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Hematogenous macrophage depletion reduces the fibrotic scar and increases axonal growth after spinal cord injury

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ABSTRACT

Spinal cord injury (SCI) leads to formation of a fibrotic scar that is inhibitory to axon regeneration. Recent evidence indicates that the fibrotic scar is formed by perivascular fibroblasts, but the mechanism by which they are recruited to the injury site is unknown. Using bone marrow transplantation in mouse model of spinal cord injury, we show that fibroblasts in the fibrotic scar are associated with hematogenous macrophages rather than microglia, which are limited to the surrounding astroglial scar. Depletion of hematogenous macrophages results in reduced fibroblast density and basal lamina formation that is associated with increased axonal growth in the fibrotic scar. Cytokine gene expression analysis after macrophage depletion indicates that decreased Tnfsf8, Tnfsf13 (tumor necrosis factor superfamily members) and increased BMP1-7 (bone morphogenetic proteins) expression may serve as anti-fibrotic mechanisms. Our study demonstrates that hematogenous macrophages are necessary for fibrotic scar formation and macrophage depletion results in changes in multiple cytokines that make the injury site less fibrotic and more conducive to axonal growth.

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Introduction

Spinal cord injury (SCI) typically leads to formation of scar tissue that can be categorized into glial and fibrotic components. This scar tissue is thought to play multiple roles including inhibition of axon regeneration and limiting infiltration of immune cells into the spinal cord parenchyma (Burda and Sofroniew, 2014; Cregg et al., 2014). Most of our current knowledge of the scar tissue has come from studies on the astroglial component, whereas our understanding of the fibrotic component is relatively lacking. In vitro assays indicate that fibroblasts are inhibitory/non-permissive for axon growth (Shearer and Fawcett, 2001) and genetic ablation of fibroblasts leads to cavitation at the injury site (Goritz et al., 2011). Therefore, a better understanding of fibrotic scar formation may provide novel therapeutic targets for SCI.

We recently reported that collagen1α1^{+} perivascular fibroblasts are a major cellular component of the fibrotic scar after SCI (Soderblom et al., 2013). While typically present around large diameter blood vessels in the normal spinal cord, after SCI, these perivascular fibroblasts detach and chronically reside in the injury site to form the fibrotic scar. However, the mechanism by which these fibroblasts are recruited from the perivascular niche into the scar is not known.

In this study, we demonstrate that macrophages are responsible for fibroblast recruitment to the injury site after SCI. Interestingly, these macrophages are of hematogenous origin rather than from microglia, which are present predominantly in the astroglial scar region surrounding the fibrotic scar. Accordingly, depletion of hematogenous macrophages from the injury site reduces fibroblast recruitment and is associated with greater axonal growth into this region. Cytokine expression analysis suggests that these effects may be due to decreased tumor necrosis factor superfamily (Tnfsf) member and increased bone morphogenetic protein (BMP) expression. Our study indicates that hematogenous macrophages recruit fibroblasts to the spinal cord injury site and macrophage depletion changes expression of multiple cytokines that makes the injury site less fibrotic and more conducive to axonal growth.

Materials and methods

Animals

The following mice were obtained from Jackson Labs: lysM-Cre (#004781), Cx3cr1^{GFP} (#005582). Col1α1^{GFP} mice were kindly donated by Dr. David Brenner and have been previously described (Yata et al., 2003). Rosa26-tdTomato reporter mice were kindly donated by Dr. Fan Wang and have been previously described (Arenkiel et al., 2011). All mice were backcrossed to C57BL/6 for at least six generations. lysM-Cre mice were bred to Rosa26-tdTomato reporter mice to generate lysM^{tdTom} mice in which lysM-Cre is hemizygous and tdTomato is homozygous. Cx3cr1^{GFP} knock-in mice were used as heterozygotes.

