Biochemical Markers of Brain Injury: Applications to Combat Casualty Care

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1.0 BACKGROUND

The Need For Biochemical Markers Of Brain Injury: Brain injury resulting from traumatic, ischemic and/or chemical etiology is a significant international health concern, representing a potentially catastrophic debilitating medical emergency with poor prognosis for long-term disability. It represents a major problem to military care, accounting for 25% of all combat casualties and is the leading cause of death (approaching 50% incidence) among our wounded soldiers reaching Echelon I medical treatment [1]**.** In civilian life, the incidence of brain injury and resultant long-term disabilities caused by traumatic insults (gunshots, automobile accidents, sports, etc.) and ischemic events (strokes, cerebral hemorrhage, cardiac arrest, etc.) are several orders of magnitude greater. There are more than 1 million TBI cases that are treated and released from an emergency department annually in the United States resulting in more than 230,000 hospitalizations, 50,000 deaths and 80,000 disabilities. The current estimation is 5.3 million Americans live with TBI-related disability. TBI is the greatest cause of death and disability in young people less than 24 years old [2].

With the exception of diuretics, supportive measures and, when appropriate, recombinant tissue plasminogen activator (tPA) [3], there are currently no approved drug treatments for traumatic or ischemic brain injury. There have been a large number of clinical trials studying potential therapies for traumatic brain injury (TBI) that have resulted in negative findings with a cost of over $200 million [4, 5]**.** Many investigators have pointed out that the absence of biochemical markers of injury could have contributed to these failures [6]. Unlike other organ-based diseases where rapid diagnosis employing biomarkers (usually involving blood tests) prove invaluable to guide treatment of the disease, no such rapid, definitive diagnostic tests exist for traumatic or ischemic brain injury to provide physicians with quantifiable neurochemical markers to help determine the seriousness of the injury, the anatomical and cellular pathology of the injury, and to guide implementation of appropriate triage and medical management.

Criteria For Biochemical/Surrogate Markers: In the course of research on biomarkers, our laboratories have developed criteria for biomarker development. As reflected in the present proposal, useful biomarkers should employ readily accessible biological material such as CSF or blood (CSF is routinely accessible in severely injured TBI patients), predict the magnitude of injury and resulting functional deficits and possess high sensitivity and specificity, have a rapid appearance in blood and be released in a time-locked sequence after injury. Ideally, biomarkers should employ biological substrates unique to the CNS and provide information on injury mechanisms, a criterion which is often used to distinguish biochemical markers from surrogate markers of injury, which usually do not provide information on injury mechanisms.

Assessments of the sensitivity of markers to specific therapeutic interventions are an effort outside of the scope of the present proposal. Potential gender- and developmentally-related differences in biomarker profiles can also be determined in later studies.

Uses Of Biomarkers: Biomarkers would have important applications in diagnosis, prognosis and clinical research of brain injuries. Triage is a major function of far-forward medical care in a combat environment or during national disasters. Simple, rapid diagnostic tools will immensely facilitate allocation of the major medical resources required to treat TBI and other brain injuries. Accurate diagnosis in acute care environments can significantly enhance decisions about patient management including decisions whether to admit or discharge or administer other time consuming and expensive tests including computer tomography (CT) and magnetic resonance imaging (MRI) scans. Biomarkers could have important prognostic functions especially in patients suffering mild TBI, which make up an estimated 80% of the 2.5 to 6.5 million individuals who suffer from lifelong impairment as a result of TBI [7, 8]. Accurate identification of these patients could facilitate development, of guidelines for return to duty, work or sports activities and also provide opportunities for counseling of patients suffering from these deficits. Biomarkers could provide major opportunities for the conduct of clinical research including confirmation of injury mechanism(s) and drug target identification. The temporal profile of changes in biomarkers could guide timing of treatment. Finally, biomarkers could provide a clinical trial outcome measure obtainable much more cheaply and readily than

conventional neurological assessments, thereby significantly reducing the risks and costs of human clinical trials.

