A Novel Marker for Traumatic Brain Injury: CSF αII-Spectrin Breakdown Product Levels

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ABSTRACT

Currently, there is no definitive diagnostic test for traumatic brain injury (TBI) to help physicians determine the seriousness of injury or the extent of cellular pathology. Calpain cleaves αII-spectrin into breakdown products (SBDP) after TBI and ischemia. Mean levels of both ipsilateral cortex (IC) and cerebral spinal fluid (CSF) SBDP at 2, 6, and 24 h after two levels of controlled cortical impact (1.0 mm and 1.6 mm of cortical deformation) in rats were significantly elevated by injury. CSF and IC SBDP levels were significantly higher after severe (1.6 mm) injury than mild (1.0 mm) injury over time. The correlation between CSF SBDP levels and lesion size from T2-weighted magnetic resonance images 24 hours after TBI as well as correlation of tau and S100β was assessed. Mean levels of CSF SBDP (r = 0.833) and tau (r = 0.693) significantly correlated with lesion size while levels of CSF S100β did not (r = 0.188). Although levels of CSF and IC SBDP and lesion size are all significantly higher after 1.6 mm than 1.0 mm injury, the correlation between CSF SBDP and lesion size was not significant following the removal of controls from the analysis. This indicates CSF SBDP is a reliable marker of the presence or absence of injury. Furthermore, larger lesion sizes 24 h after TBI were negatively correlated with motor performance on days 1–5 after TBI (r = −0.708). Based on these data, evaluation of CSF SBDP levels as a biomarker of TBI is warranted in clinical studies.

Key words: biomarker; CSF; injury magnitude; lesion size; spectrin; S100β; tau

INTRODUCTION

The difficulty of diagnosis and prediction of outcome after acute traumatic brain injury (TBI) is associated with the limitations of clinical assessment and neuroimaging (Zink, 2001). Sedatives may be used to treat patients with TBI that exhibit confusion, agitation, or non-compliance with accompanying increased brain metabolism (Mirski et al., 1995). Treatment with anti-convulsant or sedative drugs may confound information obtained from a clinical neuropsychological examination (Mirski et al., 1995). Many mild head trauma patients with a Glasgow Coma Scale (GCS) between 13 and 15 may have coincidental intoxication with drugs and alcohol that may also confound clinical neuropsychological examinations (Kelly, 1995). Head injuries may also be overlooked.
in multi-trauma patients (Buduhan and McRitchie, 2000). Clinical indicators may not predict significant intracranial trauma (Harad and Kerstein, 1992). Neurologic damage from TBI, stroke or perinatal asphyxia may precede changes seen by modern neuroimaging techniques. Although mild traumatic injury may cause long term disabilities, mild trauma may not be seen acutely with radiologic or magnetic resonance imaging (MRI). Computed tomography (CT) scanning is the quickest and most available neuroimaging, yet has low sensitivity for diffuse brain damage. In a critical care patient, cost, availability, and the time to acquire images limits use of the more sensitive measures of MRI and single photon emission CT scans. Single photon emission CT scans detect regional changes of blood flow but not necessarily structural damage. Furthermore, MRI and CT often do not predict outcome (Kido et al., 1992; Kurth et al., 1994; Wilson et al., 1995; Hanlon et al., 1999). Thus there is a need for a biochemical marker of neuronal injury to improve diagnosis and prediction of outcome after TBI.

An ideal biomarker would incorporate several properties. A good biomarker would diagnose neurologic damage before neuro-radiographic signs are evident. A biomarker of acute neuronal injury would provide a measure of injury magnitude and predict neuropsychological outcome. The biomarker would also serve as an indicator of the pathogenesis of cell death including secondary cell death and indicate a target for treatment. With earlier recognition, the window for therapeutic intervention could be extended. Furthermore, a good biomarker would allow for longitudinal monitoring of the effectiveness of therapy. A biomarker with these characteristics could be used as a surrogate marker and lower the cost of clinical trials. An ideal biomarker should also be specific to the central nervous system and provide a sensitive and specific test of neuronal injury.

Earlier biomarkers such as neuron-specific enolase, lactate dehydrogenase, or creatine kinase are not specific to the CNS and failed to reflect pathophysiology, lesion size and outcome of the injury further reinforcing the need for research into better CNS trauma indicators (Ingebrigtsen and Romner, 2002). S100β, a low molecular weight calcium-binding protein released from astrocytes, has been examined in numerous TBI studies. Serum levels of S100β have been correlated with contusion volume (Raabe et al., 1998; Herrmann et al., 2000); injury severity (Herrmann et al., 2000); neuropsychological dysfunction (Herrmann et al., 2001); GCS on admission (Elting et al., 2000); and outcome measures such as the Glasgow Outcome Score (GOS) (McKeating et al., 1998; Elting et al., 2000; Jackson et al., 2000; Raabe and Seifert, 2000; Rothoerl et al., 2000). S100β appears to be a valuable indicator of brain lesion but it is not specific to the CNS. Importantly in multitrauma patients without head injuries, S100β reached high serum levels after bone fractures and thoracic contusion (Anderson et al., 2001). Another biomarker that is being examined as an indicator of brain injury is tau (Zemlan et al., 1999). Tau is a microtubule associated protein that is expressed predominantly in axon of neurons and implicated in microtubule stability, axon elongation and axon transport (Garcia and Cleveland, 2001). In severe TBI patients, increased CSF levels of cleaved tau were found to be significant predictors of intracranial pressure and GOS at discharge (Zemlan et al., 2002), but in recent studies, CSF total tau levels did not correlate with GOS in patients with severe TBI (Franz et al., 2003) nor did serum cleaved tau levels correlate with outcome measures (Chatfield et al., 2002).

