The Relationship between Localized Subarachnoid Inflammation and Parenchymal Pathophysiology after Spinal Cord Injury

James W. Austin,^{1,3} Mehdi Afshar,³ and Michael G. Fehlings^{1–3}

Abstract

Subarachnoid inflammation following spinal cord injury (SCI) can lead to the formation of localized subarachnoid scarring and the development of post-traumatic syringomyelia (PTS). While PTS is a devastating complication of SCI, its relative rarity (occurring symptomatically in about 5% of clinical cases), and lack of fundamental physiological insights, have led us to examine an animal model of traumatic SCI with induced arachnoiditis. We hypothesized that arachnoiditis associated with SCI would potentiate early parenchymal pathophysiology. To test this theory, we examined early spatial pathophysiology in four groups: (1) sham (non-injured controls), (2) arachnoiditis (intrathecal injection of kaolin), (3) SCI (35-g clip contusion/compression injury), and (4) PTS (intrathecal kaolin+SCI). Overall, there was greater parenchymal inflammation and scarring in the PTS group relative to the SCI group. This was demonstrated by significant increases in cytokine (IL-1 α and IL-1 β) and chemokine (MCP-1, GRO/KC, and MIP-1 α) production, MPO activity, blood–spinal cord barrier (BSCB) permeability, and MMP-9 activity. However, parenchymal inflammatory mediator production (acute IL-1 α and IL-1 β , subacute chemokines), BSCB permeability, and fibrous scarring in the PTS group were larger than the sum of the SCI group and arachnoiditis group combined, suggesting that arachnoiditis does indeed potentiate parenchymal pathophysiology. Accordingly, these findings suggest that the development of arachnoiditis associated with SCI can lead to an exacerbation of the parenchymal injury, potentially impacting the outcome of this devastating condition.

Key words: arachnoiditis; blood–spinal cord barrier; chemokine; cytokine; inflammation; post-traumatic syringomyelia; spinal cord injury

Introduction

TRAUMATIC VERTEBRAL FRACTURES AND DISLOCATIONS are caused by a heterogeneous array of biomechanical forces on the spinal cord, resulting in equally divergent neurological and functional deficits. Meningeal damage and subarachnoid inflammation (arachnoiditis) following spinal cord injury (SCI) occur, although the impact of these events is unclear. The extent and resolution of post-SCI arachnoiditis is most likely determined by possible genetic and injury-related differences, leaving some patients more prone to the formation of subarachnoid scarring or adhesions that disturb cerebrospinal fluid (CSF) flow in the subarachnoid space. Although an accurate estimation of the incidence of subarachnoid scarring following SCI remains elusive, it is estimated that only 3–5% of cases

will become symptomatic with clinical evidence of post-traumatic syringomyelia (PTS), a condition characterized by extensive cysts within the cord parenchyma (Abel et al., 1999; Backe et al., 1991; Perrouin-Verbe et al., 1998). Animal studies suggest that CSF blockage by subarachnoid scarring results in altered fluid flow and pressure dynamics in the subarachnoid space, causing an influx of CSF into the spinal cord parenchyma (Brodbeck et al., 2003c; Klekamp et al., 2001).

Though symptomatic PTS is rare, recent studies suggest that the incidence of PTS is increasing (Vannemreddy et al., 2002). Currently, PTS receives little basic science research attention. Moreover, current models of SCI offer little insight into the etiological aspects of PTS due to the lack of significant arachnoiditis. Studies going back almost 80 years have used injections of kaolin into the cisterna magna to cause meningeal

¹Institute of Medical Science and ²Department of Surgery, University of Toronto, Toronto, Ontario, Canada.

³Division of Genetics and Development and Krembil Neuroscience Centre, Toronto Western Research Institute, Toronto, Ontario, Canada.

inflammation and obstruction of CSF, modelling hydrocephalus and syringomyelia (Dixon, 1932). Furthermore, groups have studied the inflammatory aspects of this model (Deren et al., 2010). The use of kaolin has been adapted for PTS research, with groups using parenchymal excitotoxic compounds combined with subarachnoid kaolin injections (Cho et al., 1994; Yang et al., 2001). This model has laid important etiological groundwork, establishing insightful mechanistic and pathophysiologic details (Brodbeck et al., 2003a,2003b,2003c). Importantly, animal models of PTS require a reproducible meningeal inflammatory event that is localized to the site of SCI, as subarachnoid scarring can be seen localized to the injury site clinically (Klekamp et al., 1997). The majority of PTS studies have looked at how the kaolin-induced scarring in the subarachnoid space leads to the entry of CSF into the spinal cord (Brodbeck et al., 2003b,2003c). However, studies of the meningeal inflammation that leads to subarachnoid scarring have been sparse, particularly in terms of how it can influence the progression of injury.

Recently, our laboratory has modified this classical PTS model (Seki and Fehlings, 2008). In our variation, animals are subjected to a clip compression/contusion injury, followed by an intrathecal injection of kaolin to induce arachnoiditis. While the literature suggests that subarachnoid scarring is associated with the formation of a syrinx, we sought to study its root cause—arachnoiditis—and how it relates to early parenchymal injury. We hypothesized that arachnoiditis associated with SCI would potentiate parenchymal pathophysiology. To this end, we studied the early spatial profile of inflammatory and scarring events in four groups of rats: (1) non-injured controls (sham), (2) arachnoiditis (intrathecal kaolin injection at T7), (3) SCI (35-g clip contusion/compression injury at T7), and (4) PTS (SCI with induced arachnoiditis at T7). We reasoned that if there was a synergistic relationship between arachnoiditis and parenchymal inflammation induced by SCI, as opposed to an additive relationship, then inflammation and scarring in the PTS group would be greater than the sum of the arachnoiditis group and SCI group combined. Overall, inflammation and scarring were greater in the parenchyma of PTS animals relative to SCI animals. However, acute parenchymal interleukin-1 α (IL-1 α) and IL-1 β production, subacute parenchymal chemokine production, blood–spinal cord barrier (BSCB) permeability, and parenchymal fibrous scarring, were much greater in SCI animals with arachnoiditis compared to the sum of these measures in animals with arachnoiditis alone and SCI alone, suggesting a synergistic relationship. Together, these findings suggest that patients with SCI associated with arachnoiditis (e.g., meningeal damage and subdural hemorrhage) can be expected to experience more severe parenchymal inflammation and scarring.

