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Intravenous immune-modifying nanoparticles as a therapy for spinal cord injury in mice

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ABSTRACT

Intravenously infused synthetic 500 nm nanoparticles composed of poly(lactide-*co*-glycolide) are taken up by blood-borne inflammatory monocytes via a macrophage scavenger receptor (macrophage receptor with collagenous structure), and the monocytes no longer traffic to sites of inflammation. Intravenous administration of the nanoparticles after experimental spinal cord injury in mice safely and selectively limited infiltration of hematogenous monocytes into the injury site. The nanoparticles did not bind to resident microglia, and did not change the number of microglia in the injured spinal cord. Nanoparticle administration reduced M1 macrophage polarization and microglia activation, reduced levels of inflammatory cytokines, and markedly reduced fibrotic scar formation without altering glial scarring. These findings thus implicate early-infiltrating hematogenous monocytes as highly selective contributors to fibrosis that do not play an indispensable role in gliosis after SCI. Further, the nanoparticle treatment reduced accumulation of chondroitin sulfate proteoglycans, increased axon density inside and caudal to the lesion site, and significantly improved functional recovery after both moderate and severe injuries to the spinal cord. These data provide further evidence that hematogenous monocytes contribute to inflammatory damage and fibrotic scar formation after spinal cord injury in mice. Further, since the nanoparticles are simple to administer intravenously, immunologically inert, stable at room temperature, composed of an FDA-approved material, and have no known toxicity, these findings suggest that the nanoparticles potentially offer a practical treatment for human spinal cord injury.

1. Introduction

Traumatic injury to the spinal cord (SCI) disrupts the blood-brain barrier and leads to a cascade of secondary responses including a rapid influx of monocytes into the injured area. Monocyte infiltration occurs in a biphasic pattern (Berton and Lowell, 1999; Boros et al., 2010; Shantsila et al., 2011) that begins within hours after SCI. The number of macrophages peaks at 7 days with a second peak at 14–28 days post injury. Infiltrating monocytes, as well as tissue resident microglia, differentiate into macrophages (Fleming et al., 2006; Beck et al., 2010; David and Kroner, 2011). Monocytes and macrophages/microglia in the injured spinal cord have both detrimental and beneficial actions, and the exact roles of these populations after SCI are yet to be fully elucidated (Meda et al., 1995; Popovich et al., 1999; Popovich et al., 2002; Majed et al., 2006; Letellier et al., 2010). Recent studies suggest that the early influx of hematogenously-derived macrophages (hMΦ), but not macrophages derived from resident microglia (mMΦ), is primarily responsible for secondary axonal dieback after SCI (Beck et al., 2010; Durafourt et al., 2012; Evans et al., 2014; Gensel and Zhang, 2015). Thus, selectively blocking hMΦ infiltration during the early phase of SCI without altering microglia could help limit secondary tissue damage while preserving the beneficial effects of mMΦ.

The tools used in prior studies of monocyte/macrophage depletion do not exclusively target only hMΦ. Clodronate liposomes, when injected intravenously, deplete circulating monocytes and improve motor functions after SCI in rodents (Popovich et al., 1999; Horn et al., 2008; Grosso et al., 2014). However, clodronate liposomes also target and destroy CNS resident microglia (Kumamaru et al., 2012; Plemel et al., 2014), which may reduce the beneficial effects exerted by microglia in the damaged spinal cord. CCR2 antagonists (Kang et al., 2011), and CCR2 small interfering RNA (Leuschner et al., 2011) target hematogenous monocytes and may spare microglia which do not express CCR2 (Jung et al., 2009; Mizutani et al., 2012). However, this approach also targets T cells and immature B

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cells that express CCR2 (Mack et al., 2001; Flaishon et al., 2004). There are similar issues with other techniques that have been used to reduce macrophage infiltration after SCI (Mabon et al., 2000; Fiore et al., 2004; Stirling et al., 2004; Lopez-Vales et al., 2005).

Here, we sought to selectively deplete hΦM after SCI by using intravenously injected biodegradable carboxylated poly(lactide-*co*-glycolide) (PLGA) immune-modifying nanoparticles (IMPs). IMPs are highly negatively charged, synthetic, 500 nm-diameter particles that bind to the macrophage receptor with collagenous structure (MARCO) on monocytes. IMPs are immunologically inert and simple to manufacture (Getts et al., 2012). We chose to use 500 nm diameter particles because a previous study reported that 500 nm diameter microparticles have a higher binding affinity for MARCO than microparticles with 20 nm, 200 nm, or 1000 nm diameters (Sanae Kanno and Hirano, 2007). Monocytes bound to IMPs no longer travel to sites of inflammation, but instead are sequestered in the spleen where they undergo caspase-3 mediated apoptosis (Getts et al., 2014). IMPs reduce tissue damage and improve outcomes in animal models of several inflammatory diseases including encephalomyelitis, lethal flavivirus encephalitis, myocardial infarction, dextran sodium sulfate–induced colitis, and thioglycollate-induced peritonitis (Getts et al., 2012; Getts et al., 2014).