αII-spectrin in the CNS is primarily localized to axons and to the presynaptic terminal of neurons (Riederer et al., 1986). In acute neuronal injury, αII-spectrin, a cytoskeletal protein, is a substrate for the calcium activated cysteine proteases, calpain (calpain-1 and -2) and caspase-3. After acute neuronal injury, calcium influx initiates a cytotoxic cascade of proteases, phospholipases, kinases and phosphatases including activation of calpain and caspases which results in necrotic and apoptotic cell death respectively. Calpain and caspase-3 both cleave the 280-kDa parent band of αII-spectrin into a 150-kDa breakdown product (SBDP150). Calpain and caspase-3 cleave signature breakdown products of 145 (SBDP145) and 120 kDa (SBDP120), respectively, in vivo and in vitro (Nath et al., 1996; Wang et al., 1998; Wang, 2000). Both the calpain-mediated SBDP 145 and SBDP 150 increased acutely in the injured cortex whereas the caspase-3 mediated SBDP 120 was absent in an unilateral controlled cortical impact (CCI) model of TBI (Pike et al., 1998). This may reflect a more prominent role of oncosis than apoptosis in the cortex in our CCI model.

αII-spectrin breakdown products (SBDP) have been used as an indicator of calpain activity in models of TBI (Newcomb et al., 1997) and ischemia (Saio et al., 1993; Roberts-Lewis et al., 1994; Bartus et al., 1998) In our laboratory, levels of SBDP have recently been found to increase in rat CSF after experimental controlled cortical impact TBI (Pike et al., 2001) and middle cerebral artery occlusion (Pike et al., 2004). In this study we extend this work by systematically comparing CSF SBDP to their counterpart in injured cortex, to injury magnitude, to CSF tau and S100α and to lesion volume (accessed by MRI). This study subjects a marker of CNS injury to rigorous preclinical examination. Based on the data we have obtained, we propose that CSF SBDP levels are a promising biomarker of injury and further study is warranted in clinical TBI.
MATERIALS AND METHODS

Animals

Three groups of adult male (280–300 g) Sprague-Dawley rats (Harlan; Indianapolis, IN) were used. For study 1, CSF was withdrawn from one group of 90 rats that were sacrificed 2, 6, and 24 h after TBI. At each time point of 2, 6, and 24 h, 9 rats received mild (1.0 mm of cortical deformation) injury, 9 rats received severe (1.6 mm of cortical deformation) injury, 8 rats received a craniotomy but no cortical deformation and 4 rats remained naive (no craniotomy or cortical deformation). For study 2, a second group of rats were sequentially scanned by MRI, subjected to CSF withdrawal, and were sacrificed at 24 h following TBI. Of the second group, 9 rats each received severe (1.6 mm) injury, mild (1.0 mm) injury or craniotomy surgery and 8 rats remained naive. One rat with severe injury was removed from the study because the CSF sample contained blood that could potentially dilute out the concentration of the marker in the CSF and introduce blood-born markers. For study 3, 35 rats were administered a rotarod test on days 1–5 after TBI and scanned by MRI at 24 h and 28 days after TBI. Of the third group of rats, 10 rats each received severe (1.6 mm) injury, mild (1.0 mm) injury or a craniotomy, and 5 rats remained naive.

Surgical Preparation and Controlled Cortical Impact Traumatic Brain Injury

As previously described (Dixon et al., 1991; Pike et al., 2001), a cortical impact injury device was used to produce TBI. Adult male rats were initially anesthetized with 4% isoflurane in a carrier gas of 1:1 O2/N2 (4 min) followed by maintenance anesthesia of 2.5% isoflurane in the same carrier gas. Core body temperature was maintained at 37 ± 1°C by placing an adjustable temperature controlled heating pad beneath the rats. Animals were mounted in a stereotactic frame in a prone position and secured by ear and incisor bars. A midline cranial incision was made, the soft tissues were reflected and a unilateral (ipsilateral to site of impact) craniotomy (7 mm diameter) was performed adjacent to the central suture, midway between bregma and lambda. The dura mater was kept intact over the cortex. Brain trauma in rats was produced by impacting the right cortex (ipsilateral cortex) with a 5 mm diameter aluminum impactor tip (housed in a pneumatic cylinder) at a velocity of 3.5 m/sec with a 150-msec dwell time (compression duration). Compression depth was set at 1.0 mm (mild), or 1.6 mm (severe). Velocity was controlled by adjusting the pressure (compressed N2) supplied to the pneumatic cylinder. Velocity and dwell time were measured by a linear velocity displacement transducer (Lucas Shaevitz™ model 500 HR; Detroit, MI) that produces an analogue signal by a storage-trace oscilloscope (BK Precision, model 2522B; Placentia, CA). Animals underwent identical craniotomy procedures but did not receive cortical compression. Naive rats did not undergo surgery or injury. Appropriate pre- and post-injury management was maintained to insure that all guidelines set forth by the University of Florida Institutional Animal Care and Use Committee and the National Institutes of Health guidelines detailed in the Guide for the Care and Use of Laboratory Animals were complied with.

