter were used as control (wild-type mice).

Spinal cord injury model

For evaluation of CST regeneration, the lateral hemisection SCI model (Goldshmit et al., 2004) was used for the histological study of axonal regeneration, in addition to behavioral testing. All the surgical procedures were approved by the University Committee of Animal Resources at the University of Rochester. The lateral hemisection SCI model was used because it produced a more consistent and reproducible SCI, with respect to the lesion size and severity, which is important for quantification of the glial scar size and the degree of injured axonal regeneration. A shortcoming however is that it is sometimes difficult to ensure all CST axons are severed, especially the CST that is close to the midline. To avoid this issue and maintain injury consistency, one of the authors, who is an experienced neurosurgeon trained in microsurgical techniques, performed all the surgeries under blinded conditions using a surgical microscope. Sectioning began at the midline and proceeded to the lateral side to ensure consistency and complete injury. Following behavioral analysis, histological assessments of each injury were performed to confirm the complete section of the CST. If it was found that the transection failed to fully extend to the midline, the mice were removed from the behavioral data. Briefly, the animals were anesthetized with intraperitoneal injections of a mixture of ketamine (8 mg/kg) and xylazine (10 mg/kg), a midline incision was made on the back region and a laminectomy was performed at T10–T11 level. Spinal hemisection at T10 was performed on the right side of the cord, using a fine corneal blade (cut twice in the same place to ensure complete section). The overlying layers of muscles and skin were closed with absorbable sutures. After surgery, bladders were manually expressed twice per day or as necessary. Animals received sulfamethoxazole (4 mg/100 g) and trimethoprim (0.8 mg/100 g) twice per day orally to prevent infection. Animal weight and hydration were carefully monitored. If dehydrated, animals received subcutaneous injections of D5-lactated Ringer's solution.

Real-time RT-PCR analysis and immunohistochemistry

Real-time quantitative PCR was used to measure levels of ephrin B2 mRNA of cultured astrocytes. Data are expressed as relative quantification units. Standard immunohistochemical staining, as described previously (Peng et al., 2009), was employed. Primary antibodies of mouse anti-GFAP (1:500, Sigma, St. Louis, MO, USA) and goat anti-ephrin-B2 (1:200, R&D Systems, Minneapolis, MN, USA) were used. For double-labeling immunohistochemistry, donkey secondary antibodies conjugated to FITC or Texas Red (Jackson ImmunoResearch) were used.

Anterograde tracing and glial scar size quantification

Anterograde labeling of the CST was done by injection of biotinylated dextran amine (BDA) (Molecular Probes, Oregon, USA) into the contralateral motor cortex 2 weeks before sacrificing the animal using standard coordinates (1.0 mm lateral, 0.5 mm deep to the cortical surface and +0.5, −0.5 mm with respect to Bregma) (Steward et al., 2008). BDA is injected into a total of 2 sites (0.5 μl per site over a 10-min time period) using a 10-μl Hamilton microsyringe tipped with a pulled glass micropipette. In our experiments, BDA was injected to the injury model 6 weeks after injury (n = 11 respectively, for wild-type and ephrin B2 knockout animals). After an additional 14-day