Current Status Of Research: Analysis of specific biochemical markers is a mandatory component of diagnosing dysfunction in a number of organs such as myocardial infarction. However, there are no biomarkers of proven clinical utility for TBI and stroke.

TBI is difficult to assess and clinical examinations are of restricted value during the first hours and days after injury. Conventional diagnoses of TBI are based on neuroimaging techniques such as CT scanning, MRI and single-photon emission CT scanning [9-11]. CT scanning has low sensitivity to diffuse brain damage, and the availability of MRI is limited [12-14]. Single-photon emission CT scanning detects regional blood-flow abnormalities not necessarily related to structural damage.

A recent review of biomarkers of TBI highlighted the need for biomarker development [15]. The most studied potential biochemical markers for TBI include creatine kinase (CK), glial fibrillary acidic protein (GFAP), lactate dehydrogenase (LDH), myelin basic protein (MBP), neuron-specific enolase (NSE) and S-100 proteins. The bulk of research in TBI has focused on NSE and S-100β. The specificity of NSE for brain is high [16], sex- and age-related variability is low [17-24], and NSE is rapidly detectible in serum after TBI [25]. However, studies relating NSE serum levels to admission GCS in patients with severe TBI show conflicting results. Similar data have been reported concerning relationships with CT scan findings, ICP and long-term outcomes. In mild TBI, NSE failed to separate patients from controls [26-28]. Thus, NSE is predominantly used as a marker for tumors [29]. NSE is also released in the blood by hemolysis, which could be a major source of error [29].

S-100β has high specificity for brain [16] although it is present in other tissues such as adipocytes and chondrocytes [30]. Investigators have reported S-100β serum levels correlate to both GCS scores, neuroradiologic findings at admission and long-term outcomes [31-33]. However, investigators have recently raised questions about the utility of S-100β reporting that high serum levels of S-100β are detectible in trauma patients not having head injuries, a factor not adequately controlled for in earlier studies [34]. In addition, serum levels of S-100β following mild TBI do not show strong correlations with neuropsychological outcome [35]. Research in this area continues and recent reports have indicated the potential utility of measures of GFAP [36] and cleaved tau protein [37] in blood following TBI.

Investigators have also generally recognized the need for more objective assessments of outcome following stroke, including biochemical markers [38, 39]. The approval of tPA as a treatment for acute stroke has additionally highlighted the potential utility of biochemical markers. Use of tPA may be hindered by diagnostic concerns because neurological deficits accompanying stroke can mimic those seen during transient ischemic attacks, complex migraine, space-occupying lesions and post-ictal paralysis. A reliable biochemical marker might give assurance to physicians considering administering thrombolitic agents for acute stroke [39].

Previously reported biomarkers of cerebral ischemia include NSE, brain specific creatine kinase enzyme (CPK-BB), S-100β and inflammatory cytokines such as IL-6 [39]. NSE and S-100β have been the most studied. After cardiac arrest, NSE elevations in serum and CSF have been correlated with neurological recovery [40-42]. Serum and CSF NSE values were reported to be elevated in rodent models of focal ischemia in proportion to the eventual infarct volume [43-45]. In clinical trials, peak serum NSE values also predicted infarct volumes as shown by CT. Correlating serum NSE values with functional outcome was less successful[43, 44, 46], possibly because functional neurological deficit is influenced by as much by location of brain injury as by infarct size [46]. S-100β protein has been studied most extensively for characterization of ischemic injuries after cardiac surgery, and several reports have documented post-operative serum elevations [47-49]. However, many of these reports do not include careful studies of neurological outcome, and several investigators have recently criticized the diagnostic utility of S-100β during cardiac surgery. [34]