CSF Withdrawal

Under anesthesia, the rat was secured in the same stereotactic frame as used in surgery. The neck was flexed to optimize exposure of the atlanto-occipital space. A mid-line incision was made over the superficial cervical muscles. A 25-gauge needle attached to polyethylene tubing was inserted into the atlanto-occipital space and CSF was gently withdrawn. CSF was immediately spun at 9,000g for 5 min at 4°C to remove any red blood cells from the cortical impact or from the tap. CSF was frozen at −80°C until examined.

Tissue Lysis

Cortical tissues were collected from naïve animals or at 2, 6, and 24 h after craniotomy or TBI. At the appropriate post-injury time-points, the animals were anesthetized with 4% isoflurane in a carrier gas of 1:1 O2/N2O (4 min) and subsequently sacrificed by decapitation. Ipsilateral (to the impact site) cortex samples were rapidly dissected and snap-frozen in liquid nitrogen. Tissue samples were stored at −80°C until further processing. Frozen samples were thawed and homogenized in a glass tube with a Teflon dounce pestle in 15 volumes of ice-cold detergent-free buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 2 mM EGTA, 0.33 M sucrose, 1 mM DTT) containing a broad-range protease inhibitor cocktail (Roche Molecular Biochemicals, no. 1-836-145) and sonicated. Homogenized samples were then centrifuged at 9000g for 5 min at 4°C. The supernatant was stored at −80°C until immunoblot analysis.

Immunoblotting

Prior to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), protein content was assayed by the Micro BCA method (Pierce, Rockford, IL) using albumin standards. For each sample, 40 μg of protein from cortical tissue or 40 μg of protein from CSF samples were added to 2 × loading buffer containing 0.2 M Tris (pH 6.8), 400 mM 2-mercapto-ethanol, 8% SDS,
0.04% Bromophenol Blue, and 40% glycerol. The amount of protein for CSF samples was optimized to identify SBDP after both mild (1.0 mm) and severe (1.6 mm) injury. The optimal amount of protein to see the 145/150 band after mild injury resulted in an amount of protein after severe (1.6 mm) injury that sometimes would make the 145 and 150 bands indistinguishable. Semi-quantitation by densitometry was used to evaluate the 145–150-kDa band together thus the blurring of the 145/150 band was not a problem. The 145–150-kDa spectrin breakdown product represents primarily calpain initiated cleavage of spectrin in our model. Consistent with a previous report that CCI in our laboratory does not produce prominent caspase-3 levels (Pike et al., 1998), caspase-3-mediated SBDP 120 was inconsistent after severe (1.6 mm) injury and absent after mild (1.0 mm) injury and was not analyzed in this set of experiments. Semi-quantitation by densitometry evaluated both the 145–150-kDa band together thus the blurring was not a problem. Samples were heated at 96°C for 10 min and then centrifuged for 1 min at 10,000 g. Samples were resolved in a vertical electrophoresis chamber for 70 min at 150 V. A 6.5% percent stacking acrylamide gel or a 4–20% Tris-Glycine gel (Invitrogen Life Technologies, Carlsbad, CA) were used. Separated proteins were either laterally transferred as a wet transfer to a nitrocellulose membrane CA) were used. Separated proteins were either laterally transferred as a wet transfer to a nitrocellulose membrane or a 4–20% Tris-Glycine gel (Invitrogen Life Technologies, Carlsbad, CA) were used. Separated proteins were either laterally transferred as a wet transfer to a nitrocellulose membrane (0.45 μM) using a transfer buffer consisting of glycine (192 mM) and tris (25 mM), (pH 8.3) with 10% methanol at a constant voltage of 100V for 70 min at 4°C or were horizontally transferred as a semi-dry transfer to an Immobilon-P polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA) using 39 mM glycine, 48 mM Tris, and 5% methanol at 20 V for 2 h at room temperature. All gels were stained with coomassie blue to confirm equal loading of protein on the gel. Selected blots were also stained with Ponceau red (Sigma, St. Louis, MO) to confirm transfer and that equal amounts of protein were loaded in each lane. Blots were blocked for one hour in 5% non-fat milk in TBST (20 mM Tris, 0.15 M NaCl, and 0.005% Tween-20). Following overnight incubation with the primary antibody, anti-α-spectrin monoclonal antibody (1:10,000 dilution for cortex and 1:5,000 dilution for CSF; Affiniti Research Products, UK) and 1% non-fat milk/TBST at 4°C temperature, the blots were incubated with goat anti-mouse secondary antibody (1:1000 for cortex and 1:5000 for CSF; Biorad) and 3% non-fat milk/TBST for 1 h. Blots were then washed for 1 h in TBST. Enhanced chemiluminescence reagents (ECL and ECL-Plus, Amersham) were used to visualize immunolabeling of cortical tissue and CSF, respectively, and developed on Kodak BioMax Light Film (Kodak). Semi-quantitative evaluation of protein levels was conducted using computer-assisted one-dimensional densitometric scanning (ImageJ, version 1.29, NIH). Data were acquired as integrated densitometric values from similarly exposed films.

