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Cerebrospinal Fluid Inflammatory Cytokines and Biomarkers of Injury Severity in Acute Human Spinal Cord Injury

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Abstract

There is an urgent need for both the scientific development and clinical validation of novel therapies for acute spinal cord injury (SCI). The scientific development of novel therapies would be facilitated by a better understanding of the acute pathophysiology of human SCI. Clinical validation of such therapies would be facilitated by the availability of biomarkers with which to stratify injury severity and predict neurological recovery. Cerebrospinal fluid (CSF) samples were obtained over a period of 72 h in 27 patients with complete SCI (ASIA A) or incomplete SCI (ASIA B or C). Cytokines were measured in CSF and serum samples using a multiplex cytokine array system and standard enzyme-linked immunosorbent assay (ELISA) techniques. Neurological recovery was monitored, and patient-reported neuropathic pain was documented. IL-6, IL-8, MCP-1, tau, S100β, and glial fibrillary acidic protein (GFAP) were elevated in a severity-dependent fashion. A biochemical model was established using S100β, GFAP, and IL-8 to predict injury severity (ASIA A, B, or C). Using these protein concentrations at 24-h post injury, the model accurately predicted the observed ASIA grade in 89% of patients. Furthermore, segmental motor recovery at 6 months post injury was better predicted by these CSF proteins than with the patients' baseline ASIA grade. The pattern of expression over the first 3 to 4 days post injury of a number of inflammatory cytokines such as IL-6, IL-8, and MCP-1 provides invaluable information about the pathophysiology of human SCI. A prediction model that could use such biological data to stratify injury severity and predict neurological outcome may be extremely useful for facilitating the clinical validation of novel treatments in acute human SCI.

Key words: traumatic spinal cord injury; biomarkers; inflammation; cerebrospinal fluid; clinical trial

Introduction

Each year, more than 10,000 individuals in North America and many thousands more around the globe are paralyzed after sustaining an acute spinal cord injury (SCI), leaving them to endure one of the most physically and psychologically devastating of injuries known to mankind. More than four decades of passionate research in this field have generated numerous therapeutic interventions that have shown promise in animal models of cord injury. Unfortunately, while a handful of these therapies have emerged from the laboratory to be tested in humans, none have succeeded in demonstrating convincing neurological benefit in large-scale clinical trials (Fehlings and Baptiste, 2005; Lammertse, 2004; Ramer et al., 2000). Without any effective treatments to offer patients who suffer this catastrophic injury, an urgent need obviously exists for not just the scientific development of novel therapeutic interventions, but also the subsequent clinical validation of these treatments in human clinical trials.

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Both of these urgent needs, however, are met with significant challenges. First, the scientific pre-clinical development of new therapies for SCI is almost entirely dependent on the use of rodent and murine models of cord injury. Such therapies aim to influence specific aspects of spinal-cord physiology and/or injury pathophysiology, such as post-traumatic neuro-inflammation, in order to improve neurological outcome. It is assumed that many of these biological and physiological aspects will be comparable between the commonly utilized animal models and the injured human spinal cord. However, the fact that no treatment to date has demonstrated convincing neurological efficacy in human clinical trials, despite showing some positive effects in animal models of cord injury, highlights the possibility that important biological differences exist between the two. Given the plethora of animal studies on the biology and pathophysiology of acute spinal cord injury and the paucity of comparable investigation in human patients, a strong translational rationale exists to study the latter in order to discern similarities and differences with animal models that are potentially important for the clinical applicability of novel experimental treatments.

Second, while it is a significant achievement for a novel experimental therapy to take the "translational leap" from bench to bedside and enter into a human SCI trial, the subsequent process of clinical validation to establish neurological efficacy is exceedingly difficult (Tator, 2006), a point often overlooked by scientists and clinicians alike. This difficulty is illustrated by the 15 years it took to evaluate the now-abandoned drug Sygen (GM-1 ganglioside), which began its clinical evaluation in 1986 (Geisler et al., 1991) and ended it in 2001 with the publication of the monumental – yet negative – 760-patient Phase 3 randomized clinical trial (Geisler et al., 2001b). A major impediment to the clinical validation of such drugs is the singular dependence upon *functional* neurological measures to classify the severity of neurological injury in SCI patients who are potential candidates for clinical trials. The American Spinal Injury Association (ASIA) grading system is universally used to classify injury severity (American Spinal Injury Association/International Medical Society of Paraplegia, 2000), and is currently the most accurate prognosticator of a patient's neurological outcome (Burns and Ditunno, 2001; Marino et al., 1999). The functional nature of this classification, however, makes it impossible to establish accurately in patients with concomitant head injuries, multi-system trauma, or drug intoxication (Burns et al., 2003). Without the ability to establish a baseline injury severity, these patients are ineligible for acute SCI clinical trials, severely limiting the pool of "recruitable" patients for such studies. Furthermore, even amongst patients with the same baseline ASIA grade, the extent of spontaneous neurological recovery is extremely variable (Fawcett et al., 2007). This imprecision with which the functional ASIA grading predicts eventual neurological outcome forces investigators to spend years enrolling large numbers of patients to achieve adequate statistical power (as witnessed in the Sygen Phase 3 trial). This fact combined with the low "recruitability" of these patients because of factors that preclude the establishment of a baseline injury severity pose enormous problems for the clinical validation of novel therapies. With promising new experimental treatments being reported on an almost weekly basis from laboratories around the world, the inability to validate them efficiently clinically will be a stifling bottleneck to the SCI community. Clearly, a new approach is needed.

In this study, we enrolled acute SCI patients into a clinical trial in which lumbar intrathecal catheters were inserted prior to surgery, and cerebrospinal fluid (CSF) samples were obtained over a period of approximately 72 h. In these samples, we measured the concentration of a series of inflammatory cytokines and neural tissue markers to describe the temporal expression of proteins that influence the regulation of the post-traumatic neuroinflammatory response. This neuroinflammation is thought to be one of the most important mediators of secondary damage after SCI (Jones et al., 2005; Trivedi et al., 2006), and it is therefore quite likely that any therapy administered in the acute SCI setting will either influence or be influenced by inflammation – a notion supported by laboratory studies of pharmacological agents (Gorio et al., 2005), growth factors (Bouhy et al., 2006), gene therapy vectors (Abdellatif et al., 2006), and cell transplants (Pearse et al., 2004; J. Yan et al., 2004). Characterizing these aspects of the inflammatory response therefore has substantial rationale as a therapeutic strategy, both for identifying targets of intervention and for determining factors that will influence the success (or failure) of experimental treatments (Demjen et al., 2004; Genovese et al., 2006; Ghirnikar et al., 2001; Gonzalez et al., 2003; Lacroix et al., 2002). Doing this in animal models is facilitated by the ability to harvest the spinal cord and directly study it. Obtaining samples of cord tissue to perform such molecular, biochemical, and histological investigations is obviously not possible in live human patients for risk of inducing further damage, and hence, the most representative tissue available for human study is the CSF that bathes the spinal cord. Animal studies that have measured cytokine concentrations in the cord, CSF, and serum after SCI have established the utility of using CSF as a biological representation of what is occurring within the spinal cord (Harrington et al., 2005; Wang et al., 1997).

In addition to our interest in describing the temporal pattern of expression of various inflammatory cytokines after acute human SCI, we sought to establish whether these inflammatory proteins could be used as biological markers of injury severity. We also evaluated structural proteins such as tau, S100β, and glial fibrillary acidic protein (GFAP) that have been previously assessed as potential biomarkers in other human neurological disorders but have not been investigated extensively in human SCI (Pouw et al., 2009). Such markers could potentially be used to stratify patients more precisely upon enrolment into clinical trials of novel therapies, and thus reduce the number of patients required to achieve adequate statistical power. The ability to measure such biological markers without requiring the patient to be able to provide a valid functional assessment could greatly enhance patient recruitment in clinical trials of acute SCI.

