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Research Paper

Comparison of subacute and chronic scar tissues after complete spinal cord transection

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ABSTRACT

Traditional views consider scar tissue formed in the lesion epicenter after severe spinal cord injury (SCI) as both a physical barrier and chemical impediment for axonal regeneration. Recently, a controversial opinion suggested that astrocyte scar formation aids rather than prevents axonal regeneration in the CNS. Here, following complete transection of the thoracic spinal cord (T8) in rats, we found that scar tissue showed greater growth factor expression at 2 weeks than 8 weeks post-SCI. Further, tandem mass tag (TMT)-based quantitative proteomic analysis revealed that the components of scar tissue formed in the subacute phase are quite different from that formed in the chronic phase. We also found significantly increased axonal regrowth of sensory axons into the lesion center after chronically formed scar tissue was removed. This indicates that scar tissue formed at the chronic phase actually inhibits axonal regeneration, and that chronic removal of scar tissue may have clinical significance and benefit for SCI repair. Taken together, our study suggests that the features and roles of subacute and chronic scar tissues formed post-SCI is different and scar tissue-targeted strategies for spinal cord regeneration cannot be generalized.

1. Introduction

It has long been thought that densely formed scar tissue in the lesion epicenter post-spinal cord injury (SCI) is not only a physical barrier (scar tissue itself) but also a chemical impediment (axonal growth inhibiting properties of the extracellular matrix [ECM] produced by scar tissue) for axonal regeneration (Asher et al., 2001; Cregg et al., 2014; Kawano et al., 2012a; Ruschel et al., 2015; Sharma et al., 2012; Silver and Miller, 2004). The main axonal growth inhibiting ECM molecules expressed by the astrocyte scar (which is derived from reactive astrocytes) belong to the chondroitin sulfate proteoglycan (CSPG) family, and include phosphacan, neurocan, brevican, and neural/glial antigen 2 (NG2) (Kawano et al., 2012a; Sharma et al., 2012; Tang et al., 2003; Yiu and He, 2006). CSPGs increase during the subacute to chronic phase after CNS injury, and studies have shown that either administration of a chondroitin sulfate (CS)-degrading enzyme, namely chondroitinase ABC (Barritt et al., 2006; Bowes et al., 2012; Bradbury and Carter, 2011; Bradbury et al., 2002; Garcia-Alias et al., 2009; Lee et al., 2010), into the lesion site, or down-regulation of the CSPG receptor, protein tyrosine phosphatase σ (PTPσ), can effectively reverse the inhibitory effect of CSPGs and promote axonal regeneration (Lang et al., 2015; Shen et al., 2009).

The Sofroniew lab published a paper in *Nature*, in which they stated that “astrocyte scar formation aids rather than prevents central nervous system axon regeneration” (Anderson et al., 2016). Although their results show that only ascending axons, and not corticospinal tracts and serotonin fibers, are “aided” by the acute astrocyte scar, their study revealed a surprising opinion, one that totally contradicts prevailing dogma. Using targeted deletion strategies, they specifically destroyed the scar formed from reactive astrocytes, acutely or chronically, and observed an enlarged lesion core but no enhanced descending or ascending axonal regeneration. Moreover, when glial scar astrocytes were acutely deleted, axonal regrowth of sensory neurons into the lesion center was decreased rather than promoted, even though intrinsic axonal regenerative ability was maximally stimulated by conditioning lesion and growth factor delivery. Taken together, the authors' concluded that astrocyte scar formation aids rather than inhibits axonal regeneration after SCI.

A commentary paper by Jerry Silver provided interpretation of the results and conclusion of the *Nature* paper from various angles, including the constitution and dynamically changed properties of the scar tissue, and role and dynamic production of CSPGs in scar formation

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(Silver, 2016). In this study, we investigated changes in scar tissue formed at 2 weeks and 8 weeks post-complete transection injury of thoracic spinal cord (T8) in rats by a tandem mass tag (TMT)-based quantitative proteomic analysis. Our results show a time-dependent decrease of cytokine expression by scar tissue after SCI, suggesting that scar tissue formed in the acute phase might be more advantageous for spinal cord regeneration, and the conclusion was in accordance with the *Nature* paper. However, this positive role of acute scar tissue seemed to be greatly decreased compared to chronic scar tissue. Moreover, we also found significantly increased axonal regrowth of sensory axons into the lesion center after the scar tissue was removed at 8 weeks post-injury, indicating that scar tissue formed at the chronic phase inhibits rather than aids axonal regeneration. Consequently, the role of scar tissue formed post-SCI is dynamic, and a scar tissue-targeted strategy for spinal cord regeneration should be time-orientated.

2. Materials and methods

2.1. Surgery and tissue processing

A total of 48 adult female Sprague Dawley rats (200 to 250 g) were housed in temperature and humidity controlled animal quarters with a 12 h light/dark cycle. The surgery procedure was slightly modified according to our previous report with a little change (Li et al., 2015). Following intraperitoneal anesthesia with sodium pentobarbital (50 mg/kg), a 2 cm midline incision was made to expose the T6–T10 vertebrae. A 2 mm-long laminectomy at T7 to T9 was performed using a no.11 blade under microscopic observation. The transaction site was irrigated with normal saline, and bleeding was controlled using cotton-tipped applicators. Following treatment, the musculature and skin were closed in separate layers with sutures, after which the animals were returned to their home cage to recover.