Methods

Animal model

All animal protocols were approved by the animal ethics board of the University Health Network, Toronto, Ontario, Canada. Injuries were induced as previously described (Seki and Fehlings, 2008). Briefly, female Wistar rats approximately 300 g in weight were anesthetized with 2% isoflurane with oxygen and NO₂. The dorsal aspect of the T6, T7, and T8

vertebrae were removed, exposing the spinal cord. The experimental groups consisted of: (1) sham group (non-injured controls, laminectomy only), (2) arachnoiditis group (5 μ L intrathecal injection of a 500-mg/mL kaolin mixture at T7 without injury), (3) SCI group (35-g clip compression injury at T7), and (4) PTS group (animals subjected to a 35-g clip compression injury at T7 followed by kaolin injection). Multilayer tissue closure was performed following the experimental manipulations.

Immunohistochemistry

Animals were perfused transcardially with ice-cold PBS and fixed by perfusion with 4% paraformaldehyde. Spinal cords were harvested and post-fixed in 4% paraformaldehyde containing 10% sucrose overnight, followed by PBS containing 20% sucrose on the following day. The cords were then frozen in OCT and sectioned longitudinally (sagittally). Sections were rinsed in PBS for 5 min and blocked in blocking solution (0.1% Triton-X 100, 1% BSA, 5% non-fat milk, and 2.5% normal goat serum in PBS) for 1 h. Primary antibodies were incubated overnight in blocking solution minus Triton-X 100 overnight at 4°C (antibody solution). Sections were rinsed 3 $\times$ 10 min in PBS, and fluorescent secondary antibodies were incubated for 2 h at room temperature in antibody solution. The sections were rinsed again in PBS (3 $\times$ 10 min) and cover-slipped in Mowiol mounting medium containing 4,6-diamino-2-phenylindole (DAPI; Vector Laboratories, Burlington, Ontario, Canada). Primary antibodies used for immunohistochemistry included: collagen IV (Abcam, Cambridge, MA), CS56 for chondroitin sulfate proteoglycans (CSPG; Sigma-Aldrich, Oakville, Ontario, Canada), glial fibrillary acidic protein (GFAP; Millipore, Billerica, MA), polymorphonuclear leukocytes (PMN; Cedarlane, Burlington, ON, Canada), and Iba-1 (Wako, Osaka, Japan). Secondary antibodies included Alexa Fluor 488 and Alexa Fluor 568 conjugates (Invitrogen, Carlsbad, CA). In all cases, the sections shown in the figures are representative of the sagittal midline of the spinal cord. All images are presented in the same orientation, with the top of the image representing the dorsal aspect of the cord, and the left side of the image representing the rostral side of the epicenter. Scale bars in all lower-magnification fluorescent images represent 1 mm. In higher-power confocal images, the scale bars represent 100 μ m.

Fresh frozen tissue sectioning

Animals were perfused transcardially with ice-cold PBS. Spinal cords were isolated and snap frozen in liquid nitrogen. For *in situ* zymography, samples were embedded in OCT and sectioned at 14 μ m using a cryostat. For enzyme-linked immunosorbent assay (ELISA) and gel zymography, samples were embedded in OCT, and dorsal meningeal isolation was carried out by sectioning off approximately 300 μ m of the dorsal surface of 0.5 cm of spinal cord tissue centered at the injury epicenter using a cryostat. This fraction contained the dorsal portion of the meninges exposed to the kaolin, and is referred to as the meningeal fraction. The remaining kaolin-free portion of the cord is referred to as the parenchymal fraction. Each isolated fraction was homogenized in RIPA buffer (Thermo Scientific, Waltham, MA).

Multiplex enzyme-linked immunosorbent assay

Samples in RIPA buffer were processed using Rat Cytokine/Chemokine multiplex ELISA assays available from Millipore. Concentrations obtained from the assay were divided by protein concentrations determined by the Lowry method, and data are expressed as picograms of cytokine or chemokine per milligram of protein.

Zymography

All gel zymography reagents were purchased from Bio-Rad (Hercules, CA) unless otherwise stated. Gel zymography was carried out using 10% polyacrylamide gels with 0.1% gelatin B (Sigma-Aldrich). Samples homogenized in RIPA buffer were prepared with equal protein concentrations following a Lowry protein assay. The sample buffer consisted of 65 mM Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, 0.01% bromophenol blue. Following electrophoresis, 2.5% Triton-X 100 (Sigma-Aldrich) in water was used to renature the gels with gentle agitation for 30 min. Zymogram developing buffer (50 mM Tris, 0.2 M NaCl, 5 mM CaCl₂, and 0.02% Brij35) was used to equilibrate the gels for 30 min at room temperature with gentle agitation, followed by the addition of fresh developing buffer overnight at 37°C. Gels were then stained with 0.5% Coomassie Blue for 30 min and destained with methanol:acetic acid:water (50:10:40). Images were taken with a Fluor-S multi-imager (Bio-Rad).

In situ zymography was performed using DQ gelatin-fluorescein (Invitrogen) as previously published (George and Johnson, 2010). Briefly, fresh frozen tissue was sectioned in OCT and sectioned at 20 μ m. Tissue was rinsed in PBS and 100 μ M of DQ gelatin-fluorescein was applied to slides overnight at 37°C. The slides were rinsed in PBS (3 $\times$ 1 min), and cover-slipped with Mowiol as a mounting medium. Areas of gelatinase activity appear fluorescent, as the non-cleaved DQ-gelatin-fluorescein remains quenched (non-fluorescent).

Myeloperoxidase activity

The myeloperoxidase activity assay was carried out per the manufacturer's instructions (Enzo Life Sciences–Stressgen, Plymouth Meeting, PA). Briefly, the animals were perfused with ice-cold PBS, and 0.5 cm of spinal cord tissue centered at the epicenter was homogenized and centrifuged at 12,000g at 4°C. Solubilization buffer was added to the pellets, followed by sonication for 30 sec, and two rounds of freeze-thaw cycles before centrifugation as above. The supernatants from this step represent the granular fractions. In the presence of H₂O₂, myeloperoxidase (MPO) contained in the samples oxidized a non-fluorescent detection reagent into a fluorescent analog, which was detected using a fluorescent plate reader (Perkin Elmer, Waltham, MA). Data are reported as units of MPO activity per gram wet tissue weight.