Methods

Spinal cord injury patient enrollment

Patients sustaining an acute SCI were recruited at a single Level 1 regional trauma institution by one of six fellowship-trained spinal surgeons, from March 2006 to March 2008. Inclusion criteria for this prospective trial included: ASIA grade A (motor and sensory complete paralysis) or B (motor complete, sensory incomplete paralysis) and C (incomplete motor and sensory paralysis) SCI upon presentation; spinal injury between C3 and T11 inclusive; within 48 h of injury; the ability

to provide a valid, reliable neurological examination. Patients were excluded if they had concomitant head injuries, concomitant major trauma to the chest, pelvis, or extremities that required invasive intervention (e.g., chest tube, internal or external fixation), or if they were too sedated or intoxicated to provide a valid neurological examination.

The clinical trial protocol was granted approval from both the university human ethics committee and the hospital clinical trials administrative body, and was registered with the US National Institutes of Health (ClinicalTrials.gov identifier NCT00135278). The clinical trial randomized patients to one of two groups: either receiving "CSF drainage" through their intrathecal catheter, in an effort to reduce intrathecal pressure, or "no CSF drainage". The drains were inserted (and the first CSF sample obtained) pre-operatively, and irrespective of randomization, the actual drainage was not initiated until after the patient was awake and neurologically examinable post-operatively. The initial CSF samples were therefore generally drawn before CSF drainage was even instituted, and so for the purpose of this analysis, we evaluated all of the patients as a single cohort. Furthermore, we discovered that, in the end, very little CSF was actually drained (due to restrictions in the protocol that mandated that CSF drainage only occur when the patients were examinable), and hence, the intrathecal pressures of the patients randomized to "CSF drainage" were essentially identical to that of the patients randomized to "no CSF drainage" (Kwon et al., 2009). This further justified the evaluation of all of the patients as a single cohort.

Nonspinal cord injury patient enrollment as "noninjured controls"

To interpret the CSF concentrations of the proteins of interest in our SCI patients, we enroled "non-injured" control individuals from whom we obtained consent to acquire CSF. We enroled individuals with hip or knee osteoarthritis who were undergoing hip or knee replacements under spinal anaesthesia, or individuals with lumbar disc herniations or stenosis who were undergoing open laminectomies. For the individuals undergoing hip and knee replacements under spinal anaesthesia, the anesthesiologist punctured the dura with a spinal needle, a 1.0–1.5 mL sample of CSF was collected, and then the anaesthetic agent was injected. For the individuals undergoing lumbar spine surgery, after the laminectomy-decompression and/or fusion was completed and the thecal sac exposed, a spinal needle was used to puncture the dura for CSF collection.

Intrathecal catheter insertion and cerebrospinal fluid collection

Prior to their spinal decompression/stabilization surgery, patients were log-rolled into the lateral position under the supervision of the spinal surgeon who maintained the cervical and thoracolumbar spine in neutral alignment during the placement of the catheter. Using a strict aseptic technique, a lumbar puncture was performed at L2/3 or L3/4, and a 3–4 mL sample of CSF was collected. Following this, an intrathecal catheter (PERIFIX® Custom Epidural Anesthesia Kit; B. Braun Medical Inc., Bethlehem, PA) was inserted and advanced 15–20 cm from the entry point on the skin surface. The catheters remained in place for approximately 72 h.

Post-operatively, CSF samples of 3-4 mL were drawn from the catheter using a strict sterile technique every 6 to 8 h (discarding the first 1 mL of CSF aspirated from the line). The sample was divided into 500 μl aliquots, centrifuged at 1000 rcf for 10 min, and the supernatant then immediately frozen in an ethanol/dry-ice bath and stored at -80°C. At the same time as the CSF sample was obtained, a 5–6 mL sample of blood was also drawn. The blood was left to clot at room temperature for 15 min and then centrifuged at 10,000 rcf for 5 min, and the supernatant frozen in ethanol/dry ice, and stored at -80°C.

Biochemical analysis

The human CSF and blood were analyzed on a Bio-Plex system (Bio-Rad, Hercules, CA) using a 25-plex human cytokine kit that included: TNF-α, TNF-R1, IL-1β, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p40, IL-13, IL-15, IL-17, IP-10, MCP-1, IFN-α, IFN-γ, eotaxin, GM-CSF, MIG, MIP-1α, MIP-1β, and RANTES (Cytokine human 25-plex panel catalogue number LHC-0009; Invitrogen, Carlsbad, CA). We added to this kit the cytokine IL-16, and also the following growth factors: BDNF, FGF-basic, GDNF, and VEGF (BDNF human singleplex, catalogue number LHC-7071, FGF-basic human singleplex, catalogue number LHG-0021, GDNF human singleplex, catalogue number LHC-7041; VEGF human singleplex, catalogue number LHG-0111; Invitrogen). We also performed standard enzyme-linked immunosorbent assays (ELISAs) on tau (Tau [Total] Human ELISA Kit, catalogue number KHB0042; Invitrogen), S100β, and GFAP (S100β Human ELISA, Glial Fibrillary Acidic Protein Human ELISA, catalog numbers RD192090100R and RD192072200R; Biovendor, Modrice, Czech Republic). Due to the inability to detect IL-1β and TNF-α with the multiplex bead technology, we also tested CSF samples taken early post injury ($<$30 h) on the Meso Scale Discovery® platform (MA2400 Human IL-1β Ultra-Sensitive Kit, catalogue number K151AGC-1, MA2400 Human TNF-alpha Ultra-Sensitive Kit, catalogue number K151BHC-1; Meso Scale Discovery, Gaithersburg, MD).

Functional analysis

The severity of neurological impairment was graded according to the ASIA standards of neurological testing, with motor scores recorded separately in the upper and lower extremities. All baseline testing and the assigning of the baseline ASIA grade (A, B, or C) were conducted by one of three study nurses to confirm the initial examination of the patients. ASIA motor assessments were conducted at 3, 6, and 12 months post injury. To assess neuropathic pain, a structured questionnaire was administered, in which patients reported on an 11-point numerical rating scale the presence and severity of sensations characteristic of neuropathic pain (Sawatzky et al., 2008). These included "dysesthetic" (numb, tingly, pins and needles, prickly), "paroxysmal" (stabbing, shooting, electric), and "other" (sensitive, achy, sharp) pain sensations (Bennett et al., 2007). The patients also reported on a scale of 0 to 10 their pain intensity, pain intolerability, pain interference with functioning, and confidence/satisfaction with pain management. Baseline pain assessments were performed within the first 6 weeks post injury when the patients were out of the ICU setting and could reasonably provide responses to the questionnaire, while follow-up pain assessments were performed at least 6 months post injury.

Statistical analysis

The CSF sample taken at around 24 h post injury was used to explore the potential of using the cytokine and protein concentrations as a method of classifying injury severity. Ordinal logistic regression was conducted on the cytokine concentrations, and then a backward selection procedure was carried out on bootstrap samples of the original data, using all of the cytokines and structural proteins measurable in the CSF. This bootstrap method produced a ranking of the importance of each measurable cytokine/protein as a predictor of injury severity (Austin and Tu, 2004). Then a series of prediction models were generated by combining each of the predictors sequentially in the order of their ranking, and the corresponding Akaike Information Criteria (AIC) was calculated. The constituents of the "final" prediction model were chosen based on the combination with the lowest AIC. Finally, the concentrations observed in each individual patient's CSF at 24 h post injury were input into the model to generate the "predicted" ASIA impairment grade (A, B, or C), based on the biochemical information. This was then compared against the observed ASIA impairment grade to determine the model's accuracy at predicting the "true" injury severity of the patient. The c-index (a predictability measurement analogous to the area under the curve [AUC] in a receiver operating characteristics [ROC] analysis for binary outcomes) was then calculated. Similar modeling was performed to predict motor recovery in the upper extremities in patients with cervical cord injury (i.e., "segmental" or "local" motor recovery). (See Appendix A, Supplemental Data, for details of statistical modeling for predicting ASIA grade and segmental motor recovery.)

