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ROBUST AXONAL GROWTH AND A BLUNTED MACROPHAGE RESPONSE ARE ASSOCIATED WITH IMPAIRED FUNCTIONAL RECOVERY AFTER SPINAL CORD INJURY IN THE MRL/MpJ MOUSE

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Abstract—Spinal cord injury (SCI) in mammals leads to a robust inflammatory response followed by the formation of a glial and connective tissue scar that comprises a barrier to axonal regeneration. The inbred MRL/MpJ mouse strain exhibits reduced inflammation after peripheral injury and shows true regeneration without tissue scar formation following an ear punch wound. We hypothesized that following SCI, the unique genetic wound healing traits of this strain would result in reduced glial and connective tissue scar formation, increased axonal growth, and improved functional recovery. Adult MRL/MpJ and C57BL/6J mice were subjected to a mid-thoracic spinal contusion and the distribution of axon profiles and selected cellular and extracellular matrix components was compared at 1, 2, 4 and 6 weeks post-injury. Recovery of hind-limb locomotor function was assessed over the same time period. The MRL/MpJ mice exhibited robust axon growth within the lesion, beginning at 4 weeks post-injury. This growth was accompanied by reduced macrophage staining at 1, 2, 4 and 6 weeks post-injury, decreased chondroitin sulfate proteoglycan staining at 1–2 weeks and increased laminin staining throughout the lesion at 2–6 weeks post-injury. Paradoxically, the extent of locomotor recovery was impaired in the MRL/MpJ mice. Close examination of the chronic lesion site revealed evidence of ongoing degeneration both within and surrounding the lesion site. Thus, the regenerative genetic wound healing traits of the MRL/MpJ mice contribute to the evolution of a lesion environment that supports enhanced axon growth after SCI. However, this response occurs at the expense of meaningful functional recovery.

Key words: regeneration, degeneration, contusion, inflammation, astrocyte, glial scar.

Tissue regenerative capacity varies across phylogeny, with complete epimorphic regeneration in planarians (Reddien and Sanchez Alvarado, 2004) and axolotls (Chernoff et al., 2003), and the loss of this capacity during metamorphosis in the tadpole (Beattie et al., 1990). Most adult mammals show no true regenerative ability, with the exception of the pinna of the rabbit ear and the wings of bats (Church and Warren, 1968; Goss and Grimes, 1975). In other tissues, injury initiates a cellular response including activation and recruitment of circulating inflammatory cells. These interact with resident cells to enhance connective tissue deposition, scar tissue formation and wound contraction to restore tissue and vascular integrity at the injury site (Werner et al., 2007).

Injury to the mammalian CNS is characterized by similar events, leading to glial activation and connective tissue scar formation at the site of injury allowing little or no axonal regeneration. Within hours to days after trauma, activated neutrophils, macrophages, mesenchymal cells and leukocytes are recruited to the site of injury (Zhang et al., 1996; Popovich et al., 1997; Sroga et al., 2003). Astrocytes vacate the lesion center, proliferate, undergo hypertrophy and accumulate at the edges of the injury site (Fitch et al., 1999; Fitch and Silver, 2008). Together with other glial cell populations, they up-regulate their expression of growth inhibitory molecules, including chondroitin sulfate proteoglycans (CSPGs), which correlate with regions of abortive axonal growth (Davies et al., 1997, 1999). The scar surrounding a CNS injury site thus creates a physical and chemical barrier that impedes successful regeneration of severed and surviving axons.

There are some conditions where true regeneration is observed in the mammalian CNS suggesting that the capacity to achieve functional connectivity may exist. For example, functional regeneration is observed after spinal cord transection in opossums and rats during early gestational development (Wang et al., 1996; Fry et al., 2003; Lane et al., 2007). In addition, long distance axon regeneration within the spinal cord is observed after a crush injury of the adult rat filum terminale (Kwiecien and Avram,

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Abbreviations: ANOVA, analysis of variance; BBB, Basso Beattie Bresnahan locomotor rating scale; BMS, Basso mouse scale for locomotor recovery in mice; CSPG, chondroitin sulfate proteoglycan; dcc, darkly stained cytoplasm; dpi, days post-injury; Fb, fibroblast; GAG, glycosaminoglycan; GFAP, glial fibrillary acidic protein; LFB, Luxol Fast Blue; MC, macrophage cluster; MMP, matrix metalloprotease; NF, neurofilament; PA, proportional area=stained area/reference area; PBS, phosphate-buffered saline; SCI, spinal cord injury; STAT3, signal transducer and activator of transcription 3; SWM, spared white matter; TCSA, total cross-sectional area.

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2008). In the exceptional cases in both the periphery and CNS, successful regeneration is supported by underlying cellular events, including limited local inflammation, the formation of a blastema or ependymal proliferation at the wound edge, and the absence of chronic glial scar or fibrotic scar tissue formation at the site of injury (Chernoff et al., 2002; Ferretti et al., 2003; Slack et al., 2004; Lane et al., 2007).