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Surgery and behavioral assessment

Mouse contusive SCI was performed as previously described (Lee and Lee, 2013). Eight- to 10-week old female mice were anesthetized (ketamine/xylazine, 100 mg/15 mg/kg i.p.) before receiving mid-thoracic (T8) contusive spinal cord injuries. Mice received a laminectomy at T8 and then the spinal column was stabilized using spinal clamps and positioned on an Infinite Horizon impactor device (Precision Systems and Instrumentation, LLC). The exposed spinal cord was visually aligned with the impactor tip, and then given a moderate (75 kDynes) contusion via computer-controlled delivery. All SCI mice received fluid supplements (Lactated Ringer's solution, 1 ml), antibiotics (Baytril, 10 mg/kg), and analgesics (buprenorphine, 0.05 mg/kg) subcutaneously for the first week (twice per day) following surgery. Twice daily bladder expressions continued for the duration of the study. Locomotor recovery was assessed using the Basso Mouse Scale (Basso et al., 2006) open field test at 1 day and weekly after injury. To deplete macrophages, clodronate encapsulated liposomes (Clodrosome, Encapsula Nanosciences, 50 mg/kg) were injected i.p. at different time points after SCI. For 7d end point, liposomes were injected at 1, 3 and 6 days after SCI; for 14d end point, liposomes were injected at 1, 3, 6 and 11 days after SCI; and for 56d end point, liposomes were given weekly after these first four doses. PBS encapsulated liposomes were used as controls. Sunitinib malate (Selleck Chemicals, 0.3 mg/kg, 100 μM in 2.5% DMSO) was delivered continuously for 2 weeks using an Alzet osmotic pump (model 1002) attached to an intrathecal catheter (Mouse Intrathecal Catheter, Alzet). After SCI, the catheter was inserted through the lumbar subarachnoid space (Lee et al., 2007) and the placement of the tip immediately caudal to the injury site was visualized through the laminectomy. The catheter and the pump were secured subcutaneously. In a separate group of animals, Evans blue dye was loaded into the osmotic pump to ensure that the drug delivery method was functional. All procedures were in accordance with University of Miami IACUC and NIH guidelines.

Histology and light sheet fluorescent microscopy (LSFM)

Mice were perfused transcardially with 4% paraformaldehyde (PFA). Brains and spinal cords were removed, post-fixed for 2 h and placed in 30% sucrose overnight. Tissue was embedded in OCT compound (Tissue-Tek) and sectioned on a cryostat. Sagittal sections, 10 μm, were cut serially from an 8 mm block surrounding the injury site and immunostained in PBS-0.3% TritonX-100 for CD11b (Invitrogen RM2800, 1:500), GFAP (Invitrogen 130300, 1:2000 or Abcam ab4674, 1:500), GFP (Abcam ab13970, 1:1000), RFP (Rockland 600-401-379S, 1:4000), laminin (Sigma L9393, 1:2000), and neurofilament (EnCor RPCA-NFM, 1:750). After primary antibody incubation, sections were incubated in the appropriate Alexa Fluor secondary antibodies (Invitrogen, 1:500). Sections were mounted in Vectashield containing DAPI (Vector Laboratories), and images were collected with a Nikon Eclipse Ti fluorescent microscope or an Olympus FluoView 1000 confocal microscope.

Tissue clearing and LSFM were performed as previously described (Erturk et al., 2012; Soderblom et al., 2013). Spinal cords were post-fixed in 4% PFA and washed in PBS (each overnight at 4 °C). After removing the dura, spinal cord was incubated in tetrahydrofuran (Sigma 401757) gradients (50%, 70%, 80%, 90%, 100%, 2 h per step and then 100% overnight) followed by 2–3 h in BABB solution (1:2 ratio of benzyl alcohol, Sigma 305197, and benzyl benzoate, Sigma B6630). Peroxides were removed from tetrahydofuran using activated aluminum oxide through column absorption chromatography as previously described (Becker et al., 2012). To label blood vessels, mice were transcardially perfused with DyLight-594-labeled *Lycopersicon esculentum* lectin (LEL) (Vector Laboratories) as previously described (Jahrling et al., 2009). After tissue clearing, spinal cords were imaged by LSFM (Ultramicroscope, LaVision Biotec) and analyzed using Imaris software (Bitplane) as described below.