ELISA

CSF S100β levels were measured using a rat specific ELISA kit, Nexus D™ Rat S100 Test Kit from SynX (Toronto, Ontario, Canada) and CSF tau was measured using a kit,Innotest™ hTau Antigen from Innogenetics, Inc. (Alpharetta, GA). The sensitivities of the S100β and tau ELISA kits were 0.02 ng/mL and 75 pg/mL, respectively.

T2-Weighted Magnetic Resonance Imaging

Animals were scanned in the Advanced Magnetic Resonance Imaging and Spectroscopy (AMRIS) facility located in the McKnight Brain Institute of the University of Florida. Animals undergoing these imaging sessions were anesthetized using isoflurane (maintenance anesthesia of 1.5–2.5% isoflurane in 1 L/min 100% O2 continuously delivered via a nose cone). Ophthalmic lubricant was used to prevent drying of the eyes during anesthesia. Anesthetized rats were placed on a custom Plexiglas cradle constructed to support the rat comfortably in the supine position. Oxygen saturation was monitored using a pulse oxymeter positioned on the left hind limb. Body temperature was monitored using a rectal fluoroptic probe and maintained using warm air. A 4.7-Tesla magnet (Oxford Instruments) and Bruker Avance Console (Bruker, Germany) and a custom built 3.3-cm (inner diameter) quadrature birdcage coil were used for all image acquisitions. T2-weighted images were acquired at 24 h and 28 days after TBI. Twelve contiguous 1.25-mm coronal slices were acquired with the following parameters: a field of view = 3.6 × 3.6 cm², repetition time (TR) = 2.1 seconds, echo time (TE) = 81 msec, matrix = 256 × 256 points per dimension (140 μm in plane). Areas of hypo-intensity on MRI were associated with hemorrhage or mechanical disruption and areas of hyper-intensity were associated with edema (Albensi et al., 2000). Lesion size was drawn using ParaVision Image Analysis tools (Bruker, Germany) similar to the methodology in (Neumann-Haefelin et al., 2000). The area of each lesion in each coronal slice was multiplied by the slice thickness and then added to calculate the total lesion size.

Neurological Functional Evaluation

Motor behavior was assessed in the sub-acute period after TBI by a blinded observer using a Rota-rod (Ugo Basile, Comerio, Italy; Hamm et al., 1994). Rats were placed on a Rota-rod, a rotating rod, which was set to
slowly accelerate from 4 to 40 rpm within 5 min. The Rota-rod requires the rat to walk as the revolving rod accelerates and maintain balance. The trial lasted until the rat fell off and tripped a plate that recorded the time or until the rat had stayed on the rod for 300 sec was reached. Rats underwent conditioning of two trials a day for three days prior to TBI. After TBI, the rats were tested for two trials a day on days 1–5. The average of the latency in seconds of the two trials was recorded.

Statistical Analysis

Means and standard errors of the means were calculated from individual rat densitometric values of the 145–150-kDa SBDP combined as one value. Two-way ANOVA was used to examine main effects and interaction effects of time and injury magnitude. One-way ANOVA with contrast to do pair-wise comparisons was used to determine significance between levels of SBDP and between lesion sizes of the corresponding experimental groups. Regression analysis was performed with lesion size as the outcome variable and CSF markers (SBDP, tau, S100β) as the predictor variable. Pearson correlations were calculated and tested using the asymptotic Z-test. Correlations were calculated in the individual animal between CSF SBDP levels and lesion size. The analysis of the correlations included animals in all groups (naive, craniotomy, 1.0 mm and 1.6 mm injury) unless stated otherwise. Repeated measures ANOVA (4 groups × 5 time points) were performed to determine individual group differences over the five time points on the Rota-rod test.

RESULTS

Injury Magnitude Is Associated with Increased Levels of SBDP in the Cortex and CSF after TBI

SBDP were measured by Western blot from the CSF and ipsilateral cortex (IC) at 2, 6, and 24 h after two magnitudes of TBI. Naive rats and rats that had undergone a craniotomy served as controls for this study. The two response variables, SBDP in the CSF and SBDP in the IC were analyzed via ANOVA with terms for injury magnitude, time, and the interaction of time and injury magnitude.