Recent studies have identified two genetically inbred strains of mice that display scarless, epimorphic healing of somatic tissues. Notably, the MRL/MpJ and its ancestral strain, LG/J, both exhibit a rapid regenerative healing response, with complete wound closure by 4 weeks after a standard 2-mm ear punch injury. Compared with several other strains, the ear wounds of these mice develop increased edema, angiogenesis, fibroblast (Fb) migration, and decreased scarring and fibrosis at the wound site. True regeneration is evident with formation of new cartilage, vessels, hair follicles and skin from a blastema-like structure formed at the edges of the injury (Clark et al., 1998; Li et al., 2001a). These mice also exhibit scarless healing after a cryogenic injury to the heart or corneal alkali burn damage (Leferovich et al., 2001; Ueno et al., 2005), although fibrosis is evident with larger wounds (Beare et al., 2006; Colwell et al., 2006). Measurements of protein and mRNA levels at the site of the healing wounds have revealed a pattern consistent with a reduced inflammatory response, earlier expression of tissue repair genes and reduced deposition of extracellular collagens in this strain (McBrearty et al., 1998; Li et al., 2001b). Because inflammation and extracellular matrix deposition are also key elements of the scar formation process that impedes CNS axonal regeneration, we tested the hypothesis that the unique regenerative traits in the MRL/MpJ mouse strain would result in the formation of a cellular and extracellular environment that would support axonal regeneration and tissue repair and improve functional recovery after spinal cord injury (SCI). We compared the extent of axon growth, the distribution of astrocytes and their processes, patterns of extracellular CSPGs, laminin and fibronectin and general ultrastructural features at the lesion site after spinal contusion injury in MRL/MpJ mice with the well-studied C57BL/6 mice. The results demonstrate the existence of an intriguing dichotomy in the cellular and tissue dynamics of the MRL/MpJ mouse strain. A marked reduction in local inflammation was associated with the formation of a permissive environment for axonal growth into the lesion at later time points. However, the MRL/MpJ mice also showed impaired functional recovery, which may be a consequence of pronounced lesion constriction and the presence of an increasingly loosely structured cellular terrain at the site of injury. These results demonstrate that genetically-encoded wound-healing traits in the MRL/MpJ mouse help reduce the growth-inhibitory nature of the glial and fibrotic scar at the lesion site; however, these events can prove detrimental to the restoration of tissue integrity and occur at the expense of functional recovery.

EXPERIMENTAL PROCEDURES

Animals, surgery, and care

A total of 74 adult female C57BL/6J ($N=35$) and MRL/MpJ mice ($N=39$) were used in these experiments (The Jackson Laboratory, Bar Harbor, ME, USA). All procedures were carried out in accordance with the Ohio State University Institutional Animal Care and Use Committee and the National Research Council Guide for the Use and Care of Laboratory Animals. Spinal cord contusion injury was performed using an electromagnetic device described in detail elsewhere (Jakeman et al., 2000; Ma et al., 2001). Mice were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg; i.p.), and a dorsal laminectomy (1.5 mm×1.7 mm) was prepared at the ninth thoracic vertebral level. The mice were injured by a rapid displacement of an impounder (1.35 mm tip diameter) 0.5 mm over an ~23 ms period (moderate severity). Subjects were age-matched to ensure similar developmental cellular maturity. Because MRL/MpJ mice were initially bred for their large size, the groups differed in total starting weight (MRL/MpJ 30.6±0.5 g and C57BL/6J=17.3±0.2 g; 10 weeks of age at time of injury). However, there were no differences in mid-thoracic spinal cord diameters of control animals or in the diameter of the spinal cords across strains at 1.5 mm rostral or caudal to the lesion center. There were no differences in peak displacement, peak force, or impulse/momentum measured at the time of spinal cord impact. Post-operative care included daily inspection, manual bladder expression, s.c. administration of 0.9% saline during the first 5 days post-injury (dpi), antibiotic (Gentacin, gentamicin sulfate 100; Vedco, Inc., St. Joseph, MO, USA; 5 mg/kg) and acidified drinking water (pH <4.0) to discourage bladder infection. Mortality across both strains was less than 5% over the duration of the study. In the course of the experiments, five MRL/MpJ mice exhibited some swelling or bite-marks on a hind limb and were treated for 1–5 days by wrapping the affected limb. Behavioral testing was not performed during treatment.

Histological analysis

Pilot studies were performed to obtain specimens at 2, 7, 14, and 28 dpi ($n=2$–3 per strain per time point). After a review of preliminary staining patterns and functional outcomes, the time course was extended. Additional specimens were obtained at 7, 14, 28 and 42 dpi ($n=6$/strain per time point) for determination of lesion size and quantitative analysis of cellular and extracellular composition. At the indicated dpi, the mice received an overdose of ketamine/xylazine anesthesia and were perfused transcardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PBS (pH 7.4). The spinal cords were post-fixed for 2 h, and transferred to 0.2 M sodium phosphate buffer overnight at 4 °C. Tissues from a 6-mm block centered on the site of impact were cryoprotected in 30% sucrose and sectioned in 10 adjacent sets at 10-μm thick. Every slide contained specimens from both strains; all slides for each stain were processed concurrently using the same working solutions. A researcher who was blind to strain and time post-injury performed analyses.

For traditional analysis of white matter sparing at the lesion epicenter, one series of transverse sections was stained with 0.1% Luxol Fast Blue (LFB) to identify phospholipids. The lesion epicenter was defined as the tissue section with the smallest cross-sectional area of normal LFB staining in the peripheral rim (Behrmann et al., 1992). The total cross-sectional area (TCSA) of the spinal cord and the peripheral boundary of residual LFB staining (spared white matter; SWM) were measured on digitized images from the epicenter and 200, 400, 600, 800, 1000 and 1500 μm rostral and caudal to this section using a computer-assisted image analysis program (MCID; Imaging Research Inc., Ontario, Canada) and manually outlining each image while viewing the tissue sections under the light microscope (Zeiss Axiophot