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INVITED REVIEW

The translational importance of establishing biomarkers of human spinal cord injury

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Abstract
The evaluation of such novel therapies for acute spinal cord injury in clinical trials is extremely challenging. Our current dependence upon the clinical assessment of neurologic impairment renders many acute SCI patients ineligible for trials because they are not examinable. Furthermore, the difficulty in predicting neurologic recovery based on the early clinical assessment forces investigators to recruit large cohorts to have sufficient power. Biomarkers that objectively classify injury severity and better predict neurologic outcome would be valuable tools for translational research. As such, the objective of the present review was to describe some of the translational challenges in acute spinal cord injury research and examine the potential utility of neurochemical biomarkers found within cerebrospinal fluid and blood. We focus on published efforts to establish biological markers for accurately classifying injury severity and precisely predict neurological outcome.

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Introduction
Acute traumatic spinal cord injury (SCI) is one of the most physically and psychologically devastating of injuries; it affects tens of thousands people of all ages around the world with incalculable personal and massive societal costs. Improvements in medical, surgical, and rehabilitative care have extended the lifespan and increased the quality of life for those who sustain a SCI. However, treatments to enhance neurologic function after acute SCI are limited. Despite many novel therapeutic interventions showing great promise in the laboratory using animal models of SCI, translating these into clinical treatments with convincing efficacy in human SCI patients has been challenging. The SCI community has undertaken a handful of large-scale clinical trials to test the efficacy of methylprednisolone and GM-1 ganglioside in acute SCI patients. These few large-scale trials have each taken many years to complete despite having multiple recruiting centers. The last decade has witnessed the initiation of a number of other smaller clinical trials to evaluate novel approaches to acute SCI; many have stopped, and none have yet been brought to completion. Both the historical and current experiences with such trials has provided many insights into the formidable challenges of conducting such human testing to evaluate the efficacy of promising approaches from the laboratory. Here, we describe some of the translational challenges in acute SCI research and outline the potential utility of neurochemical biomarkers in facilitating this difficult process.

Non-penetrating traumatic injury to the spinal cord (*e.g.*, resulting from motor vehicle accidents or falls) damages the delicate parenchymal microstructure and disrupts signal transmission that is manifested by motor, sensory, and autonomic dysfunction. While non-penetrating trauma typically does not result in complete physical transection of the spinal cord, the extent of neurologic impairment in the anatomically continuous spinal cord can be deemed "complete", with total lack of motor and sensory function below the level of the injury. Less severe injuries may result in an "incomplete" neurologic deficit with some sparing of motor and/or sensory function below the level of the injury.

Clinical Assessment of Neurologic Impairment
This concept of injury severity and the "completeness" or "incompleteness" of the neurologic impairment is critically important to the translation of acute SCI therapies into clinical trials. Currently, the most important factor that predicts neurologic outcome is how severe the neurologic injury is in the first place, making this the key factor upon which patients are enrolled and stratified within clinical trials. Assessing the severity of neurologic impairment is done with the International Standards for Neurological Classification of SCI (ISNCSCI) examination, which subjectively measures the extent of motor function in 10 myotomes and sensory function in 26 dermatomes. From this detailed assessment, the severity of neurologic injury is graded on the American Spinal Injury Association (ASIA) Impairment Scale (AIS), as either "complete" (AIS A grade), or varying

degrees of "incomplete" (AIS B, C, and D grades). This AIS grade is typically used to stratify acute SCI patients within a clinical trial, and therefore must be determined early post-injury.

Translational Implications of the Clinical Assessment of Neurologic Impairment

The reliance upon the INSCSCI examination and the necessity of establishing a baseline AIS grade have significant implications for translation in acute SCI. The INSCSCI examination is a detailed, functional examination, which relies on the conscious participation of the acutely injured patient. Therefore, it is impossible to perform and thereby impossible to establish baseline injury severity - in those with concomitant head injury, multi-system trauma, drug intoxication, or pharmacologic sedation. Such individuals are rendered ineligible for recruitment (Lee et al., 2013), thus severely limiting the pool of eligible patients for clinical trials and slowing recruitment considerably.

Limiting the pool of eligible patients for clinical trials would not be so problematic if neurologic recovery were highly predictable in those who can be examined reliably and a baseline AIS grade assigned. Unfortunately, the AIS grades predict the neurological recovery of patients with the same baseline ASI A grade with considerable variability, thus necessitating large numbers of patients to be enrolled in order to distinguish treatment effect from sheer spontaneous neurologic recovery (Fawcett et al., 2007). This forces investigators to spend years enrolling enough patients in order to achieve sufficient power to assess treatment efficacy. Even in early-stage, smaller-scale clinical trials to assess feasibility and/or safety of a treatment, variability in neurologic recovery may make it impossible to discern a treatment's efficacy. A decision to proceed to a large-scale definitive phase 3 study must then be made without important information about whether the treatment had the desired biological or functional effect.