The results indicated there was no interaction effect (\( p = 0.88 \)) or time effect (\( p = 0.12 \)) on IC SBDP levels. The analysis also indicated that injury magnitude significantly increased the level of cortical SBDP (\( p \leq 0.0001 \)). Mean levels of IC SBDP after severe (1.6 mm) injury were significantly higher than the mean levels of IC SBDP after mild (1.0 mm) injury (\( p < 0.05 \)). Mean levels of IC SBDP after both severe (1.6 mm) and mild (1.0 mm) injury were significantly greater than mean levels of SBDP after craniotomy or in naive controls (\( p < 0.0001 \)). Mean levels of IC SBDP did not differ between naive and after craniotomy. Representative gels show that levels of SBDP increased with injury magnitude in the ipsilateral cortex and the CSF (Fig. 1A). Levels of SBDP (both 145 and 150 kDa are densitometrically quantified together) were highest after 1.6-mm injury in the IC and CSF at all time points (Fig. 1B).

After severe (1.6 mm) injury, the mean levels of IC SBDP were 116.4 ± 8.9, 135.9 ± 14.1, and 135.6 ± 17.7 and after mild (1.0 mm) injury, the mean levels of IC SBDP reached 78.1 ± 9.9, 110.1 ± 19.4, and 102.8 ± 17.2 at 2, 6, and 24 h, respectively. After craniotomy, the mean levels of IC SBDP reached 22.6 ± 9.1, 40.9 ± 18.2, and 11.8 ± 4.6 at 2, 6, and 24 h, respectively. In naive rats, the mean levels of IC SBDP were 4.4 ± 1.6, 15.4 ± 7.0, and 4.4 ± 6.5 at 2, 6, and 24 h, respectively.

There was no interaction effect (\( p = 0.39 \)) or time effect (\( p = 0.13 \)) on CSF SBDP levels. The analysis also indicated that injury magnitude significantly increased the levels of CSF SBDP (\( p \leq 0.0001 \)). Mean levels of CSF SBDP after severe (1.6 mm) injury were significantly higher than the mean levels of CSF SBDP after mild (1.0 mm) injury (\( p = 0.0001 \)). Mean levels of CSF SBDP after both severe (1.6 mm) and mild (1.0 mm) injury were significantly greater than mean levels of CSF SBDP after craniotomy or in naive controls (\( p < 0.0001 \)).

Mean levels of CSF SBDP did not differ between naive and after craniotomy.

After 1.6 mm injury, the mean levels of CSF SBDP were 153.4 ± 11.3, 114.4 ± 19.1, and 91.2 ± 23.8 and after 1.0 mm injury, the mean CSF SBDP were 82.2 ± 17.3, 71.4 ± 17.3, and 64.3 ± 17.2 at 2, 6, and 24 h, respectively. After craniotomy, the mean levels of CSF SBDP reached 7.8 ± 2.7, 19.1 ± 6.0, and 10.3 ± 5.3 at 2, 6, and 24 h, respectively. In naive rats, the mean levels of CSF SBDP were 1.0 ± 0.7, 5.7 ± 3.4, and 3.0 ± 1.4 at 2, 6, and 24 h, respectively.

The Relationship of CSF SBDP Levels with Lesion Size at 24 h Post-Injury

CSF extraction to measure SBDP and T2-weighted imaging to measure lesion size was performed in the same groups of rats at 24 h after TBI. Representative T2-weighted images of a naive rat and a rat 24 h after craniotomy, mild (1.0 mm) injury and severe (1.6 mm) injury are shown in Figure 2A. Severe (1.6 mm) injury resulted in disruption of normal architecture and swelling of the ipsilateral cortex (arrow in Fig. 2A). Less disrup-
FIG. 1. Injury magnitude increases levels of SBDP in the ipsilateral cortex (IC) and CSF. (A) A representative Western blot of αl-spectrin and SBDP in the IC (left) and CSF (right) at 24 and 2 h, respectively, after TBI. Samples were collected after severe (1.6 mm) injury, mild (1.0 mm) injury, sham-craniotomy or from naive rats. Higher levels of SBDP are seen after severe (1.6 mm) injury than after mild (1.0) injury. Minimal SBDP is seen in the IC or CSF of naive rats or after sham-craniotomy in rats. (B) SBDP levels (145–150-kDa fragments) in the IC (left panel) and CSF (right panel) after sham-craniotomy, mild (1.0 mm) injury and severe (1.6 mm) injury at 2, 6, and 24 h were quantified using computer-assisted densitometric analysis (ImageJ, version 1.29×, NIH, USA). Values from naive animals were averaged as a separate time point. At each time point of 2, 6, and 24 h, 9 rats received severe (1.6 mm) injury, 9 rats received mild (1.0 mm) injury, 8 rats received a sham-cranioectomy and 4 rats remained naive. An ANOVA was performed followed by contrast with pair-wise comparisons. Data is presented as the mean plus standard error. Standard error bars on the shams are present but not easily visible. Injury magnitude significantly increased mean levels of IC and CSF SBDP over time (p < 0.0001). Mean levels of SBDP after severe (1.6 mm) injury were significantly higher from the mean levels of SBDP after mild (1.0 mm) injury (††p < 0.0001 and †p < 0.05, respectively, for CSF and IC levels of SBDP). Mean levels of IC and CSF SBDP after both severe (1.6 mm) and mild (1.0 mm) injury were significantly greater than mean levels of SBDP after sham-craniotomy or in naive controls (***p < 0.0001). Mean levels of CSF and IC SBDP did not differ between naive and sham.