To assess the relationship between the inflammatory cytokines and neuropathic pain, Pearson correlation coefficients (SCC) were calculated between the 24-h concentrations of each cytokine and the baseline and final follow-up patient-reported pain scores.

Results

A total of 27 acute SCI patients were prospectively enrolled (Table 1), and CSF samples were acquired over a period of

Table 1. Patient Demographics and Baseline Neurological Status

ID	*Mechanism of injury*	*Spinal injury*	*Age*	*Sex*	*ASIA grade*	*Level*
1	Blow to head	C5 burst fracture	29	M	C	Cervical
2	Fall from ladder	C5/6 fracture-dislocation	34	M	B	Cervical
3	MVA	T3 burst fracture	47	M	B	Thoracic
4	MVA	C6/7 fracture-dislocation	42	M	B	Cervical
5	Mountain biking	C5/6 hyperflexion with spondylosis	64	F	C	Cervical
6	Fall from standing height	C4/5 hyperextension with laminar fracture	66	M	C	Cervical
7	Fall from ladder	C6/7 fracture-dislocation	46	F	A	Cervical
8	MVA	T8/T9 fracture dislocation	37	M	B	Thoracic
9	MVA	C6/7 fracture-dislocation	33	M	A	Cervical
10	Diving	C4/5 fracture dislocation	37	M	A	Cervical
11	Fall from moving car	T9 & T10 burst fractures	55	F	B	Thoracic
12	MVA	C5/6 fracture dislocation	50	F	A	Cervical
13	Mountain biking	T3/4 fracture dislocation	40	M	A	Thoracic
14	MVA (head-on collision)	C6 teardrop fracture	23	M	A	Cervical
15	Blow to head (work-related)	C6/7 bilateral facet dislocation	23	M	A	Cervical
16	MVA (rollover)	T9 burst fracture	31	F	A	Thoracic
17	Fall down stairs	C5/6 fracture dislocation	45	F	A	Cervical
18	Fall off balcony	C5 burst fracture	46	F	A	Cervical
19	MVA (rollover)	C5/6 fracture dislocation	30	M	A	Cervical
20	Fall from ladder	C5/6 facet subluxation with spondylosis	60	M	B	Cervical
21	Bicycle accident	C5 burst fracture	46	M	C	Cervical
22	MVA (rollover)	C6/7 fracture dislocation	30	M	A	Cervical
23	Mountain biking	C5/6 fracture dislocation	28	M	A	Cervical
24	MVA	C6/7 bilateral facet dislocation	20	M	B	Cervical
25	Fall from roof	T4-T5 fracture dislocation	46	F	A	Thoracic
26	Mountain biking	C4/5 hyperextension with no fracture	39	M	C	Cervical
27	MVA	C5/6 flexion-distraction	54	M	C	Cervical
Totals			40.8 ± 2.4 years	19 male 8 female	14 A, 7 B, 6 C	21 cervical 6 thoracic

MVA, motor vehicle accident.

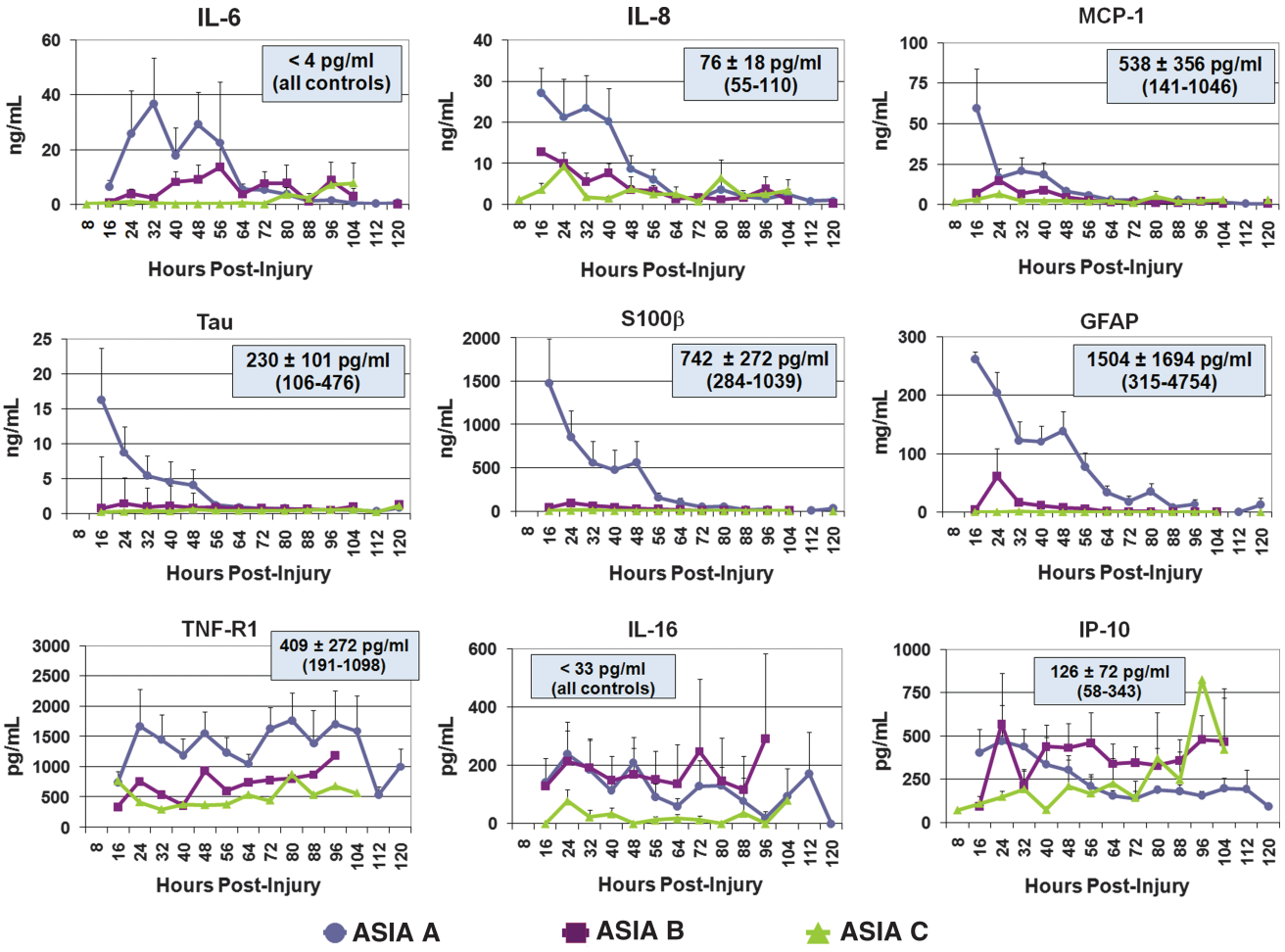

FIG. 1. Time course of expression of cytokines and neural tissue proteins within the cerebrospinal fluid (CSF) after human spinal cord injury (SCI). The concentrations of these proteins within the CSF are plotted over time according to the baseline spinal cord injury severity of the patients (ASIA A, B, or C). The color figure legend at the bottom applies to all graphs. The blue inset within each graph is the CSF concentration in 12 non-SCI, control individuals who underwent lumbar puncture for spinal anesthesia prior to hip/knee replacement surgery, or intra-operatively during lumbar decompression/fusion surgeries. The range (minimum and maximum CSF concentration) for the non-SCI control individuals is noted in parentheses within each blue inset. For IL-6 and IL-16, all non-SCI controls had concentrations "below detectable limits," which were 4 pg/mL for IL-6 and 33 pg/mL for IL-16. Note that because of the very low concentrations in the non-SCI control individuals, the units of measure in the blue inset are an order of magnitude less than on the *y* axis for IL-6, IL-8, MCP-1, tau, and glial fibrillary acidic protein (GFAP). Color image is available online at www.liebertonline.com/neu.