The translational limitations associated with being unable to 1. even conduct a reliable ISNCSCI examination in many patients and 2. accurately predict neurologic recovery in those who are examinable bring to the forefront the notion that an objective, quantifiable measurement that better predicted outcome would be extremely helpful for testing novel therapies in acute SCI. It is here that interest in biomarkers of SCI has emerged. "Biomarkers" or "biological markers" are defined as objectively measured indicators of normal or pathological processes or pharmacological responses to a therapeutic intervention. In this regard, biomarkers of SCI that could be measured in all patients to establish injury severity (regardless of their level of consciousness) would increase the number of eligible subjects that could be enrolled in a clinical trial. Biomarkers of SCI that could more accurately predict neurologic recovery would reduce the number of subjects required to enroll in order to have sufficient statistical power. Finally, biomarkers of SCI that were responsive to treatment could be used as a surrogate outcome measure to evaluate a treatment's biological/physiological effect, thus informing decisions about pursuing and planning definitive phase 3 trials. Taking these into consideration, biomarkers of SCI have the potential to greatly facilitate the translation of novel therapies for acute SCI.

Biomarkers of Spinal Cord Injury

We and others have been interested in the establishment of neurochemical biomarkers of SCI, utilizing either blood or cerebrospinal fluid (CSF). CSF is intuitively more representative of the parenchymal injury given its proximity to the spinal cord, and it has been utilized in the investigation of biomarkers for other neurologic conditions, including traumatic brain injury, stroke, and other neurodegenerative disorders. Due largely to the fact that CSF is not routinely collected in the clinical management of SCI (unlike in TBI, where CSF is frequently accessed and drained with intraventricular catheters), work in the area of biomarker discovery within CSF has been relatively limited. Yokobori et al. (2015), for example, reported on 7 acute SCI patients whose CSF was evaluated to measure ubiquitin B-terminal hydrolase-L1 (UCH-L1), spectrin breakdown products (SBDP), myelin basic protein (MBP), and glial fibrillary acidic protein (GFAP) (Yokobori et al., 2015). They found transient elevations of all four proteins within the CSF, and identified a correlation between GFAP levels and SCI injury severity and improvement. More recent work from this same group has taken a broader proteomics approach to biomarkers discovery in both rodent and human CSF samples, and have identified additional protein candidates that are elevated in moderate-severe injuries (Moghieb et al., 2016). Moreover, Singh et al. (2016) investigated the concentration of nitric oxide (NO) within the CSF of 40 acute SCI patients, 15 of whom had serial CSF samples at 1, 2, and 4 weeks post-injury. The authors described an elevated CSF concentration of NO within Frankel A and B patients (those with motor complete paraplegia/quadriplegia) as compared to those with Frankel C and D patients (those with some spared motor function), at 2 weeks post-injury. While the time points for CSF collection were not standardized per se, the demonstration that NO increases over time in severely injured patients is consistent with the concept of utilizing biomarkers to define injury severity and predict outcome.

Our interest in biomarker discovery began in 2007 when we initiated a clinical trial of CSF drainage after acute SCI (ClinicalTrials.gov: NCT01279811). The clinical trial included patients with cervical or thoracic AIS A, B, and C severities of injury (A representing motor and sensory complete paralysis, B being motor complete, sensory incomplete paralysis, and C having modest motor and sensory sparing). Intrathecal catheters were installed at the time of surgery and were used to obtain CSF samples over 3 days (Kwon et al., 2009). While the CSF drainage was intended to improve spinal cord perfusion, the CSF was collected for biochem-

ical evaluation. Using ELISA and Luminex bead assays, we quantified different cytokines, growth factors, and structural proteins in serially collected CSF samples. The majority of cytokines and growth factors were not detectable. However, we were able to measure and characterize the temporal pattern expression of a series of inflammatory cytokines (*e.g.*, interleukin (IL)-6, IL-8, and monocyte chemotactic protein (MCP)-1) and structural proteins (tau, S100β, and glial fibrillary acidic protein) which typically peaked around 24–36 hours post-injury and then decreased to nearly normal levels by 72 hours post-injury (Kwon et al., 2010). When evaluating the CSF concentrations at 24 hours post-injury, we found that these proteins were distinct between AIS grades, and could be used in an ordinal logistic regression model to classify AIS grade with an accuracy of 89%. They could also be used in cervical SCI patients to predict segmental motor recovery with better accuracy than utilizing the baseline AIS grade. We also reported that TNF-R1 levels were closely correlated to the patients' reporting of neuropathic pain.

These were encouraging findings from what, at the time, represented the largest series of CSF samples from acute human SCI patients. We continued to enroll acute SCI patients into a prospective observational study which subsequently became a multi-center initiative. In 2016, we reported on the first 50 acute SCI patients recruited to our site: a collection of 32 cervical SCI and 18 thoracic SCI (Kwon et al., 2016). Here, we focused on the 24 hour post-injury timepoint and the extent to which the CSF biomarkers (IL-6, IL-8, MCP-1, tau, S100β, and GFAP) could classify injury severity and predict neurologic recovery in cervical *versus* thoracic SCI. With the larger number of patients, we sought to determine if the biomarkers could predict neurologic recovery with regards to improvement in AIS grade and total motor score.