SCI leads to scarring at the lesion site that includes both fibrotic and gliotic responses (Goritz et al., 2011; Soderblom et al., 2013; Zhu et al., 2015a; Zhu et al., 2015b). The lesional scar inhibits axonal regeneration through a number of mechanisms including accumulation of molecules that are inhibitory to axonal outgrowth, such as chondroitin sulfate proteoglycans (CSPGs), and acting as a physical impediment to axon elongation. The relative roles of gliosis and fibrosis in inhibiting axon outgrowth are unclear. Traditionally astrogliosis has been viewed as an impediment to axon outgrowth, but some evidence suggests that certain populations of astrocytes could enhance regeneration after SCI (Bush et al., 1999; Faulkner et al., 2004; Anderson et al., 2016). The fibrotic response appears to arise from perivascular fibroblasts, which secrete the majority of fibronectin in spinal lesions (Goritz et al., 2011; Soderblom et al., 2013; Zhu et al., 2015a; Zhu et al., 2015b). Secreted fibronectin dimers are then assembled into an insoluble fibronectin matrix via an integrin dependent mechanism. The assembled matrix is characterized by abundantly crosslinked fibronectin that fails to be successfully remodeled and has been shown to remain even at chronic time points after SCI (Zhu et al., 2015b). In toto these observations suggest that both gliosis and fibrosis at the lesion site influence regenerative responses after SCI.

In this paper, we report that IMPs administered intravenously (iv) after SCI significantly reduced numbers of intralesional inflammatory macrophages and other hematogenous inflammatory cells and decreased the proportion of M1-polarized inflammatory macrophages. IMPs did not bind to resident microglia, and IMPs treatment after SCI did not change the number of microglia in the injured spinal cord. hMΦ depletion via IMPs treatment diminished fibrotic scarring and collagen accumulation in the injured sites, suggesting a relationship between hematogenous monocytes and chronic fibrotic scarring. IMPs treatment did not alter glial scarring but did reduce accumulation of chondroitin sulfate proteoglycans in the scar. Moreover, IMPs treatment significantly improved recovery of motor function in mouse models of both moderate and severe SCI. IMPs are simple to administer intravenously, are stable at room temperature for up to several months, are composed of an FDA-approved material and have no known animal toxicity. The outstanding translatability of IMPs, combined with our animal data, suggests that IMPs potentially offer a practical treatment for human SCI.

2. Materials and methods

2.1. Experimental design

The present work adheres to the ARRIVE guidelines and the Minimal Information about a Spinal Cord Injury Experiment reporting standards (Kilkenny et al., 2010; Lemmon et al., 2014). The objectives of this study were to define the role of hMΦ in glial and fibrotic scarring and to determine whether IMPs treatment improves anatomic and functional recovery after SCI in mice. All experiments in this paper were conducted with the researchers blinded to the identity of the treatment groups. One researcher, who did not participate in the behavioral scoring or tissue analyses, randomized and administered the treatment injections and maintained the blinding key. Samples were unblinded only after all measurements were completed. The number of animals in each group necessary for each measurement was determined by a power analysis done with G*Power 3.1.9.2 using the standard deviations and effect sizes from our preliminary studies, an alpha of 0.05, and a power (1-β) of 0.80. Flow cytometry data, except for M1 and M2 macrophage analysis, was replicated with three independent experiments. M1 and M2 macrophage flow cytometry was done twice.

2.2. Mouse spinal cord injury and care

All animal procedures were performed in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and were approved by the Northwestern University Institutional Animal Care and Uses Committee. Eight-week old female C57BL/6 mice (Charles River) were anesthetized using 2.5% isoflurane gas in oxygen. A laminectomy was performed to expose the spinal cord at the T11 level. A severe injury was performed using the Infinite Horizons Spinal Cord Impactor system (IH-0400 Precision Systems and Instrumentation) with 100 kdyn of impact force and a dwell time of 60 s. A moderate injury was induced with 60 kdyn of impact force and zero dwell time. After the injuries, the skin was sutured using 9 mm wound clips (BD Biosciences) and the animals were allowed to recover on a heating pad to maintain body temperature. Buprenorphine anesthetic (0.05 mg/kg, subcutaneously in 1 ml sterile saline) was administered daily for two days after injury. Baytril antibiotic (2.5 mg/kg, subcutaneously in 1 ml sterile saline) was administered daily for three days after injury to reduce the risk of infection. Bladders were manually expressed daily.

2.3. IMPs injection

Five-hundred-nanometer carboxylated PLG microparticles were obtained from Phosphorex, Inc. (Fall River, MA) and diluted in sterile phosphate buffered saline (PBS, pH = 7.4) to a final concentration of 4.7 mg/ml. 200 μl of the dilute IMPs was injected via tail vein 2–3 h after SCI. Additional injections were performed at 24 and 48 h post injury. Control animals received equivalent volume injections of sterile PBS at the same time points.

2.4. Open field testing

Fifty mice (25 per group) were injured for each experiment. Behavioral scoring was performed 24 h after injury using Basso Mouse Scale (BMS) open field scoring by two researchers blinded to the identity of the treatment groups. Any animal with a combined BMS score (left hindlimb + right hindlimb) greater than one at 24 h post injury was excluded from further analysis. Thereafter, BMS scores were assessed weekly by two blinded investigators. Graphs presenting detailed behavioral recovery data and score distributions were prepared in the style of (Estrada et al., 2014).

2.5. Flow cytometry

Mice were anesthetized with 50 mg/kg Nembutal followed by cardiac perfusion with 30 ml of PBS. Injured sections of spinal cords were pooled (four to six mice/experiment), minced with a razor, pushed through a 100 μm filter and digested at 37 °C for 60 min in a PBS solution containing 40 U/ml of Liberase R1 (Roche) and 50 mg/ml DNase I. The