FIG. 2. Lesion size on T2 weighted images increases with injury magnitude 24 h after TBI. (A) Representative serial T2-weighted magnetic resonance images of a naive rat and a rat 24 h after sham-craniotomy, mild (1.0 mm) and severe (1.6 mm) injury are shown. Twelve contiguous coronal 1.25 mm slices were acquired with the following parameters: a field of view = 3.6 × 3.6 cm², repetition time (TR) = 2100 sec, echo time (TE) = 81 msec, matrix = 256 × 256 points per dimension (140 μm in plane). Four of the 12 coronal slices for each rat are shown. Severe (1.6 mm) injury resulted in disruption of normal architecture and swelling of the ipsilateral cortex (arrow). Less disruption of normal architecture is noted after mild (1.0 mm) injury (arrowhead). Sham-craniotomy resulted in varying amounts of hyper-intensity in the ipsilateral cortex (arrowhead). (B) Lesion size was drawn using ParaVision Image Analysis tools (Bruker, Germany) similar to the methodology in (Neumann-Haefelin et al., 2000). The area of each lesion in each coronal slice was multiplied by the slice thickness and then added to calculate the total lesion size. One-way ANOVA with contrast to do pair-wise comparisons was used to determine difference between lesion sizes of the treatment groups. Nine rats each received severe (1.6 mm) injury, mild (1.0 mm) injury or sham surgery and 8 rats remained naive. The lesion after severe (1.6 mm) injury is significantly greater than the lesion size after mild (1.0 mm) injury (**p < 0.001) and both are significantly greater than after sham-craniotomy (††p ≤ 0.001) or naive animals (***p ≤ 0.001) Sham injury is greater than the absence of a lesion in naive animals (*p < 0.05).
tion of normal architecture is noted after mild (1.0 mm) injury (arrowhead in Fig. 2A). Craniotomy resulted in varying amounts of hyper-intensity in the ipsilateral cortex (arrowhead in Fig. 2A). Average lesion size was $0.044 \text{ cm}^3 \pm 0.00058$ after craniotomy, $0.100 \text{ cm}^3 \pm 0.010$ after 1.0 mm injury, and $0.166 \text{ cm}^3 \pm 0.016$ after 1.6 mm injury (Fig. 2B). Mean lesion size was significantly different between 1.6 mm and 1.0 mm injury groups and
between both injury groups and after craniotomy ($p \leq 0.001$; Fig. 2B). Mean levels of CSF SBDP significantly correlated with lesion size ($r = 0.833$, $p < 0.0001$) when including all 4 groups (1.6 mm and 1.0 mm injury, craniotomy, and naive rats) (Fig. 3A). This correlation was not significant if craniotomy and sham rats were not considered in the analysis. To explore the ability of SBDP to predict lesion size, a regression analysis was run with lesion size as the outcome variable and CSF SBDP as the predictor variable from individual rats from all 4 groups. The regression weight for CSF SBDP was estimated to be 1059.86, and the parameter estimate of the intercept was 10.707. The regression analysis revealed CSF SBDP contributed significantly to predicting lesion volume ($p < 0.0001$).

Levels of CSF tau significantly correlated with lesion size ($r = 0.693$, $p < 0.0001$) (Fig. 3B) as levels of CSF S100β did not ($r = 0.188$) (Fig. 3C). The regression weight for CSF tau was estimated to be 0.00001258, and the parameter estimate of the intercept was 0.03485. CSF levels of tau significantly contributed to the prediction of lesion volume ($p < 0.0001$). Neither CSF tau or CSF S100β were correlated if just the 1.6-mm and 1.0-mm injured rats were used for analysis.

A regression analysis was performed to determine which marker or combination of markers (SBDP, tau and S100β) best predicted lesion size. A full regression model indicated the only significant variable was SBDP ($p < 0.0001$). S100β was eliminated from the model and the regression re-run looking at SBDP and tau as predictors of lesion size. CSF SBDP was again the only significant predictor of lesion size ($p < 0.0001$). CSF SBDP and CSF tau are significantly correlated ($r = 0.750$, $p < 0.0001$) and CSF SBDP has a higher correlation with CSF tau than CSF tau’s correlation with lesion size.

![Graphs showing correlation between CSF markers and lesion size](image-url)

**FIG. 3.** The relationship of levels of CSF SBDP and tau with lesion size 24 h after TBI. Regression analysis was performed with lesion size as the outcome variable and levels of CSF markers (SBDP, tau, S100β) 24 h after TBI as the predictor variable. (A) Levels of CSF SBDP correlate with lesion size after TBI ($r = 0.83$, $p \leq 0.0001$). A linear regression equation showed that CSF SBDP significantly contributed to prediction of lesion size ($p \leq 0.0001$). (B) Levels of CSF tau correlate with lesion size after TBI ($r = 0.690$, $p < 0.001$). A linear regression equation showed that CSF tau significantly contributed to prediction of lesion size ($p \leq 0.0001$). The correlation with CSF SBDP and tau was not significant if craniotomy and sham rats were not considered in the analysis. (C) Levels of CSF S100β did not correlate with lesion size ($r = 0.188$). ■, rats after 1.6 mm injury; ◆, rats after 1.0 mm injury; *, rats after sham-craniotomy; △, naive rats.
Injury Magnitude Is Associated with Decreased Performance on the Rota-Rod Test and Increased Lesion Size