Quantification

Quantification of immunohistochemical images were performed by unbiased observers using the Nikon IS software or ImageJ 1.47v. To quantify fibroblast and CD11b$^+$ macrophage density at injury site after clodronate treatment, immunoreactivities of Col1α1^{GFP} and CD11b were determined by thresholding above background level and calculating the area covered by the thresholded regions using imageJ. Immunoreactivities of GFP and CD11b were then normalized to the area of GFAP$^-$ region. To quantify the number of lysM^{tdTom} or Cx3cr1^{GFP} cells and neurofilament$^+$ axons, 50 μm square grids were generated over the entire image. Regions were determined based on GFAP staining; GFAP$^-$ regions were considered the fibrotic scar and GFAP$^+$ regions considered the astroglial scar. Every 6th square was quantified. For quantification of cell density, only DAPI$^+$ cells were counted and cells touching the left and bottom limits of a square were disregarded. For quantification of axon density, only neurofilament signal that is linear and at least 1 μm in length was counted as one axon. Axon density was only quantified in the GFAP$^-$ regions (fibrotic scar). Co-localization was determined using Olympus FV1000-ASW 3.0 viewer software to examine each of the ten one-micron Z-stack slices. For each animal, sections including the injury epicenter and two adjacent sagittal sections spaced 100 μm apart were quantified, and the counts from each section were averaged.

Quantifications of LSFM images were performed using specific tools in the Imaris software. The number of fibroblasts in a region of interest centered at the injury epicenter was determined using Imaris's spots tool at the automatically detected threshold level. Blood vessels at the same region of interest were quantified using Imaris's surface tool to calculate the volume of automatically thresholded vessels.

Bone marrow transplantation

Bone marrow transplantation was performed as previously described (Ashbaugh et al., 2013). Female lysM^{tdTom} donor mice (8–12 weeks old) were anesthetized as described above and euthanized by cervical dislocation. Bone marrow cells from femur and tibia were flushed out with sterile HBSS using a 27.5 G needle attached to a 10 ml syringe. Red blood cells from bone marrow were lysed with Tris-buffered ammonium chloride (140 mM NH_4Cl and 17 mM Tris, pH 7.65) for 1 min at 37 °C. After three washes with sterile HBSS, bone marrow cells were passed through 40 μm cell strainer to prepare single cell suspension. The number of live cells was estimated by trypan blue staining. Donor cells were injected through the tail vein (5×10^5 cells/mouse) of female Cx3cr1^{GFP} recipient mice (6–8 weeks old) that received lethal whole-body irradiation (900 rad, Gammacell 40, 137Cs source) 1d earlier. These lysM^{tdTom} > Cx3cr1^{GFP} chimeras were kept on antibiotics (gentamicin in drinking water, 0.5 mg/ml) for 2 weeks after irradiation/transplantation and allowed at least 8 weeks for reconstitution before receiving SCI as described above. To test for chimerism efficiency, bone marrow from CD45.1 C57BL/6 donor mice (Jackson #002014) were injected into CD45.2 C57BL/6 recipient mice to generate CD45.1 > CD45.2 chimeras in parallel with generating lysM^{tdTom} > Cx3cr1^{GFP} mice. Chimerism efficiency were tested using flow cytometry as described below.

Flow cytometry

For calculation of chimerism efficiency, 150 μl of blood was collected from uninjured CD45.1 > CD45.2 or lysM^{tdTom} > Cx3cr1^{GFP} mice through the tail vein and mixed with heparinized HBSS (1 IU/100 μl). Immune cells from blood were enriched with Ficoll-Paque (GE Healthcare) according to manufacturer's instructions. Cell suspensions

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