Blood–spinal cord barrier permeability

Disruption in the BSCB was measured by extravasation of Evans blue (EB) as previously described (Yu et al., 2008), with few modifications. Briefly, 4% EB was injected intravenously and the animals were kept anesthetized for 30 min before sacrifice. The animals were then perfused with saline containing 10 U/mL heparin. Cords were removed (0.5-cm sections centered at the epicenter), and weighed prior to

homogenization in *N,N*-dimethylformamide. Homogenized cords were left at 50°C for 3 days before centrifugation at 20,000g for 20 min. Absorbance at 620 nm was measured using a NanoDrop spectrophotometer (Thermo Scientific). Data are reported as the amount of EB per wet tissue weight (μ g/g).

Statistical analysis

Data are presented as means and standard deviations (SD). Statistical analyses were done using StatPlus:mac Version 2009 Software (AnalystSoft Inc., Alexandria VA). Bonferroni *post-hoc* tests were carried out for analyses of variance (ANOVA).

Results

Cellular meningeal inflammation in PTS animals

When kaolin was injected into the subarachnoid space rostral to the site of injury, it remained present and recruited local and systemic inflammatory cells. Figure 1 shows cellular meningeal inflammation in response to SCI and induced arachnoiditis (PTS group). Figure 1A shows a schematic of the region where the images were taken. Global representations in Figure 1 are tiled fluorescence microscope images, whereas higher-power images are confocal micrographs. At 2 days post-injury there was evidence of both microglia/macrophages (Iba-1, labeled green in the left panel), and neutrophils (PMN, labeled green in the right panel) in the meningeal layers (dense layer of nuclei) surrounding the kaolin in PTS animals (Fig. 1B). By 7 days post-injury, the meningeal layers were more difficult to discern due to the inflammation and scarring in the area; however, some neutrophils (labeled red) remained around the injury epicenter at the dorsal aspect of the spinal cord (Fig. 1C), whereas there was little evidence of these cells surrounding the kaolin directly. In contrast, macrophages/microglia (labeled red) completely surrounded the kaolin, and were even found in the meninges at remote sites from the injury epicenter (Fig. 1D). GFAP staining for astrocytes (green) was included to delineate the parenchymal boundary.

Meningeal cytokine and chemokine expression

To determine spatial information regarding the expression of inflammatory mediators, kaolin-exposed meningeal fractions were isolated and analyzed separately from the remaining cord tissue (see Fig. 2 for a schematic of sectioning). Multiplex ELISA was used to assay cytokines (IL-1 α , IL-1 β and IL-6) and chemokines (MCP-1, MIP-1 α and GRO/KC) in these fractions. The results are shown in Figure 2 and are reported as pg/mg protein (n=3 per group; 2-way ANOVA, $p < 0.05$ for each molecule). Non-injured sham animals are represented by hatched lines on the graphs. All cytokines and chemokines in meningeal fractions from the arachnoiditis group were significantly increased relative those from sham animals at 1 day post injury (* $p < 0.05$, Bonferroni *post-hoc* test). SCI alone resulted in significant increases in meningeal IL-6, MCP-1, and GRO/KC at 1 day post injury relative to shams ($p < 0.05$). Additionally, relative to sham animals, the combination of SCI and induced arachnoiditis (PTS group) resulted in significant increases in all of the meningeal cytokines and chemokines reported at 1 day post injury ($p < 0.05$). Relative to the SCI group, there was a significant increase in

all inflammatory mediators reported at 1 day post injury in the PTS group ($p < 0.05$). Relative to the arachnoiditis group, the PTS group had a significant increase in only MCP-1 at 1 day post injury ($p < 0.05$). At 3 days post injury, the arachnoiditis group had higher chemokine levels as compared to both the sham and SCI groups ($p < 0.05$).

Parenchymal cytokine and chemokine expression

Spinal cord tissues free from areas of kaolin/arachnoiditis were also analyzed for cytokine and chemokine expression. Parenchymal fractions from the arachnoiditis group contained significantly more MIP-1 α and GRO/KC at 1 day post-injury compared with sham animals ($p < 0.05$ by Bonferroni *post-hoc* test; Fig. 2, bottom panel), with no significant differences seen at 3 days post-injury. SCI alone resulted in a significant increase in IL-6, MCP-1, MIP-1 α , and GRO/KC at 1 day post-injury ($p < 0.05$), and IL-1 α , MCP-1, MIP-1 α , and GRO/KC at 3 days post-injury, compared to sham controls ($p < 0.05$). Relative to sham animals, PTS animals exhibited significant increases in all of the parenchymal cytokines and chemokines reported at 1 day and 3 days post-injury ($p < 0.05$). Relative to the SCI group, there was a significant increase in IL-1 α and IL-1 β at 1 day post-injury ($p < 0.05$), and a significant increase in each chemokine at 3 days post-injury ($p < 0.05$) in PTS animals. Relative to arachnoiditis alone, PTS animals had a significant increase in all inflammatory mediators reported at 1 and 3 days post-injury ($p < 0.05$).

Neutrophil extravasation

The relative extent of neutrophil extravasation in animals was determined using an MPO activity assay and qualitative immunohistochemistry. Figure 3A shows results from an MPO assay that analyzed the enzyme activity in granular fractions, measured in units per gram of whole cord tissue ($n = 5$ per group). Non-injured sham animals are represented by the black-hatched bars. Arachnoiditis animals did not contain significantly more MPO activity than sham animals at 1 and 3 days post-injury ($p < 0.05$ by two-way ANOVA and Bonferroni *post-hoc* test). The SCI group and PTS group had significantly more MPO activity relative to sham animals at 1 and 3 days post-injury ($p < 0.05$ for each). Relative to the SCI group, PTS animals did not have a significant increase in MPO activity at 1 day post-injury ($p = 1.0$), but did exhibit an increase at 3 days ($p < 0.05$). Relative to arachnoiditis alone, the PTS group had a significant increase in MPO activity at 1 and 3 days post-injury ($p < 0.05$).