We found that IL-6 and GFAP were the most distinct between the three injury severities (AIS A, B, and C) in both cervical and thoracic SCI. In cervical SCI, tau and S100β were also significantly different between injury severities. A prediction model was generated using discriminant function analysis and had an 84% accuracy at classifying correct AIS grade across all 50 patients. With regards to predicting neurologic recovery, we found that the CSF biomarkers could be incorporated into a model that had an 83.3% accuracy at predicting AIS improvement over 6 months. Importantly, the concentrations of IL-6, MCP-1, S100β, and GFAP were significantly different amongst AIS A patients who did or did not improve over time, suggesting that the biology (as represented by the CSF biomarkers) could help to discern the heterogeneity within this single AIS grade of injury. Finally, all the biomarkers were significantly correlated with motor score improvement over time – a phenomenon that was particularly strong in the cervical SCI patients. With regards to neurologic improvement, the CSF markers were particularly good at predicting who would not spontaneously improve an AIS grade or who would not achieve motor score recovery. This ability to identify those who are unlikely to recovery function would be especially useful in early stage clinical trials during which discerning treatment effect from spontaneous recovery is challenging.

Our biomarker studies have been limited to a specific set of proteins within CSF (Kwon et al., 2010, 2016). While CSF may be the most representative of the injured spinal cord, clearly, the ability to define biomarkers within blood would have huge practical advantages. In our first study (Kwon et al., 2010), we reported that the biomarker concentrations within CSF at 24 hours post-injury were in some cases many orders of magnitude higher than their concentrations within blood. A broader analysis of blood samples is ongoing to determine if the markers we have identified (IL-6, IL-8, MCP-1, tau, S100β, and GFAP) may still have some utility as biomarkers of SCI even if their concentrations are far lower than in the CSF. Others have evaluated blood samples from acute SCI patients and have identified potential biomarkers. For example, Kuhle et al. (2015) recently reported in 27 acute SCI patients that serum neurofilament light chain (NFL) concentrations were closely correlated with injury severity and that the NFL levels were decreased in patients treated with minocycline (Kuhle et al., 2015). This study is important because it not only reveals the potential utility of biomarkers for distinguishing injury severity, but also as surrogate measures of outcome. Ahadi et al. (2015) reported in 35 acute SCI patients that GFAP, phosphorylated neurofilament heavy chain (pNF-H), and NSE were elevated in the serum, and that GFAP levels were correlated with injury severity.

Future Considerations

These findings bring to light a few issues. Firstly, the list of candidate biomarkers that may be useful for classifying injury severity and predicting neurologic outcome is potentially huge and may be defined largely by what is actually empirically studied in human SCI. Our focus has been on the CSF concentrations of a small set of proteins, but we are also actively engaged in broader studies of the proteomic, metabolomic, lipidomic and genomic responses to injury with the notion that there may be other biomolecules that can serve as useful biomarkers in acute SCI. Recently, for example, we reported on our metabolomic evaluation of blood and CSF samples from acute human SCI patients, in which we could discern distinct responses in both fluid samples for the AIS A, B, and C patients (Wu et al., 2016).

Another issue is that there is expectantly a time course for these biological events and so characterizing their temporal pattern of expression is useful for providing the scientific community with a description of these complex biological responses to traumatic injury. By characterizing what features are measurable and how they change over time, we may define biomarkers that are targets of therapies and could be used as biological surrogate outcome measures in a clinical trial of therapeutic for acute SCI.

Furthermore most of these have been described in animal models of SCI and little has been documented from empirical studies of human SCI. The lack of direct biological studies of human SCI makes it difficult to determine whether the pathological responses that are targeted in the animal setting are similarly modulated in the human SCI condition.

Alongside this, the identification of useful biological markers in human SCI could also be promoted by the parallel identification of biomarkers in animal SCI. Currently, the outcome measures used to study treatments in rodent models of SCI (*e.g.*, BBB score, white matter tissue sparing) have no measurable correlate in human SCI. Biomarkers that are shared between different animal species and humans would enable us to monitor specific biological effects in humans that are predicted by the animal experiments – thus helping in the translation of novel therapies. We are currently engaged in a proteomic, metabolomic, lipidomic and genomic evaluation of CSF and blood from our porcine model of SCI with the goal of identifying biological markers that may be relevant to both the animal and human condition after SCI. We should point out that while our studies to date have largely focused on CSF samples, we recognize that the establishment of biomarkers within blood (as has been described by Kuhle et al. (2015) and Ahadi et al. (2015)) would have considerable practical significance. Hence, we are actively studying parallel serum samples that were obtained from our acute SCI patients who also provided CSF samples. Ultimately, the establishment of biomarkers for SCI will require validation in larger scale studies, and in this regard, work in this particular area will suffer many of the same vagaries as other novel therapies that struggle to recruit sufficient numbers of patients. However, given the reliance on functional measures to evaluate SCI patients in clinical trials of novel therapies, and the growing recognition of the obstacle that this imposes on the validation of such treatments, new approaches are clearly needed. We contend that biomarkers have the potential to facilitate the validation of novel therapies, and in this regard have a critical role in translational research in SCI.

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