αII-Spectrin Degradation-A Prototype Biomarker: Our research program to develop biomarkers for TBI has focused on α-spectrin degradation as a prototypical biochemical marker [50-52]. αII-spectrin is the major structural component of the cortical membrane cytoskeleton and is particularly abundant in axons and presynaptic terminals [53, 54] Importantly, αII-spectrin is a major substrate for both and calpain and caspase-3 cysteine proteases [55]. Our laboratory has provided considerable evidence that αII-spectrin is processed by calpains and/or caspase-3 to signature cleavage products *in vivo* after TBI [56-59] and in *in vitro* models of mechanical stretch injury [60]. Immunoblots of αII-spectrin degradation thus provide concurrent information on the activation of calpain and caspase-3, potentially important regulators of cell death following TBI. The calcium sensitivity and low basal levels of calpain optimize its utility as a marker of cell injury. Although not found in erythrocytes and thus robust to confounding by blood contamination, αII-spectrin is not specific to the CNS [54]**.** We have generated considerable laboratory data on the utility of αII-spectrin degradation as a biomarker for TBI. Preliminary human data are also promising. Recent collaborative studies conducted at the University of Florida and WRAIR have also indicated that αII-spectrin degradation is a useful biomarker for ischemic injury and potentially capable of distinguishing ischemic vs. mechanical brain insults (see below).

1.1 RESULTS OF STUDIES

To date, we have conducted three significant studies examining the potential role of biomarkers following acute traumatic brain injury (TBI) and focal cerebral ischemia. The first study examined accumulation of α-II spectrin and calpain-cleaved α-II spectrin breakdown products in cerebrospinal fluid (CSF) after experimental TBI in rats produced by closed cortical impact [50]. As we previously demonstrated, cleavage of α-II spectrin by calpain and caspase-3 resulted in accumulation of protease-specific spectrin breakdown products (SBDPs) that can be used to monitor the magnitude and temporal duration of protease activation. However, accumulation of α-II spectrin and α-II SBDPs in CSF after TBI had never been examined. Following a moderate level (2.0 mm) of controlled cortical impact TBI in rodents, native α-II spectrin was decreased in brain tissue and increased in CSF from 24 h to 72 h after injury. In addition, calpain-specific SBDPs were observed to increase in both brain and CSF after injury. Increases in the calpain specific 145 kDa SBDP in CSF were 244%, 530% and 665% of sham-injured control animals at 24 h, 48 h and 72 h after TBI, respectively. The caspase-3-specific SBDP was observed to increase in CSF in some animals but to a lesser degree. Importantly, levels of these proteins were undetectable in CSF of uninjured control rats. These results indicate that detection of α-II spectrin and α-II SBDPs is a powerful discriminator of outcome and protease activation after TBI. In accord with our previous studies, results also indicated that calpain may be a more important effector of cell death after moderate TBI than caspase-3.

The second study examined the accumulation of calpain and caspase-3 proteolytic fragments of α-II spectrin in CSF after middle cerebral artery occlusion (MCAO) in rats [61]. This investigation examined accumulation of calpain- and calpase-3-cleaved α-II SBDPs in CSF of rodents subjected to 2 hrs of transient focal cerebral ischemia produced by MCAO followed by reperfusion. After MCAO injury, full-length α-II spectrin protein was decreased in brain tissue and increased in CSF from 24 to 72 hrs after injury. Whereas α-II SBDPs were detectable in sham-injured control animals, calpain but not caspase-3 specific α-II SBDPs were significantly increased in CSF after injury. However, caspase-3 α-II SBDPs were observed in CSF of some injured animals. These results indicated that α-II SBDPs detected in CSF after injury, particularly those mediated by calpain, may be useful diagnostic indicators of cerebral infarction that can provide important information about specific neurochemical events that have occurred in the brain after acute stroke.