As neutrophils are the main source of post-SCI MPO, we qualitatively assessed neutrophil accumulation 1 day following injury using immunohistochemistry. Representative images were taken from the sagittal midline of spinal cords and are shown in Figure 3B. Note the significant increase in

PMN (green) immunoreactivity in the parenchyma of the SCI and PTS groups compared to animals in the arachnoiditis group. Further, the number of parenchymal neutrophils in the PTS group was increased compared to the SCI group, suggesting that increases in MPO activity might correlate with an increase in neutrophil influx.

Blood–spinal cord barrier permeability

In order to determine BSCB permeability, EB was injected intravenously into injured animals and its extravasation into the spinal cord was imaged visually in fixed frozen tissue via fluorescence, and measured in homogenized tissue by absorbance spectrophotometry. Figure 4A shows representative EB fluorescence (red) images from arachnoiditis, SCI, and PTS animals counterstained with DAPI (blue), and taken 2 days following injury (with equal exposure and intensity settings). These images demonstrate that there is evidence of parenchymal EB extravasation in the arachnoiditis group, in addition to EB fluorescence in the meninges. Also note that the area of EB fluorescence in the parenchyma is larger in the PTS group than in the SCI group. Further, there is a strong fluorescence signal in the meninges in PTS animals, which includes areas remote from the injury epicenter and on the ventral side of the spinal cord. Figure 4B shows the level of EB detected in whole tissue (0.5 cm) homogenates at 1 and 3 days post-injury. Animals in the arachnoiditis group did not exhibit significantly more EB extravasation than sham animals at 1 or 3 days post-injury ($p < 0.05$ by two-way ANOVA and Bonferroni *post-hoc* test). The SCI group had a significant increase in EB extravasation compared to sham animals at 1 day following injury ($p < 0.05$), but not at 3 days ($p = 1.0$). In contrast, PTS animals exhibited a significant increase in EB extravasation at 1 and 3 days post-injury compared to sham animals ($p < 0.05$ for each day). Relative to the SCI group, PTS animals did not exhibit increased EB extravasation at 1 day post-injury ($p = 0.18$), but did show significantly more at 3 days ($p < 0.05$). Additionally, PTS animals exhibited significantly more EB extravasation compared to arachnoiditis animals at 1 and 3 days post-injury ($p < 0.05$ for each).

As matrix metalloproteinase-9 (MMP-9) has been implicated in promoting acute blood vessel permeability associated with injury, we assessed its spatial activity at 2 days post injury. Figure 4C shows a representative gel zymogram of MMP-9 activity in meningeal and parenchymal fractions. MMP-9 activity was not detected in sham animals, and was only very slightly increased in arachnoiditis animals in both fractions. Similarly, there was slight MMP-9 activity in the meninges of animals in the SCI group, and the parenchymal activity was more pronounced. When induced arachnoiditis was associated with SCI (PTS group), there was an increase in MMP-9 activity in both meningeal and parenchymal fractions

FIG. 1. Cellular meningeal inflammation. (A) Images were taken from sagittal midline spinal cord sections and are oriented with the dorsal aspect of the cord at the top, and the rostral end of the epicenter to the left. (B) At 2 days post-PTS (2d-PTS) injury there was evidence of both microglia/macrophages (left panel; Iba-1), and neutrophils (right panel; PMN) in the meningeal layers surrounding the kaolin. (C) At 7 days post-injury (7d-PTS) some neutrophils (PMN) remained around the injury epicenter, whereas there was little evidence of these cells surrounding the kaolin directly. (D) Microglia/macrophages (Iba-1) completely surrounded the kaolin, and were even found in the meninges at very remote sites from the injury epicenter (right panel; scale bar = 1 mm in low-power images and = 100 μ m in high-power images; PTS, post-traumatic syringomyelia; PMN, polymorphonuclear leukocyte; GFAP, glial fibrillary acidic protein). Color image is available online at www.liebertonline.com/neu

FIG. 2. Spatial cytokine and chemokine expression. The dorsal meninges were removed from the parenchyma and each fraction was analyzed for cytokine/chemokine expression with multiplex ELISA. A schematic of the sectioning is shown at center. Non-injured sham animals are represented with hatched lines on the graphs. **(A)** Meningeal cytokines/chemokines. All cytokines and chemokines in the dorsal spinal cord meningeal fractions of the arachnoiditis group were significantly increased relative to sham animals at 1 day post-injury. There were significant increases in meningeal IL-6, MCP-1, and GRO/KC at 1 day in SCI animals compared to sham animals ($p < 0.05$). Relative to sham animals, PTS animals had significant increases in all of the meningeal cytokines and chemokines at 1 day post-injury ($p < 0.05$). Relative to the SCI group, the PTS group had a significant increase in all inflammatory mediators at 1 day post-injury ($p < 0.05$). Relative to the arachnoiditis group, the PTS group had a significant increase only in MCP-1 at 1 day post-injury ($p < 0.05$). **(B)** Parenchymal cytokines/chemokines. The spinal cord fractions free of dorsal meninges from arachnoiditis animals contained significantly more MIP-1 α and GRO/KC at 1 day post-injury compared with sham animals. SCI significantly increased IL-6, MCP-1, MIP-1 α , and GRO/KC at 1 day post-injury, and IL-1 α , MCP-1, MIP-1 α , and GRO/KC at 3 days post-injury, compared to non-injured sham animals. Similarly, PTS animals contained significantly higher parenchymal cytokine and chemokine levels at 1 day and 3 days post-injury compared to shams. Relative to SCI alone, PTS significantly increased IL-1 α and IL-1 β at 1 day post-injury, and significantly increased each chemokine at 3 days post-injury. Relative to arachnoiditis alone, PTS animals had a significant increase in all inflammatory mediators reported at 1 and 3 days post-injury ($n = 3$ per group; $p < 0.05$ by two-way ANOVA for each molecule; $*p < 0.05$ by Bonferroni *post-hoc* testing; ANOVA, analysis of variance; ELISA, enzyme-linked immunosorbent assay; MCP-1, monocyte chemoattractant protein-1; MIP-1 α , macrophage inflammatory protein-1 α ; IL-6, interleukin-6; SCI, spinal cord injury; IL-1 α , interleukin-1 α ; IL-1 β , interleukin-1 β). Color image is available online at www.liebertonline.com/neu