72 h. Importantly, careful monitoring of the patients during these 72 h revealed no device-related adverse events such as nausea/vomiting (related to CSF leakage), meningitis, or neurological deterioration. CSF samples were also obtained via a single lumbar puncture from 12 non-SCI patients whose CSF served as the "normal" uninjured controls.

Biochemical analysis of cerebrospinal fluid

The analysis of the CSF samples revealed that IL-6, IL-8, IP-10, MCP-1, IL-16, TNF-R1, tau, S100β, and GFAP were present in measurable concentrations. Plotting the raw protein concentrations (mean ± SEM) according to the baseline severity of paralysis (ASIA A, B, or C) that was documented at the time of patient presentation revealed a severity-dependent expression of IL-6, IL-8, MCP-1, tau, S100β, and GFAP early in the post-injury period (Fig. 1). In general, the concentrations of these proteins were highest during the first 24 to 36 h, and then decreased to low levels by 72 h post injury. In the non-SCI control patients, the concentrations of these six proteins were very low, and in many cases below detectable limits (Fig. 1). The remaining cytokines and growth factors that were included on the Multiplex kit were not expressed at measurable levels in the CSF at any time point.

Many of the cytokines and all of the growth factors that were included in our multiplex analysis were not expressed at measurable levels at any time point. These included IL-1β, IL-2, IL-4, IL-5, IL-7, IL-10, IL-12p40, IL-13, IL-15, IL-16, IL-17, IFN-α, IFN-γ, eotaxin, GM-CSF, MIG, MIP-1α, MIP-1β, RANTES, TNF-α, IL-2R, BDNF, FGF-basic, GDNF, and VEGF. The observation that both IL-1β and TNF-α were below detection levels with the multiplex bead assay (approximately 1.5 pg/mL and 0.25 pg/mL respectively) was somewhat surprising to us, given their reported importance in the early

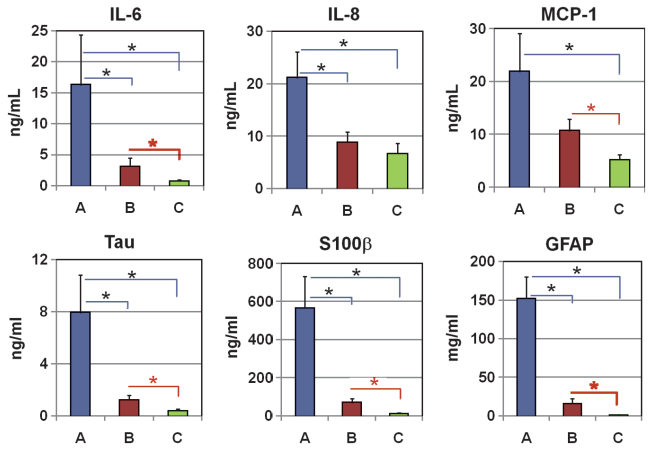

FIG. 2. Cerebrospinal fluid (CSF) concentrations of IL-6, IL-8, MCP-1, tau, S100*β*. and glial fibrillary acidic protein (GFAP) are increased according to injury severity at 24 h post injury. Plotting the concentrations of IL-6, IL-8, MCP-1, tau, S100*β*, and GFAP from the CSF sample around 24 h post injury (a time point that would be clinically feasible to obtain such a sample in future patients) revealed substantial differences between patients with ASIA A, B, or C injury severities. This figure simply illustrates the injury-severity-dependent pattern of expression for these proteins. While statistically significant differences are indicated (*), the statistical modeling approach to classifying injury severity utilizes a combination of different proteins, and therefore is not dependent upon the significant differences within ASIA impairment grades for a single cytokine/protein (*$p < 0.05$, Wilcoxon signed-rank test). Color image is available online at www.liebertonline.com/neu.

neuroinflammatory response to injury. We repeated this analysis on samples drawn within 36 h of injury utilizing the Meso Scale technology, which has greater sensitivity for both IL-1*β* and TNF-α than the Multiplex technology, and again were unable to detect these two pro-inflammatory cytokines.

Classifying injury severity with cerebrospinal fluid proteins

The raw cytokine data at 24 h post injury revealed that substantial differences existed between the ASIA A, B, and C patients for IL-6, IL-8, MCP-1, tau, S100*β*, and GFAP (Fig. 2). A comparison between the CSF and serum concentrations of these proteins at 24 h post injury revealed that the CSF concentrations were at least 10 times greater than in serum; in ASIA A patients in particular, the CSF levels were often orders of magnitude greater than in serum (Fig. 3). While significant differences were noted for individual cytokines/proteins in the 24-h post-injury CSF sample between ASIA A, B, and C patients (Fig. 2), we explored the potential of combining these CSF markers to create a prediction model that would be stronger at predicting the baseline severity of neurological deficit. Ordinal logistic regression was applied to CSF concentrations of IL-6, IL-8, MCP-1, tau, S100*β*, and GFAP from the 24-h post-injury sample, and a backward step-wise predictor selection was repeated 1000 times ("bootstrapping") to identify those proteins that most frequently predicted injury severity. These were S100*β*, GFAP, and IL-8, which were then included in the final prediction model. By adding the actual concentrations of these proteins from the 24-h post-injury sample into the model, a "predicted" ASIA impairment grade (A, B, or C) was generated, and this was compared against the "observed" ASIA impairment grade. The biochemical prediction model accurately classified the patients' ASIA impairment grade in 24 out of 27 cases (88.9%; Table 2). The c-index of this model was 0.987 (with a model no better than sheer chance having a c-index of 0.5, and a perfect test having a c-index of 1.0).

Recognizing that the performance of this prediction model is enhanced by the fact that the model itself was derived from the data that is then used to assess its accuracy at predicting ASIA impairment grade, we performed a 2:1 "internal assessment" by establishing the model on the first 18 patients (the "derivation cohort"), and then testing it on the last nine patients (the "validation cohort"). The model correctly classified the observed ASIA impairment (A, B, or C) in seven of nine patients (for an accuracy rate of 78%). We additionally generated 1000 bootstrap samples of a nine-patient "validation" data set and tested the prediction model on this data set to establish the variability with which the model accurately classified injury severity. In the 1000 bootstrap samples, the accuracy rate was 77%, with a 95% CI of 0.44 to 1.00. (Fig. 4)

Predicting neurological recovery with cerebrospinal fluid proteins

It is well established that the baseline severity of neurological impairment after SCI is the most important determinant of eventual neurological recovery. It is therefore reasonable to expect that a valid biomarker of injury severity would also be able to predict neurological recovery. Conceptually, the extent of motor recovery immediately around the injury site (termed "local" or "segmental" recovery) is the most representative of the extent of local damage, but because this is not measurable in thoracic SCI patients, this analysis of segmental recovery is limited to cervical SCI patients. Like the prediction model for classifying the ASIA grade, bootstrap modeling was done to generate a model to predict the extent of segmental upper-extremity motor recovery at 6 months post injury using S100*β*, GFAP, and IL-8. The 6-month post-injury time point has been shown to be the time at which segmental recovery in tetraplegics plateaus (Waters et al., 1993, 1994), and it has been used as the endpoint for neuroprotective trials because, beyond that, other factors unrelated to the initial injury likely influence motor function (e.g., community physiotherapy resources). Given these considerations, we compared our cytokine prediction model to how well the standard clinical classification of ASIA grade (ASIA A, B, or C) predicted local motor recovery at 6 months post injury. We found that our biochemical prediction model was comparable (if not marginally better) than the current clinical ASIA grading scale at predicting the extent of local motor recovery at 6 months post injury (accuracy of 70% vs. 65% for the biomarkers vs. the functional ASIA grade, with a c-index of 0.867 and 0.773 respectively; Table 3).