The final study examined whether α-II SBDP levels were associated with injury magnitude and predicted lesion size [62]. Injury magnitude following closed cortical impact injury in rats significantly elevated the mean levels of both ipsilateral cortex (IC) and cerebral spinal fluid (CSF) SBDP at 2, 6, and 24 hours after two levels of lateral controlled cortical impact (1.0 mm and 1.6 mm of cortical deformation) in

rats. CSF SBDP levels were significantly higher after severe (1.6 mm) injury than mild (1.0 mm) injury. CSF SBDP levels were significantly correlated to IC levels in individual rats at 2, 6 and 24 hours after TBI. We also assessed the correlation between CSF SBDP levels and lesion size from T2-weighted magnetic resonance images (MRI) at 24 hours after TBI as well as correlation of two additional biomarkers, tau and S100β. Mean levels of CSF SBDP (r = 0.833) and tau (r= .693) significantly correlated with lesion size while levels of CSF S100β did not (r = 0.188). In a model to determine which marker or combination of markers (SBDP, tau, S100β) best predicted lesion size, CSF SBDP levels were the only significant predictor of lesion size. Furthermore, larger lesion sizes 24 hours after TBI were negatively correlated with decreased motor performance on days 1-5 after TBI (r = -0.708). Based on this data, we propose that CSF SBDP levels are a novel and promising biomarker of TBI and other acute CNS injuries.

1.1.1 DISCUSSION

Studies to date have demonstrated CSF levels of SBDP have three properties of a good biomarker: 1) association with injury magnitude, 2) reflection of pathophysiology in the brain, 3) significant contribution to prediction of outcome as measured by lesion size. Not only do these studies strongly support the utility of CSF SBDP as a biomarker of acute neuronal injury, they provide further evidence of the relationship between injury magnitude and biochemical outcome measures. This study is also the first rigorous preclinical evaluation of a biomarker of acute neurological injury. The contribution of this work is a foundation for future studies assessing the utility of this marker in human brain injury.

Injury magnitude significantly increased CSF and cortical levels of SBDP over the two control groups, sham-craniotomy and naïve rats. CSF levels of SBDP were significantly higher after severe (1.6 mm) injury than mild (1.0 mm) injury at 2, 6 and 24 hours after TBI reflecting injury magnitude.

Increased levels of calcium after TBI have been shown in several models [77, 87, 96, 97]. After TBI, calcium initiates a cytotoxic cascade of proteases including calpain which breaks down the cytoskeletal protein, spectrin. Higher levels of injury magnitude increased mRNA levels of calpain-1 and calpain-2 in the injured cortex and hippocampus (unpublished data). Similar to our study, varying injury magnitude by depth or by velocity of impact, significantly effected lesion size [79]. Injury magnitude also significantly increased peak intracranial pressure and hippocampal neuron loss in similar models of TBI [76, 79]. Temporal increases in intracellular calcium were correlated with injury magnitude after controlled cortical impact TBI in rats [77]. The corresponding increase in calcium after more severe TBI may explain the association between injury magnitude and SBDP levels in the IC and CSF. In the acute time period following TBI, CSF SBDP significantly correlated with cortical levels of SBDP and both increased with injury magnitude. Calpain-mediated SBDP have been extensively examined and shown to increase in *in vivo* and *in vitro* models of neuronal injury [70, 88, 89, 93]. Recently it has been shown that CSF SBDP increased in models of TBI [50] and ischemia [61]. The increased levels of SBDP 150/145 are primarily associated with calpain activation in our CCI model. Although caspase-3 may also cleave spectrin to SBPD 150, similar to prior work in our laboratory [91], the caspase-3 signature SBDP 120 was not significant in our CCI model, suggesting a much less relevant role of caspase-3 in the production of SBDP in this model. Calpain inhibitors have been neuroprotective in models of TBI [73, 94], ischemia [71, 84, 86], and spinal cord injury [69]. The ability of CSF levels of SBDP to reflect the pathophysiology of acute neuronal injury may provide a therapeutic target for treatment of TBI and an effective way to monitor treatment of TBI.

CSF levels of SBDP significantly contributed to prediction of lesion size after TBI. Other biomarkers have shown varying correlations with lesion size. Serum levels of creatine kinase isoenzyme BB did not correlate with CT findings in patients with mild TBI [85]. Two clinical studies of serum levels of S100β revealed a correlation with contusion volume [81, 92], while in a study of mild TBI, serum S100β levels did not correlate with MRI or CT scans [80]. S100β may be released from damaged glial cells, and this variable may not change consistently with the magnitude of injury.