FIG. 3. Neutrophil extravasation. (A) Neutrophil extravasation was determined by assessing MPO activity in granular fractions at 1 and 3 days post-injury (measured in units per gram of tissue). There was a significant increase in MPO activity in SCI and PTS animals compared to sham animals and arachnoiditis animals at 1 and 3 days post-injury. At 3 days following injury, there was a significant increase in MPO activity in PTS animals compared to SCI animals ($n=3$ per group; $p<0.05$ by two-way ANOVA and Bonferroni *post-hoc*). (B) Longitudinal immunohistochemical images taken at 2 days post-injury show neutrophils stained with a PMN antibody (green) in arachnoiditis, SCI, and PTS animals. The images were taken from sagittal midline spinal cord sections, and are oriented with the dorsal aspect of the cord at the top and the rostral side of the epicenter to the left. Note the increased number of labeled cells in PTS animals (scale bars=1 mm in low-power images and =100 μ m in high-power images; PTS, post-traumatic syringomyelia; PMN, polymorphonuclear leukocyte; MPO, myeloperoxidase; ANOVA, analysis of variance; GFAP, glial fibrillary acidic protein; SCI, spinal cord injury; DAPI, 4,6-diamino-2-phenylindole). Color image is available online at www.liebertonline.com/neu

compared to the SCI group and arachnoiditis group. Additional spatial data are demonstrated in Figure 4D, which shows representative results from *in situ* zymography. Fresh tissue was incubated with a fluorescent gelatin conjugate 2 days post-injury. When cleaved, the previously quenched substrate becomes fluorescent, demonstrating areas of gelatinase (MMP) activity. Animals in the arachnoiditis group exhibited gelatinase activity in the meninges and slight activity in the parenchyma, though this activity was not extensive compared to sham animals. In contrast, animals from the SCI group exhibited more pronounced gelatinase activity in

the parenchyma with little in the meninges. Also note the increase in gelatinase activity in the parenchyma and meninges of animals in the PTS group compared to the SCI group.

Fibrous scarring

Next we set out to determine the impact of arachnoiditis on subacute fibrous scarring. Assessment of fibrous scarring was carried out immunohistologically on midline sagittal sections using collagen IV (red) and CSPG (green) at 7 days following

FIG. 4. Blood–spinal cord barrier permeability. (A) Evans blue (EB) extravasation into the spinal cord was determined in injured animals. Representative EB (red) fluorescence images from arachnoiditis, SCI, and PTS animals 2 days following injury show a larger area of extravasation in PTS animals. Note the intense fluorescence in the meninges of PTS animals. The slides were counterstained with DAPI (blue). (B) EB extravasation was measured in homogenized tissue by absorbance spectrophotometry. There was a significant increase in the amount of EB in SCI and PTS animals compared to sham and arachnoiditis animals at 1 day post-injury. PTS animals contained significantly more EB relative to SCI animals at 3 days post-injury ($n=3$ per group; $p<0.05$ by two-way ANOVA and Bonferroni *post-hoc* test). (C) PTS animals exhibited an increase in matrix metalloproteinase-9 (MMP-9) activity in both parenchymal and meningeal fractions at 2 days post-injury. (D) *In situ* zymography was used to determine spatial MMP-9 activity at 2 days following injury. Note the increase in fluorescence in the parenchyma and meninges in PTS sections compared to SCI and arachnoiditis animals. Images were taken from sagittal midline spinal cord sections, and are oriented with the dorsal aspect of the cord at the top and the rostral side of the epicenter to the left (scale bar = 1 mm; PTS, post-traumatic syringomyelia; ANOVA, analysis of variance; SCI, spinal cord injury; DAPI, 4,6-diamino-2-phenylindole). Color image is available online at www.liebertonline.com/neu

injury. Figure 5 shows representative images revealing extensive scarring in the meninges caudal to and surrounding the kaolin in the arachnoiditis group and the PTS group. While there was little parenchymal collagen IV and CSPG immunoreactivity in arachnoiditis animals, the SCI and PTS groups contained extensive parenchymal fibrous scarring. Note the large increase in parenchymal fibrous scarring in animals from the PTS group compared to the SCI group.

Discussion

Our study demonstrated that the induction of arachnoiditis following SCI dramatically worsens the intramedullary pathology, and is associated with evidence of increased inflammation, BSCB permeability, and fibrous scarring. To our knowledge, this is the first study to separately examine meningeal and parenchymal inflammation and study the

FIG. 5. Fibrous scarring. Shown are longitudinal fluorescence immunohistochemical images from SCI and PTS animals at 7 days post-injury labeled for collagen IV (red, Col IV) and CSPG (green). Note the extensive scarring in the meninges caudal to and surrounding the kaolin. Additionally, this image shows an increase in parenchymal CSPG and collagen IV immunoreactivity (scale bar = 1 mm; PTS, post-traumatic syringomyelia; SCI, spinal cord injury; CSPG, chondroitin sulfate proteoglycan). Color image is available online at www.liebertonline.com/neu

contribution of arachnoiditis to the early pathophysiology of SCI. Animals subjected to SCI alone experienced some dorsal meningeal inflammation, including increased IL-6 and MCP-1 expression, MMP-9 activity, and permeability of blood vessels. When this meningeal inflammation was synthetically increased by the introduction of kaolin (PTS group), the animals exhibited greater parenchymal cytokine/chemokine expression, neutrophil extravasation, MMP-9 activity, BSCB permeability, and fibrous scarring compared to SCI alone. In some cases, these increases were not merely additive, suggesting that arachnoiditis potentiates parenchymal inflammation synergistically. Based on our data, we propose that if sufficient arachnoiditis were to exist following SCI, parenchymal inflammation would be increased, and patients would have a poorer prognosis and possibly be more susceptible to the development of syringes.

Though a synthetic means was used to induce a more robust and local meningeal inflammatory response following SCI, it is modeling an event that is strongly associated with the development of PTS: localized subarachnoid scarring (Brodelt and Stoodley, 2003; Klekamp et al., 1997). The literature suggests that kaolin exhibits some toxicity towards endothelial cells *in vitro* (Murphy et al., 1993), and its presence in the subarachnoid space results in the formation of a granuloma (Cho et al., 1994; Yamada et al., 1996). Taken together, these suggest that kaolin might cause inflammation in the meninges by direct endothelial cell lysis, increased BSCB permeability,

and increased recruitment of inflammatory cells that attempt to isolate the foreign substance. Importantly, we recognize that there are limitations associated with using kaolin to stimulate a meningeal inflammatory response, and it is unlikely to produce the exact post-injury response seen in humans with SCI. Although kaolin is a foreign substance and does not replicate injury-related arachnoiditis precisely, results from a 23-plex ELISA demonstrated that kaolin did not increase the expression of any inflammatory mediator that was not already increased by SCI alone; it just increased the magnitude (Fig. 2 and additional data not shown). Of note, the amount of kaolin injected was based on previous studies (Seki and Fehlings, 2008; Stoodley et al., 2000), and is capable of causing a more pronounced syrinx at 6 weeks following injury when combined with SCI (PTS), compared to SCI alone (Seki and Fehlings, 2008).