Correlation between inflammatory cytokines and neuropathic pain

Neuropathic pain is a common and often intractable problem for acute and chronic SCI patients (Siddall et al., 1999, 2003). While substantial evidence from animal models of SCI points to the post-traumatic neuro-inflammatory

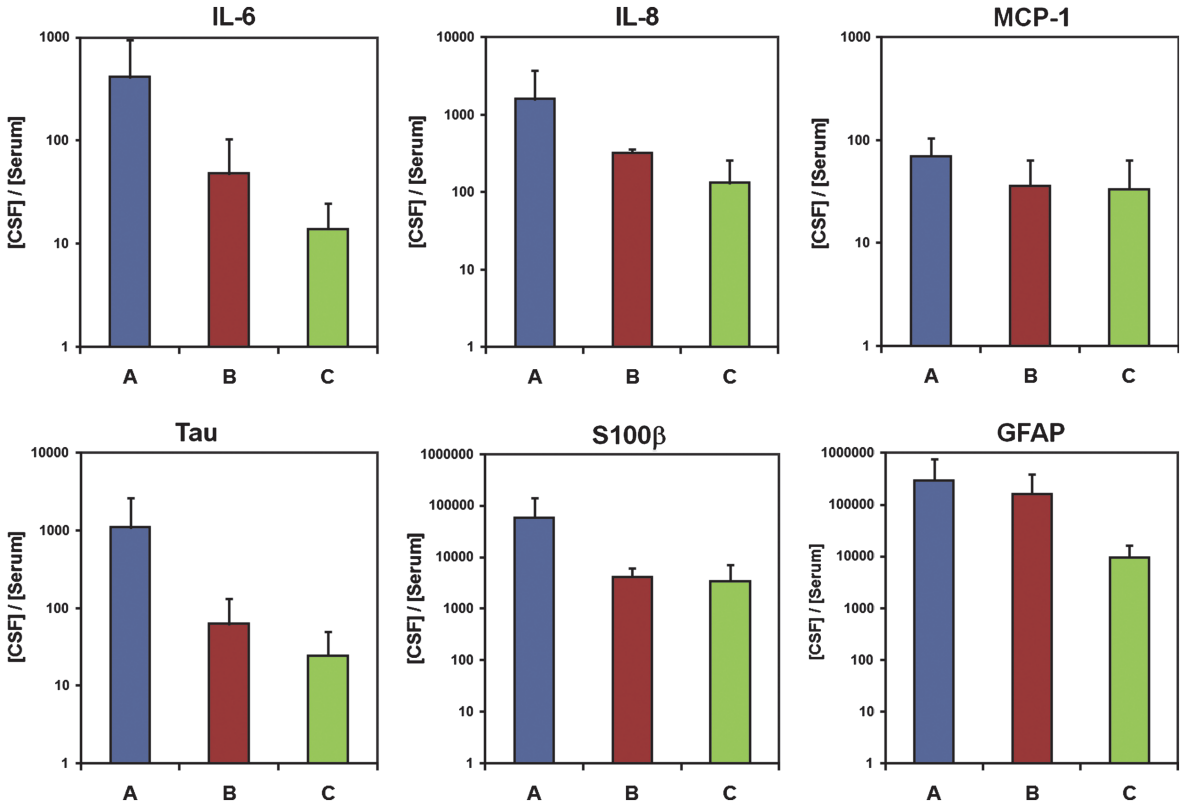

FIG. 3. Cerebrospinal fluid (CSF) concentrations of IL-6, IL-8, MCP-1, tau, S100β, and glial fibrillary acidic protein (GFAP) far exceed serum concentrations. Blood samples were drawn at the same time as CSF samples to compare CSF and serum concentrations. Here, the ratio between CSF and serum concentrations of IL-6, IL-8, MCP-1, tau, S100β, and GFAP at 24 h post injury are plotted on a logarithmic scale to illustrate that the CSF concentrations represent a CNS-specific process. Even in the least severely injured ASIA C patients, the CSF concentrations are at least 10 times that of the serum. Color image is available online at www.liebertonline.com/neu.

response as an important contributor to the genesis of neuropathic pain (Moalem and Tracey, 2006), the pathophysiology in human patients is poorly understood. Elucidating the mechanisms by which such pain is initiated is a matter of great interest, as it may lead to better treatments for an often intractable problem for these patients (Burchiel and Hsu, 2001; Siddall and Middleton, 2006). The patients reported the intensity of their pain and the interference of their pain with their function, and were asked to report specifically the extent

Table 2. Biochemical Model for Predicting ASIA Impairment Grade with Cerebrospinal Fluid (CSF) Levels of S100β, GFAP, and IL-8 at 24 h Post Injury

		Predicted ASIA grade			
		A	*B*	*C*	*Total*
Observed ASIA grade	*A*	14	0	0	14
	B	1	5	1	7
	C	0	1	5	6
	Total	15	6	6	27

GFAP, glial fibrillary acidic protein.

The biochemical model that utilizes the patient's CSF levels of S100β, GFAP, and IL-8 at 24 h post injury correctly classifies the patient's baseline ASIA impairment grade with an accuracy of 89% [$(14+5+5)/27 = 88.9\%$]. The prediction model has a c-index of 0.987.

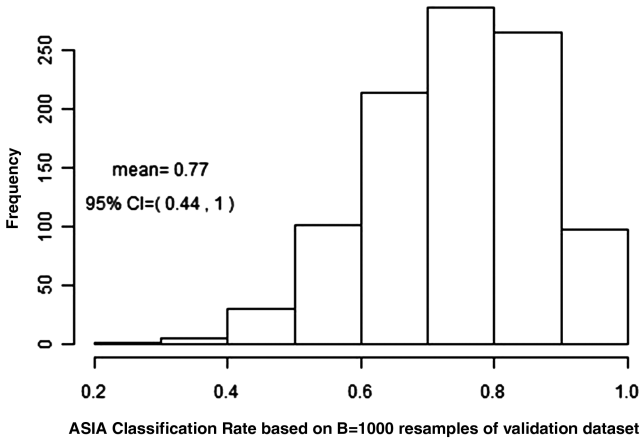

FIG. 4. Validation of biochemical model for predicting baseline ASIA grade. To assess how well the biochemical model based on the first 18 "derivation" patients would perform on an independent sample of nine "validation" patients, 1000 bootstrap samples of a nine-patient validation data set were generated. The accuracy rate for predicting injury severity in these 1000 samples was 77%, with a 95% CI of 0.44 to 1.00.

Table 3. Prediction of Upper-Extremity Motor Recovery with Either the 24-h post-injury Cerebrospinal Fluid Concentrations of S100β, GFAP, and IL-8, or the Initial ASIA Grade of the Patient

	Predicted upper-extremity motor recovery at 6 months post injury(ASIA motor points)							
	Biomarker model (utilizing S100β, GFAP, IL-8)[a]				*Functional model (utilizing patients' observed ASIA grade)*[b]			
Observed upper-extremity motor recovery (ASIA motor points)	*<5 points*	*5–10 points*	*>10 points*	*Total*	*<5 points*	*5–10 points*	*<10 points*	*Total*
<5 points	7	0	1	8	7	0	1	8
5–10 points	2	0	2	4	1	0	3	4
>10 points	1	0	7	8	2	0	6	8
Total	10	0	10	20	10	0	10	20

[a]Accuracy rate: 14/20 = 70%; c-index = 0.867. [b]Accuracy rate: 13/20 = 65%; c-index = 0.773. GFAP, glial fibrillary acidic protein.