Importantly in multi-trauma patients without head injuries, S100β reached high serum levels after bone fractures and thoracic contusion and also increased after burns and minor bruising [66]. Numerous studies examined the use of S100β to mark cerebral damage after cardio-pulmonary bypass surgery [65] but S100β was found to be released from the mediastinum of cardiopulmonary bypass patients [66]. After stroke, higher serum S100β levels were associated with larger infarcts and more severe neuropsychological deficits [63, 68, 74]. However Hill and colleagues [83] found only 32% of stroke patients had elevated serum S100β on admission. Early identification of stroke is necessary for optimal treatment within three hours.

CSF c-tau levels were significant predictors of outcome measures (intracranial pressure and GOS at discharge) [98] supporting the finding of a significant correlation between CSF tau and lesion size in our study. On the other hand, Franz *et al.*, 2003 [78]showed that CSF levels of total tau did not correlate with injury severity (initial GCS) nor with outcome (GOS). The wide range of tau levels in that study was thought to be due to distance of the white matter lesion from the ventricles. Lesion variability is less in a model of CCI than in a clinical study of TBI. Initial examination of serum c-tau indicated the presence of serum c-tau increased the odds of an intracranial injury and a greater chance of a poor out-come [95], however, later work indicated serum cleaved tau levels did not correlate with outcome measures [75]. After acute stroke, tau increased in the CSF [82] and serum [72], and serum tau levels correlated to lesion size and severity. Similar to S100B, however, it increased in less than 50% of stroke patients [72]. Our study did not examine serum SBDP levels but further work will be important to establish if SBDP crosses the blood-brain barrier and reflects SBDP levels in the CSF and brain.

Changes in high resolution MRI have been shown to correlate well with histology in a lateral fluid percussion model [64] and a closed head injury model [67] of TBI. Areas of hypo-intensity on MRI were associated with hemorrhage or mechanical disruption and areas of hyper-intensity were associated with edema [64]. Twenty-four hours after rats underwent sham-craniotomy, varying amounts of hyper-intensity were noted, most likely due to edema associated with the changes in cranial pressures. In the closed head injury model, areas of hyper-intensity decreased between 2 and 7 days after TBI likely representing resolution of edema [67]. Similarly in our study, the overall size of the lesion decreased between 24 hours and 28 days, although a significant correlation was maintained between lesion size in individual rats at the two time points.

We also examined *in vivo* lesion size and the correlation to neuromotor function. Higher levels of injury magnitude significantly increased lesion size and decreased motor performance. In a stroke model, lesion size from T2-weighted images at 2 and 7 days after ischemia was significantly correlated with an average of individual neurological score [90]. Similarly in our study, the larger the lesion size, the worse the performance on the motor function test. Because lesion size at 24 hours was highly correlated with lesion size at 28 days and significantly negatively correlated with motor performance, it is suggestive that acute levels of SBDP might correlate with both acute motor performance and chronic lesion size. Because withdrawal of CSF is a terminal procedure in our laboratory at this time, the correlation is only speculative.

In conclusion, the results of these studies show that injury magnitude is associated with the levels of SBDP in the IC and CSF over acute time periods after TBI. We also showed the levels of CSF SBDP correlate with IC SBDP levels supporting the idea that CSF SBDP levels reflect the pathophysiology in the cortex at that time. We further showed that 24 hours after TBI, CSF SBDP significantly correlate with lesion size. In a model to determine which marker or combination of markers best predict lesion size, we found CSF SBDP levels to be the best predictor. αII-spectrin is not found in red blood cells [50] although it is found in very low levels in other organs systems (Pike, Flint, Wang, Hayes, unpublished data). Future work could confirm that CSF levels of SBDP have diagnostic utility for prediction of outcome after TBI in humans.

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