PTS can occur in the acute, subacute, or chronic phase of traumatic SCI in clinical cases. Indeed, several studies have described cases developing within several weeks to months following SCI (Carroll and Brackenridge, 2005; Sgouros and Sharif, 2008; Vannemreddy et al., 2002), a time course that is mimicked by the model used in the present study. While the presentation of PTS occurs in a delayed fashion following injury, it is quite plausible that the inflammatory events leading to the development of subarachnoid scarring progress as part of the acute/subacute response to the pathophysiology of SCI.

Arachnoiditis, inflammation, and SCI

At the basic science level, little has been studied regarding meningeal inflammation following SCI. Some studies of relevance demonstrate that the presence of blood in the CSF following injury can stimulate cells in the arachnoid layers to act as antigen-presenting cells and to initiate an inflammatory response (Xin et al., 2010). Additionally, when inflammatory mediators are introduced into the subarachnoid space, they cause a breakdown in the blood–cerebrospinal fluid barrier and potentiate inflammation (Ichikawa et al., 2011). Following human SCI, inflammatory mediators have been detected in the CSF (Kwon et al., 2010), though the source of these molecules is likely from a combination of parenchymal and meningeal cells. Importantly, we acknowledge the limitations of our spinal cord sectioning procedures, and acknowledge that it is unlikely that the “meningeal” fraction was completely devoid of parenchymal cells.

To our knowledge, no study has specifically isolated the meninges and looked at the expression of inflammatory mediators. As such, data from the literature are typically representative of a combination of products from parenchymal and meningeal cells. Inflammatory cytokine mRNAs (tumor necrosis factor- α [TNF- α], IL-1 β , and IL-6) are elevated within minutes after SCI, peak after several hours, and return to basal levels after 3 days (Bartholdi and Schwab, 1997; Basu, 2004; Donnelly and Popovich, 2007; Pineau and Lacroix, 2007; Yang et al., 2004). In light of this fact, our assay is most likely assessing post-peak production of cytokines. Additionally, maximal expression of chemokines is achieved between 12 and 24 h post-SCI (Lee et al., 2000b; Pineau and Lacroix, 2009; Tonai, 2001), suggesting that we were seeing peak chemokine production at our 1-day post-injury time point.

Maximal BSCB permeability reported in the literature is reached 1 day following injury, with some permeability still evident at 1 week (Noble and Wrathall, 1989; Popovich et al., 1996). Our results are consistent with these results (Fig. 4). However, in PTS animals there was no decrease in EB extravasation from 1 to 3 days, as was the case in the SCI group, suggesting that there is prolonged BSCB permeability in these animals. This increase was likely due to a combination of meningeal and parenchymal blood vessel permeability. It should be noted that we did not examine other vasoactive substances that could have influenced BSCB, such as reactive oxygen species, kinins, histamines, nitric oxide, and elastases (Fleming et al., 2006; Noble et al., 2002). The increased permeability that was associated with kaolin + SCI can partly be explained by tight-junction breakdown due to increased MMP-9 activity. Following SCI, MMP-9 activity has been identified to contribute to BSCB permeability (Noble et al., 2002), and reduced MMP-9 activity has been associated with attenuation of tight junction degradation and BSCB permeability (Lee et al., 2012).

Upon the association of arachnoiditis with SCI (PTS animals), some parenchymal inflammatory mediators detected were merely additive (i.e., the inflammation seen was equal to that of arachnoiditis alone plus SCI alone). However, others were greater than that of each combined, suggesting a synergistic increase. These include IL-1 α and IL-1 β at 1 day post-injury (Fig. 2), the inflammatory chemokines reported at 3 days post-injury (Fig. 2), BSCB permeability at 3 days (Fig. 4), and the extent of parenchymal scarring (Fig. 5). It should be

noted that the number of animals per group in most figures is small, and thus negative findings may reflect inadequate power. In the majority of cases, the variability is reasonable and we feel that additional numbers would not alter the interpretation of our data.

Translation to human SCI

There was some modest dorsal meningeal inflammation induced by the moderate clip compression/contusion injury used in our study (SCI group). From this observation, we postulate that not all SCI patients develop PTS because (1) arachnoiditis is either cleared in a timely fashion following injury, or (2) it is not severe enough to lead to alterations in parenchymal pathophysiology or cause subarachnoid scarring. The clip contusion/compression injury causes extensive parenchymal hemorrhaging, as is seen in humans following SCI. It is likely that blood products released from damaged vessels in the parenchyma can diffuse into the CSF and stimulate meningeal inflammation.

Our data demonstrated that the induction of arachnoiditis following SCI can have a significant impact on parenchymal pathophysiology. This increase could make the spinal cord more susceptible to the development of syringes, as it has been demonstrated that increased severity of parenchymal lesions leads to an increased incidence of syrinx formation (Brodbeck et al., 2003a). Furthermore, studies suggest that complete—presumably more severe—injuries are more prone to syrinx development in humans (Vannemreddy et al., 2002). In parallel studies, we have demonstrated that a bioengineered hydrogel containing hyaluronan can reduce arachnoiditis caused by kaolin injection and improve histological and functional outcomes following SCI (Austin et al., 2012).

There is most likely a reciprocal inflammatory relationship when arachnoiditis is associated with SCI. Inflammatory cells from the parenchyma can migrate to sources of inflammation in the meninges, and vice versa. Similarly, local inflammatory mediators can flow via extracellular fluid/CSF to alter inflammation in areas remote to the site of the inflammatory stimulus. It is believed that approximately 30% of CSF is derived from ECF (Brodbeck and Stoodley, 2007), allowing for movement of such macromolecules from the parenchyma to the subarachnoid space and vice versa. We did not examine meningeal fibroblasts in this study, though they are expected to play a role in cytokine and chemokine production, as well as deposition of the fibrous scar in the parenchyma. Indeed, others have demonstrated that following SCI, proliferative meningeal cells can enter the spinal cord parenchyma (Brazda and Muller, 2009; Parr et al., 2007).