Because CSF SBDP correlated with lesion size at 24 hours, we looked at the relationship between lesion size and motor performance. Motor performance was assessed in the same (study 3) rats that lesion size was measured at 24 h and 28 days (Fig. 4A). Similar to 24 h, at 28 days lesion size varied with injury magnitude. Lesion size at 24 h in the individual animal was significantly correlated with lesion size at 28 days \( (r = 0.881, p < 0.0001) \). Assessment of Rota-rod performance prior to treatment revealed no significant differences between groups. Injury magnitude had a significant effect on Rota-rod performance \( (p < 0.0001) \). Mean Rota-rod scores were significantly lower after 1.6-mm injury at all time points (1–5 days after TBI) compared to mild (1.0 mm) injury, craniotomy, or naive rats \( (p = 0.05) \). After 1.0-mm injury, mean Rota-rod scores were significantly lower than in naive rats \( (p < 0.01) \) and showed a trend toward being lower than after craniotomy (Fig. 4A). Naive rats averaged close to a perfect score of 300 sec at all time points. Naive rats had significantly higher scores than rats after craniotomy \( (p < 0.05) \). Furthermore, larger lesion sizes were associated with decreased performance on the Rota-rod (Fig. 4B). In the individual rat, the average of the 5 days of Rota-rod scores correlated negatively with lesion size at 24 h \( (r = -0.708; p < 0.0001) \).

DISCUSSION

This paper examined the relationship between IC and CSF levels of SBDP and injury magnitude and outcome measures. The results show that SBDP levels in both CSF and IC SBDP increase with injury magnitude. Although both IC and CSF levels of SBDP increased, they did not parallel each other. Levels of CSF SBDP peaked at 2 h and decreased over time, while IC levels of SBDP slowly increased over the first 6 h after TBI. The correlation between lesion size and CSF levels of SBDP supported CSF SBDP as an indicator of injury. Correlational analysis of relationships between lesion size and CSF levels of SBDP indicated that CSF SBDP is a reliable marker of the presence or absence of injury but failed to be a reliable marker of injury magnitude. Although both CSF and IC SBDP levels and lesion size were significantly higher after 1.6-mm injury than after 1.0-mm injury, the correlation between CSF SBDP and lesion size was not significant following the removal of the control groups (naive and craniotomy) from the analysis. Further study is needed to show if CSF SBDP levels are a useful predictor of outcome such as lesion size.

Factors that affect brain derived protein levels in the CSF after injury have not been extensively explored and are likely to affect the variability of CSF SBDP levels and correlations with CSF SBDP. Petzold et al. (2003) suggest the main determinants of brain tissue proteins in the CSF are the extent of the primary lesion, the total pathological severity causing imbalance of brain home-
ostasis, and the onset and duration of the brain injury. The CCI model that we have used in these studies has some inherent variability in impact force that affects lesion size, lesion severity and the location of pathology. Injured cells in the subarachnoid space can directly release protein into the CSF while protein from cells in the parenchyma must be transported to the CSF by flow of interstitial fluid or edema (Hans et al., 1999). The molecular flux/CSF flow theory suggests that changes in diffusion across the blood-brain and brain-CSF barriers are primarily predicated on CSF flow (Reiber and Peter, 2001; Reiber, 2003). If CSF flow rate is decreased after injury, then ventricular concentration of brain derived proteins is increased (Reiber, 2003). Variation of lesion impact might also cause variation in CSF flow rate and in the distance of brain-derived proteins from the CSF. As more is learned about factors effecting CSF levels of brain-derived proteins, the ability of CSF SBDP and other biomarkers to predict outcome may improve.

Hans et al. (1999) conducted one of the first studies to rigorously analyze a potential biomarker examining distribution and upregulation of mRNA and protein levels of IL-6 in tissue, and bioactivity of IL-6 in CSF and serum in a model of TBI. Similar to previous work in our lab examining mRNA of calpain-1 and calpain-2 (Ringer et al., 2004), IL-6 mRNA was upregulated after injury. Similar to our study, CSF levels of IL-6 peaked within 2–4 h after injury (Hans et al., 1999). IL-6 protein as seen on immunohistochemistry increased by 1 hour and persisted for 24 h, similar to the increase in IC SBDP on western blots at 2, 6, and 24 h after TBI. Hans et al. (1999) suggested that the increased tissue protein immunoreactivity reflected the increased IL-6 activity in the CSF. CSF levels of IL-6 were higher than serum levels between 2 and 8 h after injury. The CSF levels of IL-6 appear higher within 8 h of injury than in the CSF of sham animals, however the paper did not address this important question statistically.

