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High mobility group box-1 (HMGB1) is increased in injured mouse spinal cord and can elicit neurotoxic inflammation

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ABSTRACT

Inflammation is a ubiquitous but poorly understood consequence of spinal cord injury (SCI). The mechanisms controlling this response are unclear but culminate in the sequential activation of resident and recruited immune cells. Collectively, these cells can exert divergent effects on cell survival and tissue repair. HMGB1 is a ubiquitously expressed DNA binding protein and also a potent inflammatory stimulus. Necrotic cells release HGMB1, but HMGB1 also is actively secreted by inflammatory macrophages. A goal of this study was to quantify spatio-temporal patterns of cellular HMGB1 expression in a controlled mouse model of experimental SCI then determine the effects of HMGB1 on post-SCI neuroinflammation and recovery of function. We documented SCI-induced changes in nuclear and cytoplasmic distribution of HMGB1 in various cell types after SCI. The data reveal a time-dependent increase in HMGB1 mRNA and protein with protein reaching maximal levels 24–72 h post-injury then declining toward baseline 14–28 days post-SCI. Although most cells expressed nuclear HMGB1, reduced nuclear labeling with increased cytoplasmic expression was found in a subset of CNS macrophages suggesting that those cells begin to secrete HMGB1 at the injury site. *In vitro* data indicate that extracelluar HMGB1 helps promote the development of macrophages with a neurotoxic phenotype. The ability of HMGB1 to elicit neurotoxic macrophage functions was confirmed *in vivo*; 72 h after injecting 500 ng of recombinant HMGB1 into intact spinal cord ventral horn, inflammatory CNS macrophages co-localized with focal areas of neuronal killing. However, attempts to confer neuroprotection after SCI by blocking HMGB1 with a neutralizing antibody were unsuccessful. Collectively, these data implicate HMGB1 as a novel regulator of post-SCI inflammation and suggest that inhibition of HMGB1 could be a novel therapeutic target after SCI. Future studies will need to identify better methods to deliver optimal concentrations of HMGB1 antagonists to the injured spinal cord.

1. Introduction

Microglial and macrophage activation is a prominent feature of many central nervous system (CNS) disorders including spinal cord injury (SCI). The net effect of this activation is debatable, however, several studies show benefits from blocking the migration of or depleting monocytes and macrophages early after SCI (Blight, 1994; Giulian and Robertson, 1990; Gris et al., 2004; Popovich et al., 1999). Data from our laboratory and others indicate that intraspinal macrophage phenotype predicts their effector functions (Gensel et al., 2017; Gensel et al., 2015; Kigerl et al., 2009; Kroner et al., 2014; Miron et al., 2013; Shechter et al., 2013). *In vivo*, macrophages that adopt phenotypes that mimic *in vitro* classically activated (M1) macrophages are neurotoxic while those that resemble alternatively activated (M2) macrophages promote axon growth and repair (Kigerl et al., 2009). The signals that polarize CNS macrophages to become M1 macrophages after SCI are unknown.

HMGB1 is a highly conserved ubiquitous nuclear protein that aids in transcription factor initiation (Javaherian et al., 1978; Read et al., 1994; Sutrias-Grau et al., 1999). HMGB1 is also a potent regulator of inflammation. Originally described as a serum biomarker of macrophage activation in a mouse model of sepsis (Wang et al., 1999), HMGB1 has since been found to mediate many inflammatory disease processes (Andersson and Tracey, 2011; Harris et al., 2012). HMGB1 is passively released from necrotic cells (Rovere-Querini et al., 2004; Scaffidi et al., 2002) and also can be secreted by activated macrophages (Jiang and Pisetsky, 2006;

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Lamkanfi et al., 2010; Rendon-Mitchell et al., 2003; Tang et al., 2007 et al.). Once released from a cell, HMGB1 can activate innate immune receptors including TLR4, TLR2, and the receptor for advanced glycation end products (RAGE) (Hori et al., 1995; Kokkola et al., 2005; Park et al., 2006, 2004; Yu et al., 2006). These receptors are expressed by macrophages and glia and are important in regulating inflammation, gliosis, and demyelination after SCI (Church et al., 2016, 2017; Gensel et al., 2015; Kigerl et al., 2014, 2007; Stirling et al., 2014; Stivers et al., 2017).

In the diseased and injured brain, HMGB1 contributes to neuron death. In the ischemic brain HMGB1 is released from necrotic neurons and drives neuroinflammation by acting on microglia and macrophage RAGE and Mac-1 receptors (Faraco et al., 2007; Gao et al., 2011; Muhammad et al., 2008). shRNA-mediated blockade of HMGB1 (Kim et al., 2006) and neutralizing antibodies against HMGB1 (Liu et al., 2007; Muhammad et al., 2008; Zhang et al., 2011) reduce brain infarct size and blood–brain barrier disruption in stroke models. HMGB1 also is involved in epilepsy pathogenesis (Maroso et al., 2010) and becomes concentrated at sites of trauma or inflammation in other CNS pathologies including multiple sclerosis and SCI (Andersson et al., 2008; Kawabata et al., 2010; Sun et al., 2015).