After the operation, each rat routinely received antibiotics and glucose intravenously for 5 days and its bladder had to be emptied manually every 12 h for 2 weeks due to a lack of normal micturition reflexes.

Six rats were killed at 2 and 8 weeks post injury, respectively to assay growth factor expression profiles; Nine rats were killed at 2 and 8 weeks post injury, respectively, and the scar tissues formed at the lesion site were isolated for quantitative proteomics analysis.

At 8 weeks post injury, rats in the chronic spinal cord injury phase received anesthesia with sodium pentobarbital (50 mg/kg). The primary longitudinal incision was reopened and the tissue was separated to expose the spinous process and canalis vertebralis. The scar tissues deposited in the spinal lesion site were carefully removed by micro scissors under a microscope (Zeiss). 18 rats were allocated into three groups: Control group without scar tissue removal (n = 6); Only scar tissue removal group (n = 6) and Scar tissue removal along with growth factor (5 μg BDNF and 5 μg NT3) delivery group (n = 6). Choleratoxin B (CTB) (List Biological Laboratory, Campbell, CA) 1 μl of 1% wt/vol in sterile water was injected into both sciatic nerves three days before perfusion to visualize ascending sensory tract (AST) axons similar to Anderson et al. (2016).

2.2. Quantitative proteomics analysis of scar tissue

Scar tissues isolated from rats at 2 and 8 weeks post injury were immediately lysed using Qproteome mammalian buffer, and proteins were precipitated using chloroform/methanol. The process of TMT based quantitative proteomics analysis was similar to Belin et al. (2015).

2.3. Histological analysis

The spinal cords from rats in this study were retrieved and fixed in 4% (v/v) formaldehyde for 24 h. Then, the segments were transferred to 20% sucrose (overnight at 4 °C) and then 30% sucrose (72 h at 4 °C). Then, the tissue samples were embedded in Tissue-Tek O.C.T. compound (Sakura Finetechnical Co., Japan) and sectioned on a cryostat set at a thickness of 10 μm (Leica Microsystems GmbH, Germany). For immunofluorescence staining, the primary antibodies were applied to the sections and incubated overnight at 4 °C. The primary antibodies were against the following: bFGF (1:500, MABS72, Millipore), PDGF (1:800, ab111310, Abcam) and VEGF (1:800, ABS82, Millipore). Sections were then incubated with secondary antibody (Alexa Fluor 594, 1:800; Invitrogen). Cell nuclei were stained with DAPI (1:1000, sigma) and images were taken under the Leica TCS SP8 Confocal Microscope (Leica Microsystems, Germany). Image-Pro Plus software (Media Cybernetics LP, Maryland, USA) was used to quantify immunostaining-positive signals by selecting at least 3 fields per sample at the lesion center.

2.4. Growth factors analysis

The subacute and chronic rat spinal scar tissues were isolated for quantitative growth factors analysis. bFGF, VEGF and PDGF in the scar tissues were analyzed with the Mouse/Rat FGF basic Quantikine ELISA Kit (R&D systems, Catalog Number MFB00), Rat VEGF Quantikine ELISA Kit (R&D systems, Catalog Number RRV00), and Abcam's PDGF-AA Rat ELISA Kit (Abcam, Catalog Number ab155464).

2.5. Statistics

Data were presented as mean values ± standard deviation. SPSS 13.0 was used for all statistical analysis. Comparisons of quantitative immunohistochemistry data were performed with one-way ANOVA (S-NK). The LSD test was used for post-hoc analysis to correct for multiple comparisons. P values < 0.05 were considered significant.

2.6. Ethics approval

Animal experiments were performed in accordance with Guide for the Care and Use of Laboratory Animals from the National Institutes of Health, and approved by the Animal Care and Use Committee of the Institute of Genetics and Developmental Biology, the Chinese Academy of Sciences.

3. Results

To investigate growth factor secretomics of subacute and chronic scar tissue, we first performed immunofluorescent staining to compare expression profiles of basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF) in scar tissue at 2 weeks and 8 weeks post-injury (these time points are in accordance with the 2016 *Nature* paper by the Sofroniew lab). As shown in Fig. 1A, all detected growth factors showed significantly higher secretion levels in scar tissue at 2 weeks post-injury than 8 weeks. Expression levels of these growth factors significantly decreased during the subacute to chronic phase of scar tissue formation. Meanwhile, we also isolated the rat spinal scar tissues from 2 and 8 weeks post injury and quantitatively analyzed the contents of bFGF, VEGF and PDGF in the scar tissues. Our ELISA results showed that the contents of the three growth factors in the scar tissue at 2 weeks post injury were 26, 67, and 83 pg/mg, respectively, which were significantly higher than those in scar tissue formed at 8 weeks post injury (Fig. 1B). The result is also in accordance with that in the immunofluorescent staining assay described above. This indicates that subacute scar tissue formed post-SCI might provide a more permissive regenerative environment, whereas chronic scar tissue with an altered growth factors expression profile may be an impediment for axonal growth, partly due to decreased neurotrophic support. Indeed, this might partly explain the observed worsening of axonal regeneration