Segmental motor recovery in the upper extremity at 6 months post injury (as measured by ASIA motor points) was predicted with either the concentration of S100β, GFAP, and IL-8 in the 24-h post-injury CSF samples or the initial ASIA grade. The biochemical model was comparable to the initial ASIA grade at predicting segmental motor recovery, with a slightly higher c-index.

of such characteristic neuropathic symptoms as "stabbing/shooting/electric sensations, and "tingling/numbness/pins andneedles" sensations. We were particularly interested in patients with cervical SCI, in which "at level" neuropathic pain extends down into their upper extremities. None of the CSF inflammatory cytokines or structural markers that demonstrated severity-dependent patterns of expression (i.e., IL-6, IL-8, MCP-1, tau, S100β, or GFAP) were found to be positively correlated with neuropathic pain sensations at baseline testing (25.0 ± 4.6 days post injury). However, a strong positive association was observed between TNF-R1 levels in the CSF at the 24-h post-injury time point and all descriptors of neuropathic pain (Table 4). At a later follow up (211.8 ± 34.6 days post injury), there were no signficant associations between any of the CSF proteins and the patients' self-reported symptoms of neuropathic pain.

Cellular analysis of cerebrospinal fluid

Hemocytometer counts were performed on daily samples of CSF, providing a unique description of the temporal change in both erythrocytes and leukocytes within the CSF after human SCI (Fig. 5). In the first 24 to 48 h post injury, there were significant numbers of erythrocytes present in the CSF, particularly in the ASIA A and to a lesser extent the ASIA B patients, indicating that the most severe injuries are associated with the greatest extent of bleeding within the intrathecal space (Fig. 5A). Large numbers of leukocytes were also present, also most notably in the ASIA A patients (Fig. 5B). A strong relationship between CSF red and white blood cells indicates that, for the most part, the presence of leukocytes within the CSF is the result of bleeding. A random-effect model analysis was conducted to discern the influence of injury severity on the leukocyte response. This analysis revealed that while the leukocyte count is strongly influenced by the erythrocyte count ($F = 37.76$, $p < 0.0001$), injury severity, as represented by the ASIA grade of the patient, also has a statistically significant effect ($F = 3.45$, $p = 0.048$). This suggests that the leukocyte response is indeed quantitatively influenced by the severity of the injury. The percentage of neutrophils, lymphocytes, and monocytes was consistent between the groups, suggesting that the leukocyte response in these three injury severities is qualitatively similar. As described by Weaver and colleagues, the neutrophils are the predominant leukocyte in the early inflammatory response to human SCI (Fleming et al., 2006).

Discussion

The challenges facing the scientific community seeking to improve the neurological function of sufferers of SCI are twofold: developing effective treatments in the research laboratory, and then validating them in clinical trials. History has revealed that neither is trivial. That treatments showing great promise in the laboratory have universally gone on to fail in clinical SCI trials (Tator, 2006) raises the possibility that the biological processes successfully targeted in animal SCI models are sufficiently different from that which is occurring in the human condition, a consideration being similarly voiced by stroke researchers, who have suffered far greater disappointment in the clinical translation of promising therapies (O'Collins et al., 2006). This challenge is difficult to address, given the paucity of data on the biology and pathophysiology of human SCI. Additionally, the outcome instruments that clinicians depend upon to evaluate and then validate novel therapies in human SCI trials are gross and imprecise measures of cord physiology, requiring the enrolment of hundreds of patients to achieve statistical validity (Fawcett et al., 2007; Lammertse et al., 2007). While this practical clinical consideration may seem trivial compared to the ethereal mechanistic intricacies of cord biology, the fact that the clinical evaluation of GM-1 ganglioside (Sygen) for acute SCI took almost a decade and a half to complete (and the combined effort of 28 neurotrauma institutions) (Geisler et al., 1991; Geisler et al., 2001a, 2001b) infers that many decades may be necessary to complete the evaluation of only those therapies that are currently in early clinical trials or are about to begin, let alone the many promising treatments that are in the scientific pipeline. Approaches to reduce this inevitable clinical bottleneck on the growing stream of emerging therapies are sorely needed.

Our study attempts to address both of these challenges. Our study is the first description of the temporal pattern of inflammatory cytokines and structural proteins such as tau, S100β, and GFAP released from the spinal cord in a series of living SCI patients, and thus differs in scope from the detailed

Table 4. TNF-R1 Levels in the CSF at 24 h Post Injury Correlate with Neuropathic Pain Symptoms, Pain Intensity, Intolerability, and Pain Interference

		Stabbing, shooting electrical sensations		*Tingling, numbness, pins & needles sensations*		*"Other" (sensitive, achy, sharp) sensations*		*Pain intolerability*		*Pain intensity*		*Pain interference*	
		PCC	*Prob*	*PCC*	*Prob*	*PCC*	*Prob*	*PCC*	*Prob*	*PCC*	*Prob*	*PCC*	*Prob*
Il-6	Baseline	0.260	0.314	0.058	0.826	0.211	0.416	−0.076	0.780	0.211	0.417	0.192	0.461
	Follow-up	−0.156	0.478	0.137	0.532	−0.153	0.487	−0.286	0.186	−0.190	0.385	−0.168	0.443
IL-8	Baseline	0.119	0.648	0.032	0.904	0.064	0.807	−0.108	0.690	0.063	0.812	0.285	0.267
	Follow-up	−0.135	0.540	0.192	0.380	−0.171	0.436	−0.096	0.662	−0.012	0.958	0.013	0.953
IP-10	Baseline	0.079	0.764	0.412	0.101	0.279	0.278	−0.035	0.896	0.075	0.774	0.184	0.479
	Follow-up	−0.023	0.917	0.163	0.457	−0.029	0.897	0.047	0.831	−0.053	0.811	0.201	0.357
MCP-1	Baseline	0.138	0.596	0.116	0.657	0.142	0.586	−0.045	0.868	0.004	0.988	0.282	0.272
	Follow-up	0.049	0.824	0.033	0.882	−0.095	0.667	0.304	0.158	0.296	0.170	0.380	0.073
TNF-R1	Baseline	0.583	**0.029**	0.565	**0.035**	0.793	**0.001**	0.594	**0.032**	0.565	**0.035**	0.688	**0.007**
	Follow-up	−0.211	0.385	0.181	0.458	−0.184	0.452	−0.282	0.241	−0.209	0.390	−0.104	0.671
IL-16	Baseline	0.089	0.762	0.135	0.645	0.340	0.234	0.638	0.019	0.524	0.055	0.396	0.161
	Follow-up	−0.003	0.991	0.203	0.405	−0.053	0.828	−0.198	0.417	−0.166	0.497	−0.027	0.914
Tau	Baseline	−0.037	0.888	−0.354	0.163	−0.239	0.355	−0.291	0.274	−0.355	0.163	−0.269	0.297
	Follow-up	0.143	0.515	−0.102	0.645	−0.046	0.835	−0.288	0.182	−0.104	0.638	−0.277	0.201
S100β	Baseline	−0.092	0.725	−0.203	0.434	−0.165	0.527	−0.194	0.472	−0.123	0.638	0.049	0.852
	Follow-up	−0.185	0.409	0.039	0.863	−0.232	0.299	−0.214	0.338	−0.172	0.445	−0.201	0.370
GFAP	Baseline	−0.200	0.442	−0.328	0.199	−0.207	0.426	−0.192	0.475	−0.112	0.669	0.051	0.847
	Follow-up	−0.012	0.958	−0.086	0.703	−0.224	0.316	−0.026	0.909	−0.023	0.918	−0.110	0.625

PCC, Pearson correlation coefficient; Prob, p value for the null hypothesis of "no correlation."