As we begin to recognize the heterogeneity in SCI, models like these are needed to properly mimic the permutations seen in certain subpopulations of patients. Previous studies from our lab demonstrated that our PTS model significantly decreased locomotor recovery and increased neuropathic pain (Seki and Fehlings, 2008). This suggests that arachnoiditis could have a substantial impact on patient quality of life.

Conclusions

Our model of PTS used a synthetic means to induce a more robust and local meningeal inflammatory response following SCI, an event that is thought to contribute to subarachnoid scarring and eventual parenchymal influx of CSF. The

induction of arachnoiditis potentiated parenchymal inflammation and scarring following injury, suggesting that if sufficient arachnoiditis were to exist following SCI, patients might experience a more severe injury and might be susceptible to the development of syringes. Specific models like this one are needed to address the biological permutations seen in certain populations of patients in order to develop targeted treatment options.

Acknowledgments

The authors would like to thank Mr. Hoang Nguyen for his technical assistance and support. This work was funded by the Physicians Services Incorporated and the Canadian Syringomyelia Network. Support was also provided by the Ontario Graduate Scholarship (J.W.A.), CIHR Vanier Graduate Scholarship (J.W.A.), and the Krembil Chair in Neural Repair and Regeneration (M.G.F.).

Author Disclosure Statement

No conflicting financial interests exist.

References

- Abel, R., Gerner, H.J., Smit, C., and Meiners, T. (1999). Residual deformity of the spinal canal in patients with traumatic paraplegia and secondary changes of the spinal cord. *Spinal Cord* 37,14–19.
- Austin, J.W., Kang, C.E., Baumann, M.D., Didiodato, L., Satkunendrarajah, K., Wilson, J.R., Stanisz, G.J., Shoichet, M.S., and Fehlings, M.G. (2012). The effects of intrathecal injection of a hyaluronan-based hydrogel on inflammation, scarring and neurobehavioural outcomes in a rat model of severe spinal cord injury associated with arachnoiditis. *Biomaterials* 33, 4555–4564.
- Backe, H.A., Betz, R.R., Mesgarzadeh, M., Beck, T., and Clancy, M. (1991). Post-traumatic spinal cord cysts evaluated by magnetic resonance imaging. *Paraplegia* 29, 607–612.
- Bartholdi, D., and Schwab, M. (1997). Expression of pro-inflammatory cytokine and chemokine mRNA upon experimental spinal cord injury in mouse: an in situ hybridization study. *Eur. J. Neurosci.* 9, 1422–1438.
- Basu, A., Krady, J., and Levison, S. (2004). Interleukin-1: A master regulator of neuroinflammation. *J. Neurosci. Res.* 78, 151–156.
- Brazda, N., and Muller, H.W. (2009). Pharmacological modification of the extracellular matrix to promote regeneration of the injured brain and spinal cord. *Prog. Brain Res.* 175, 269–281.
- Broadbelt, A., and Stoodley, M. (2007). CSF pathways: a review. *Br. J. Neurosurg.* 21, 510–520.
- Broadbelt, A.R., and Stoodley, M.A. (2003). Post-traumatic syringomyelia: a review. *J. Clin. Neurosci.* 10, 401–408.
- Broadbelt, A.R., Stoodley, M.A., Watling, A., Rogan, C., Tu, J., Brown, C.J., Burke, S., and Jones, N.R. (2003a). The role of excitotoxic injury in post-traumatic syringomyelia. *J. Neurotrauma* 20, 883–893.
- Broadbelt, A.R., Stoodley, M.A., Watling, A.M., Tu, J., Burke, S., and Jones, N.R. (2003b). Altered subarachnoid space compliance and fluid flow in an animal model of posttraumatic syringomyelia. *Spine* 28, E413–E419.
- Broadbelt, A.R., Stoodley, M.A., Watling, A.M., Tu, J., and Jones, N.R. (2003c). Fluid flow in an animal model of post-traumatic syringomyelia. *Eur. Spine J.* 12, 300–306.
- Carroll, A.M., and Brackenridge, P. (2005). Post-traumatic syringomyelia: a review of the cases presenting in a regional spinal injuries unit in the north east of England over a 5-year period. *Spine (Phila Pa 1976)* 30, 1206–1210.
- Cho, K.H., Iwasaki, Y., Imamura, H., Hida, K., and Abe, H. (1994). Experimental model of posttraumatic syringomyelia: the role of adhesive arachnoiditis in syrinx formation. *J. Neurosurg.* 80, 133–139.
- Deren, K.E., Packer, M., Forsyth, J., Milash, B., Abdullah, O.M., Hsu, E.W., and McAllister, J.P., 2nd. (2010). Reactive astrogliosis, microgliosis and inflammation in rats with neonatal hydrocephalus. *Exp. Neurol.* 226, 110–119.
- Dixon, W.H., H. (1932). Experimentelle Hypertonie durch Erhöhung des intrakraniellen Druckes. *Arch. Exp. Pathol. Pharmacol.* 166, 265–275.
- Donnelly, D., and Popovich, P. (2007). Inflammation and its role in neuroprotection, axonal regeneration and function recovery after spinal cord injury. *Exp. Neurol.* 209, 378–388.
- Fleming, J.C., Norenberg, M.D., Ramsay, D.A., Dekaban, G.A., Marcillo, A.E., Saenz, A.D., Pasquale-Styles, M., and Dietrich, W.D. (2006). The cellular inflammatory response in human spinal cords after injury. *Brain* 129, 3249–3269.
- George, S.J., and Johnson, J.L. (2010). In situ zymography. *Methods Mol. Biol.* 622, 271–277.
- Ichikawa, H., Ishikawa, M., Fukunaga, M., Ishikawa, K., and Ishiyama, H. (2011). Quantitative evaluation of blood-cerebrospinal fluid barrier permeability in the rat with experimental meningitis using magnetic resonance imaging. *Brain Res.* 1321, 125–132.
- Klekamp, J., Batzdorf, U., Samii, M., and Bothe, H.W. (1997). Treatment of syringomyelia associated with arachnoid scarring caused by arachnoiditis or trauma. *J. Neurosurg.* 86, 233–240.
- Klekamp, J., Volkel, K., Bartels, C.J., and Samii, M. (2001). Disturbances of cerebrospinal fluid flow attributable to arachnoid scarring cause interstitial edema of the cat spinal cord. *Neurosurgery* 48, 174–185; discussion 185–176.
- Kwon, B.K., Stammers, A.M., Belanger, L.M., Bernardo, A., Chan, D., Bishop, C.M., Slobogean, G.P., Zhang, H., Umedaly, H., Giffin, M., Street, J., Boyd, M.C., Paquette, S.J., Fisher, C.G., and Dvorak, M.F. (2010). Cerebrospinal fluid inflammatory cytokines and biomarkers of injury severity in acute human spinal cord injury. *J. Neurotrauma* 27, 669–682.
- Lee, J.Y., Kim, H.S., Choi, H.Y., Oh, T.H., Ju, B.G., and Yune, T.Y. (2012). Valproic acid attenuates blood-spinal cord barrier disruption by inhibiting matrix metalloproteinase-9 activity and improves functional recovery after spinal cord injury. *J. Neurochem.* 121, 818–829.
- Lee, Y.L., Shih, K., Bao, P., Ghirnikar, R.S., and Eng, L.F. (2000b). Cytokine chemokine expression in contused rat spinal cord. *Neurochem. Int.* 36, 417–425.
- Murphy, E.J., Roberts, E., and Horrocks, L.A. (1993). Aluminum silicate toxicity in cell cultures. *Neuroscience* 55, 597–605.
- Noble, L.J., Donovan, F., Igarashi, T., Goussev, S., and Werb, Z. (2002). Matrix metalloproteinases limit functional recovery after spinal cord injury by modulation of early vascular events. *J. Neurosci.* 22, 7526–7535.
- Noble, L.J., and Wrathall, J.R. (1989). Distribution and time course of protein extravasation in the rat spinal cord after contusive injury. *Brain Res.* 482, 57–66.
- Parr, A.M., Kulbatski, I., and Tator, C.H. (2007). Transplantation of adult rat spinal cord stem/progenitor cells for spinal cord injury. *J. Neurotrauma* 24, 835–845.