In rat models of traumatic and ischemic SCI, acute but transient increases in HMGB1 occur together with increases in proinflammatory cytokines (e.g., TNFα), TLRs and RAGE (Chen et al., 2011; Gong et al., 2012; Kawabata et al., 2010). High concentrations of extracellular HMGB1 also become enriched in the extracellular matrix of chronically-injured rat spinal cord (Didangelos et al., 2016). In humans, HMGB1 is increased in the blood after SCI and may contribute to systemic inflammatory complications (Papatheodorou et al., 2017). Thus, post-SCI regulation of HMGB1 appears to be conserved across species, although the sources and functional significance of these changes are unknown.

Here, we extended previous studies by characterizing the temporal distribution and cellular localization of SCI-induced HMGB1 (mRNA and protein) in a mouse model of controlled moderate contusion SCI. New data indicate that HMGB1 levels increase in the injured spinal cord, likely from multiple cellular sources including dying cells and secretion from activated CNS macrophages. Data from *in vivo* and *ex vivo* assays show that extracellular HMGB1 elicits neurotoxic inflammation and restricts axonal growth/plasticity. Intraperitoneal injections of an HMGB1 blocking antibody were used in an effort to block HMGB1 during the time of peak HMGB1 expression after SCI. Neutralizing HMGB1 via this route of injection had no effect; quantitative measures of intraspinal pathology and locomotor recovery were not different from SCI mice receiving control injections (vehicle or non-specific antibodies). Together, these data indicate that HMGB1 promotes intraspinal inflammation and is an important regulator of macrophage-mediated neurotoxicity and axon die-back after SCI. Future attempts to inhibit these deleterious effects of extracellular HMGB1 will likely require that antagonists are delivered directly into the spinal cord (intraparenchymal or intrathecal) or that new inhibitors are created that easily bypass the blood-spinal cord barrier.

2. Methods

2.1. Animals and spinal cord injury

All surgical and post-operative care procedures were performed in accordance with the Ohio State University Institutional Animal Care and Use Committee. Animals were housed in ventilated cages and were maintained on a 12-h light/dark cycle with ad libitum access to food and water. Ambient room temperature was maintained at 70° F ± 4° with 30–70% humidity. Female C57BL/6 mice were anesthetized with an i.p. cocktail of ketamine (80 mg/kg)/xylazine (10 mg/kg) after which a partial laminectomy was performed at T9. All mice received a spinal contusion injury (75 kdyn) using the Infinite Horizons injury device. Post-operatively, animals were hydrated with 2 mL Ringer's solution (s.c.) and were given prophylactic antibiotics (0.1 mL Gentacin/s.c.) for 5 days. Bladders were voided manually at least twice daily for the duration of the study.

2.2. Anti-HMGB1 neutralizing antibodies

Anti-HMGB1 neutralizing antibody was generated as previously described (Qin et al., 2006) kindly provided by Dr. Kevin Tracey (anti-HMGB1 2G7). The specificity of this antibody has been characterized elsewhere (Andersson and Tracey, 2011; Gao et al., 2010; Qin et al., 2006; Yang et al., 2010). Mice were randomly assigned to treatment or control groups using a random number generator provided by QuickCalcs on the GraphPad Software website. Antibody was injected daily via i.p. injection (50 μg/day; n = 6/group) starting 1 day prior to SCI and continuing for 7 days. Antibody dose was based off effective dosing in a murine sepsis model (Valdés-Ferrer et al., 2015). Equivalent amounts of PBS or IgG2a antibodies (isotype control) were also delivered via daily i.p. injection. All experimenters were unaware of group designations.

2.3. Quantitative analysis of spared myelin and Immunohistochemistry

Eriochrome cyanine (EC) staining was used to visualize myelin. Frozen sections cut through the rostro-caudal extent of the lesion were incubated in EC for 30 min at 20 °C, washed in dH2O then were differentiated in 5% iron alum then borax-ferricyanide for 5–10 min. The injury epicenter was defined visually as the spinal cord section with the smallest visible rim of spared myelin. That section and those immediately rostral/caudal were analyzed from each animal/group then were averaged. To calculate the area of spared myelin and lesion volume, digital images of equidistant EC-stained sections spanning the injury epicenter were captured using a Zeiss Axioplan 2 Imaging microscope. A point grid of known area was overlaid with random orientation onto printed digital images and myelin sparing was calculated according to the Cavalieri method using the formula: $(V = T \bullet a/p \bullet n\sum p)$ where T equals the distance between sections, a/p equals calculated area per point, and $n\sum p$ equals the sum of points counted across all sections (Kigerl et al., 2006).

2.4. Analysis of locomotor function

Open-field locomotor function was assessed using the Basso Mouse Scale for Locomotion (BMS; (Basso et al., 2006)) at 1, 3, 7, 14, 21, 28, 35, and 42dpi. General indices of locomotion and activity were assessed with an AccuScan activity monitor (AccuScan Intruments, Columbus OH). Mice were recorded using the AccuScan system for 30 min prior to SCI and then again at 42dpi.

2.5. Intraspinal microinjection

Mice were anesthetized with a cocktail of ketamine/xylazine (80 and 10 mg/kg, respectively). Using aseptic technique, a laminectomy was performed at the $T_{12\text{-}13}$ vertebral level after which the spinal column was secured via the spinous processes adjacent to the laminectomy site using Adson forceps fixed in a spinal frame. Sterile glass micropipettes (pulled to an external diameter of ~25 μm and pre-filled with sterile recombinant HMGB1 (R&D Systems; 500 ng/mouse; n = 6) or sterile PBS (n = 6) were positioned at 0.4 mm lateral from midline. From the

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