Pearson correlation coefficients were calculated between the CSF concentrations of all cytokines/proteins that were measurable in the 24-h post-injury sample, and the patients' self-reported pain symptoms at baseline (25.0 ± 4.6 days post injury) and follow-up (211.8 ± 34.6 days post injury). At baseline, only TNF-R1 levels were consistently associated with neuropathic pain symptoms (in bold).

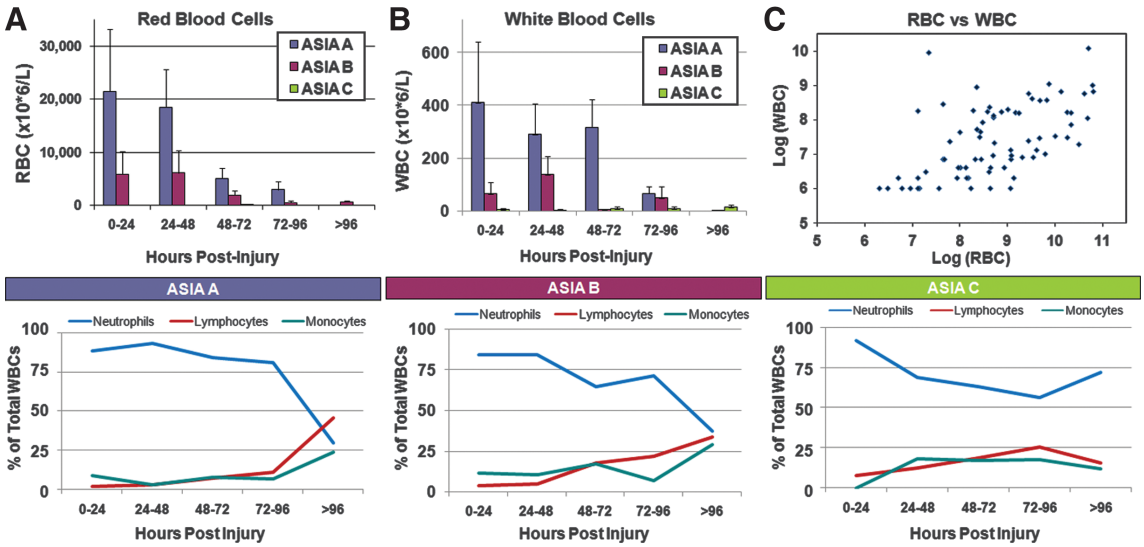

FIG. 5. Cellular analysis of cerebrospinal fluid (CSF) after acute spinal cord injury. Hemocytometry counts revealed an injury-severity-dependent increase in both red and white blood cells, particularly early post injury (**A** and **B**). A strong correlation was noted between the RBC and WBC counts (**C**), reflective of bleeding within the intrathecal space. Despite this, ASIA injury severity also had a significant influence on WBC count. The relative proportion of neutrophils, lymphocytes, and monocytes were comparable across all three injury severities. Note that the neutrophils are the predominant early leukocyte, with lymphoctyes and monocytes emerging after 48 h. Color image is available online at www.liebertonline.com/neu.

post-mortem description of the cellular response to SCI reported by Weaver and colleagues (Fleming et al., 2006). Our results suggest that IL-6, IL-8, MCP-1, IP-10, IL-16, and TNF-R1 are released in quantities that are measurable with multiplex bead or standard ELISA technology. While we did not detect the majority of the cytokines and all of the growth factors included in our multiplex kit, this is most likely to be a measurement phenomenon related to technological limitations, the dilutional effect of the CSF, and possibly the breakdown of some cytokines while in the intrathecal space. We were nonetheless surprised that IL-1β and TNFα —— two cytokines with important roles in the early inflammatory response – were not detected despite detection limits of approximately 1.5 pg/mL and 0.25 pg/mL respectively. These analyses for IL-1β and TNFα were repeated using the MSD Multi-Array® microplate platform (Meso Scale Discovery), but, again, we were unable to measure either cytokine (unpublished results).

The finding of elevated CSF levels of IL-6, IL-8, and MCP-1, IP-10, IL-16, and TNF-R1 are notable given that each has been reported in animal SCI models to be involved in the complex pathophysiological cascade of acute secondary damage, and therapeutic strategies may be developed to target their activity (Ghirnikar et al., 2000, 2001). IL-6 mRNA expression, for example, is significantly increased within hours of injury in both rodent (Nakamura et al., 2003) and human SCI (Yang et al., 2004), and antibody-blockade of its receptor IL-6R has been shown to reduce glial scarring, neutrophil and monocyte/macrophage invasion, and improve functional recovery after SCI (Nakamura et al., 2005; Okada et al., 2004). Kerr and colleagues reported that IL-6 was dramatically elevated in the CSF of patients during the acute onset of idiopathic transverse myelitis, and that IL-6 infused intrathecally in a rodent model was sufficient to induce axonal degeneration and demyelination (Kaplin et al., 2005). Interestingly, while these authors described their patients' CSF IL-6 levels to be "among the highest reported in any human disease (up to 4209 pg/mL)," we observed CSF levels in our ASIA A patients that exceeded 114,000 pg/mL, with an average peak IL-6 level of 29,378 pg/mL in this most severely injured subset of patients. These exceedingly high levels, and the important role that IL-6 appears to have in the mediating secondary damage in animal models of SCI, are of therapeutic interest, given that a humanized IL-6 receptor antibody is currently available and already engaged in clinical evaluation for rheumatoid arthritis (Nishimoto et al., 2009). Such an approach using function-blocking antibodies to MCP-1, IP-10, and CINC-1 (rat analogue of IL-8) has also been reported to have therapeutic benefit in models of acute SCI and cerebral ischemia-reperfusion injury (Glaser et al., 2004, 2006; Gonzalez et al., 2007; Ousman and David, 2001; Yamasaki et al., 1997). From a clinical perspective, characterizing the temporal pattern of expression in human SCI of these particular inflammatory cytokines, which have previously been evaluated in animal models, has a number of translational implications: establishment that these individual cytokines are in fact measurable in human SCI using relatively non-invasive techniques; confirmation of the potential human clinical relevance of a therapeutic approach utilizing specific antagonists to that which we have characterized, particularly given the availability of specific antibodies against these cytokines, and the demonstration of the efficacy of a treatment approach involving antibody administration – either intrathecally as is currently being done for the anti-Nogo antibody (Freund et al., 2006), or intravenously as have been investigated for the anti-CD11d antibody (Ditor et al., 2006); establishment of the time window during which these cytokines appear to be most elevated (for planning inclusion criteria around how late one would enroll patients for a clinical trial involving a specific cytokine antagonist); establishment that the severity-

dependent nature of expression for some cytokines and not others, which would influence the inclusion criteria of what ASIA categories of patients to include in such a clinical trial of a specific antagonist.