- Perrouin-Verbe, B., Lenne-Aurier, K., Robert, R., Auffray-Calvier, E., Richard, I., Mauduyt de la Greve, I., and Mathe, J.F. (1998). Post-traumatic syringomyelia and post-traumatic spinal canal stenosis: a direct relationship: review of 75 patients with a spinal cord injury. *Spinal Cord* 36, 137–143.
- Pineau, I., and Lacroix, S. (2007). Proinflammatory cytokine synthesis in the injured mouse spinal cord: multiphasic expression pattern and identification of the cell types involved. *J. Comp. Neurol.* 500, 267–285.
- Pineau, I., Sun, L., Bastien, D., and Lacroix, S. (2009). Astrocytes initiate inflammation in the injured mouse spinal cord by promoting the entry of neutrophils and inflammatory monocytes in an IL-1 receptor/MyD88-dependent fashion. *Brain Behav. Immun.* 24, 540–553.
- Popovich, P.G., Horner, P.J., Mullin, B.B., and Stokes, B.T. (1996). A quantitative spatial analysis of the blood-spinal cord barrier. *Exp. Neurol.* 142, 258–275.
- Seki, T., and Fehlings, M.G. (2008). Mechanistic insights into posttraumatic syringomyelia based on a novel in vivo animal model. Laboratory investigation. *J. Neurosurg. Spine* 8, 365–375.
- Sgouros, S., and Sharif, S. (2008). Post-traumatic syringomyelia producing paraplegia in an infant. *Childs Nerv. Syst.* 24, 357–360; discussion 361–354.
- Stoodley, M.A., Jones, N.R., Yang, L., and Brown, C.J. (2000). Mechanisms underlying the formation and enlargement of noncommunicating syringomyelia: experimental studies. *Neurosurg. Focus* 8, E2.
- Tonai, T., Shiba, K., Taketani, Y., Ohmoto, Y., Murata, K., Muraguchi, M., Ohsaki, H., Takeda, E., and Nishisho, T. (2001). A neutrophil elastase inhibitor (ONO-5046) reduces neurologic damage after spinal cord injury in rats. *J. Neurochem.* 78, 1064–1072.
- Vannemreddy, S.S., Rowed, D.W., and Bharatwal, N. (2002). Posttraumatic syringomyelia: predisposing factors. *Br. J. Neurosurg.* 16, 276–283.
- Xin, Z.L., Wu, X.K., Xu, J.R., and Li, X. (2010). Arachnoid cell involvement in the mechanism of coagulation-initiated inflammation in the subarachnoid space after subarachnoid hemorrhage. *J. Zhejiang Univ. Sci. B* 11, 516–523.
- Yamada, H., Yokota, A., Haratake, J., and Horie, A. (1996). Morphological study of experimental syringomyelia with kaolin-induced hydrocephalus in a canine model. *J. Neurosurg.* 84, 999–1005.
- Yang, L., Blumbers, P., Jones, N., Manavis, J., Sarvestani, G., and Ghabriel, M. (2004). Early expression and cellular localization of proinflammatory cytokines interleukin-1B, interleukin-6, and tumour necrosis factor- α in human traumatic spinal cord injury. *Spine J.* 29, 966–971.
- Yang, L., Jones, N.R., Stoodley, M.A., Blumbergs, P.C., and Brown, C.J. (2001). Excitotoxic model of post-traumatic syringomyelia in the rat. *Spine (Phila Pa 1976)* 26, 1842–1849.
- Yu, F., Kamada, H., Niizuma, K., Endo, H., and Chan, P.H. (2008). Induction of MMP-9 expression and endothelial injury by oxidative stress after spinal cord injury. *J. Neurotrauma* 25, 184–195.

Address correspondence to:
 Michael G. Fehlings, M.D., Ph.D.
 Toronto Western Hospital
 399 Bathurst Street, 4WW-449
 Toronto, Ontario, Canada M5T 2S8
 E-mail: michael.fehlings@uhn.on.ca