The finding of elevated TNF-R1 levels within the CSF and its association with neuropathic pain is intriguing, in part because of the strong associations across the board in all specific descriptors of neuropathic pain, the lack of association between neuropathic pain and any other measurable cytokine, and the severity-independent pattern of both TNF-R1 expression and the suffering of neuropathic pain symptoms. Mounting evidence exists that links TNFα to the genesis of neuropathic pain behavior in animal models of SCI (Detloff et al., 2008; Peng et al., 2006), and the clinically available TNFα blocker etanercept (Enbrel®; Immunex, Thousand Oaks, CA) has recently been shown to reduce mechanical allodynia in an animal model of SCI (Marchand et al., 2009). TNFα is recognized as an important factor in the development of neuropathic pain after peripheral nerve injury, and this has been attributable to signaling through TNF-R1 but not TNF-R2 (Sommer, 1999). The local expression of TNF-R1 increases rapidly after contusion SCI in animal models (Harrington et al., 2005; P. Yan et al., 2003), and our observed correlation between neuropathic pain and the CSF levels of TNF-R1 suggests that such an increase may also be an important phenomenon with respect to the development of these pain symptoms in SCI patients.

Outside of providing a description of the inflammatory response to human SCI, we studied other non-inflammatory neural markers such as tau, S100β, and GFAP because of their potential use as biomarkers of injury severity. Interest in these markers within CSF has been particularly intense in traumatic brain injury (TBI) and during thoracolumbar aortic aneurysm surgery, where intrathecal drains are frequently inserted and thus provide access to CSF samples. Increased levels of tau, a microtubule associated protein, have been measured in the CSF of TBI patients, with a worsened long-term outcome correlating with higher tau levels (Ost et al., 2006). S100β is a calcium-binding protein that has been found to be elevated in both serum and CSF after TBI, although its release from adipose tissue may limit its utility as a serum biomarker of injury (Chatfield et al., 2002). GFAP released from injured glial cells and the light subunit of neurofilament protein (NFL) released from axons after trauma were evaluated by Guez and associates (2003) in a small series of six SCI patients who underwent lumbar punctures to obtain single samples of CSF. The authors did report that increased levels of GFAP and NFL correlated with severity of paralysis, but this was based on only three patients with CSF samples obtained acutely (the rest were obtained at 3 weeks post injury).

The biomarker model that we established is based upon the simple concept that cord damage and the spilling of neural tissue proteins such as tau, S100β, and GFAP would be proportional to the severity of parenchymal damage and subsequent neurological impairment (as reviewed recently by Pouw et al., 2009). In our study of 27 patients, the addition of some inflammatory cytokines such as IL-8 to the model reflects how the local inflammatory response and cellular invasion may in some ways be "titrated" to the extent of cord damage, a phenomenon that has been demonstrated in contusive models of SCI (Yang et al., 2005), and we too confirmed it here with the three cytokines IL-6, IL-8, and MCP-1. While the model includes a combination of inflammatory cytokines (e.g., IL-8) and structural proteins (e.g., GFAP), it is unclear at this point whether either inflammatory cytokines alone or structural proteins alone would be the better instrument to assess the "biological effect" of a particular treatment for human SCI. A particular treatment may have anti-inflammatory effects after human SCI, but whether this is best detected by measuring levels of IL-6, IL-8, and MCP-1, or by measuring downstream changes in terms of neuronal and/or astrocytic responses with tau, S100β, or GFAP, is unknown. There is no doubt that the accessibility of blood would make serum biomarkers far easier to use as a clinical tool, and future efforts to identify such markers are still warranted. We nevertheless observed that the serum concentrations of our proteins of interest were far below that measured in the CSF (Fig. 3), confirming the somewhat intuitive fact that the CSF is more specifically representative of what is biologically occurring within the spinal cord. Our experience mirrors that of Ost and colleagues (2006), who reported that CSF levels of tau correlated strongly with 1-year outcomes after traumatic brain injury, but that serum tau levels were not even detectable.

It is worth noting that because the proposed biomarker panel utilizes the 24-h post-injury CSF concentrations to classify ASIA grade, and then compares this to the observed ASIA grade, the "performance" of the biomarkers will never exceed that of the baseline assessment of neurological impairment (which, in this case, represents the comparative "gold standard"). Our intention was to demonstrate that the biochemical data could in fact accurately predict the functional impairment, recognizing that, in many cases, obtaining a valid functional examination is either difficult or impossible. At our institution, we estimate that in more than half of acute SCI patients, a valid baseline neurological status cannot be established because of associated head injury, intoxication, or pharmacological sedation. Therefore, our biochemical model, which classifies ASIA impairment with almost 90% accuracy and a c-index of 0.98, is a promising step forward for developing a new approach for biologically stratifying acute SCI patients. Arguably of greater importance is the fact that the biomarker panel was superior at predicting the extent of segmental motor recovery than the initial functional injury severity. Here, the biomarkers profile is evaluated not for its ability to classify an already established outcome (the baseline injury severity), but rather for its ability to predict an outcome 6 months down the road. While the abilities of the biomarker model and the standard ASIA classification at predicting segmental motor recovery 6 months post injury were not dramatically different, the fact that the biomarker model was even slightly better is encouraging for the future development of such a system whereby biological measures are used to predict outcome better, rather than traditional gross functional measures.

There is indeed biological variability in the CSF protein concentrations, as shown in Figures 1 and 2, and this contributes to the less than 100% accuracy of the model. As illustrated in Figure 2 with the 24-h post-injury CSF concentrations, there is a large difference between ASIA A (completely paralyzed) patients and the ASIA B and C (incompletely paralyzed) patients, but less of a distinction between the ASIA B and the ASIA C patients. Consistent with this, the biochemical model was best at identifying the ASIA A patients, but less successful at distinguishing ASIA B and C

patients. All 14 ASIA A patients were correctly predicted by their S100b, GFAP, and IL-8 levels to be ASIA A, but there were mistakes in distinguishing the ASIA B and C patients. Patient 1 was an ASIA C who was predicted to be an ASIA B; patient 2 was an ASIA B predicted to be an ASIA A; and patient 25 was an ASIA B predicted to be an ASIA C. Further work will be necessary to determine if better distinction of ASIA B and C patients can be achieved.

An additional limitation in our study is that the ability of the biomarker model to classify injury severity was evaluated on the cohort of patients from which the data was accrued. Therefore, while it is fairly evident that there are differences between the ASIA A, B, and C patients in CSF cytokine/protein concentrations at 24 h post injury (as shown in Fig. 2), the biomarker model's performance at actually distinguishing between these injury severities is naturally optimal when tested against the data upon which it was derived. This is demonstrated to some extent by dividing the 27-patient cohort into the initial 18-patient "derivation" data set and the final nine-patient "validation" data set, where the accuracy at predicting outcome was 78% (seven out of nine patients). This accuracy could easily have been 89% with one more correct classification, or 66% with one more mistaken classification, although the distribution of 1000 accuracy rates shown in Figure 4 reveals that the majority were in the 70–100% range. In a sense, it is fortuitous for the observed accuracy to be 78%, as it illustrates the important point that the model works best on data that was used to derive it, and we should anticipate that it might not work as well on an "independent" sample. Clearly, the validity of the biomarker model needs to be confirmed using a larger, independent sample of SCI patients. We have continued to recruit SCI patients locally for this, and have recently launched a similar multi-center initiative across Canada to assist in patient accrual.

In conclusion, our study provides a unique description of the changes that occur in the concentration of a number of inflammatory cytokines during the early post-injury phase of acute SCI. A number of these were expressed in an injury-severity-dependent manner, and when combined with other neural markers (tau, S100β, and GFAP), a biochemical model was established to classify injury severity. The CSF concentrations of these cytokines were able to classify the injury severity of the patients accurately, and importantly, could be used to predict motor outcome better at 6 months than the standard functional classification. As indicated earlier, our field is in dire need of new approaches for classifying injury severity and improved methods of predicting outcome if we are to break the gridlock that is imminent with the emergence of many new SCI therapies that are seeking validation in clinical trials.

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Author Disclosure Statement

No competing financial interests exist.

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