Increased levels of calcium after TBI have been observed in several models (Fineman et al., 1993; Nadler et al., 1995; Verweij et al., 1997; Xiong et al., 1997). 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 (Ringer et al., 2004). Similar to our study, varying injury magnitude by depth or by velocity of impact, significantly effected lesion size (Goodman et al., 1994). Injury magnitude also significantly increased peak intracranial pressure and hippocampal neuron loss in similar models of TBI (Cherian et al., 1994; Goodman et al., 1994). Temporal increases in intracellular calcium were correlated with injury magnitude after lateral fluid percussion model of TBI in rats (Fineman et al., 1993). The corresponding increase in calcium after more severe TBI in the Fineman study may explain the association between injury magnitude and SBDP levels in the IC and CSF in our study.

In the acute time period following TBI, both CSF and IC SBDP significantly 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 (Bartus et al., 1995; Nath et al., 1996; Saatman et al., 1996a; Newcomb et al., 1997). Recently, it has been shown that CSF SBDP increased in models of TBI (Pike et al., 2001) and ischemia (Pike et al., 2004). The increased levels of SBDP150/145 are primarily associated with calpain activation in our CCI model. Although caspase-3 may also cleave spectrin to SBPD150, similar to prior work in our laboratory (Pike et al., 1998), the caspase-3 signature SBPD120 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 (Saatman et al., 1996a,b; Buki et al., 2003), ischemia (Bartus et al., 1994; Hong et al., 1994; Markgraf et al., 1998), and spinal cord injury (Banik et al., 1998). The ability of CSF levels of SBDP to reflect increased IC SBDP levels after acute neuronal injury may provide a therapeutic target for treatment of TBI and an effective way to monitor treatment of TBI if CSF is available.

CSF cleaved tau levels were significant predictors of outcome measures (intracranial pressure and GOS at discharge; Zemlan et al., 2002) supporting the finding of a significant correlation between CSF tau and lesion size in our study. On the other hand, Franz et al. (2003) 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.

An advantage of a serum biomarker is that it can be measured by less invasive methods than CSF biomarkers. The disadvantage of serum markers is that measurable serum levels of brain tissue proteins must cross the blood–brain and the CSF–blood barriers. Use of serum tau as a biomarker has produced conflicting results. Initial examination of serum cleaved tau indicated that the presence of serum cleaved tau increased the odds of the presence of an intracranial injury and a greater chance of a poor out-come (Shaw et al., 2002). Later work indicated serum cleaved tau levels did not correlate with outcome measures (Chatfield et al., 2002). After acute stroke, total tau increased in the CSF (Hesse
SBDP, A NOVEL MARKER OF TBI

e et al., 2001) and serum (Bitsch et al., 2002), and serum tau levels correlated to lesion size and severity. Serum tau levels, however, increased in less than 50% of stroke patients during the first 5 days after stroke (Bitsch et al., 2002).

Analysis of S100β has primarily been from the serum in clinical studies. Two clinical studies of serum levels of S100β revealed a correlation with contusion volume (Raabe et al., 1998; Herrmann et al., 2000), while in a study of mild TBI, serum S100β levels did not correlate with MRI or CT scans (Herrmann et al., 1999). S100β may be released from damaged glial cells, and this variable may not change consistently with lesion volume.

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 (Anderson et al., 2001). Numerous studies examined the use of S100β to mark cerebral damage after cardio-pulmonary bypass surgery (Ali et al., 2000), but S100β was found to be released from the mediastinum of cardiopulmonary bypass patients (Anderson et al., 2001). After stroke, higher serum S100β levels were associated with larger infarcts and more severe neuropsychological deficits (Aurell et al., 1991; Abrahã et al., 1997; Buttner et al., 1997). Yet despite these promising correlations, Hill et al. (2000) found only 32% of stroke patients had elevated serum S100β on admission similar to serum tau levels (Bitsch et al., 2002). Early identification of stroke is necessary for optimal treatment within three hours.

The utility of SBDP as a serum marker has not been examined in clinical cases or models of stroke or TBI to the author’s knowledge. Our study did not examine serum SBDP levels but further work will be important to establish if SBDP crosses the blood–brain and blood–CSF barrier and reflects SBDP levels in the CSF and brain. α1-spectrin is not found in red blood cells (Pike et al., 2001) although it is found in very low levels in other organs systems (Pike, Flint, Wang, and Hayes, unpublished data). The utility of SBDP as a marker would also benefit from knowledge of serum levels of SBDP in multi-trauma patients without head injuries and acutely after stroke.

Changes in high resolution MRI have been shown to correlate well with histology in a lateral fluid percussion model (Albensi et al., 2000) and a closed head injury model (Assaf et al., 1997) of TBI. Areas of hypo-intensity on MRI were associated with hemorrhage or mechanical disruption and areas of hyper-intensity were associated with edema (Albensi et al., 2000). At 24 h after rats underwent 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 (Assaf et al., 1997). Similarly in our study, the overall size of the lesion decreased between 24 h and 28 days, although a significant correlation was maintained between lesion size in individual rats at the two time points.

This study 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 (Palmer et al., 2001). Similarly in our study, the larger the lesion size, the worse the performance on the motor function test. Because lesion size at 24 h 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 this study show that levels of SBDP in the IC and CSF are significantly higher after 1.6-mm injury than after 1.0-mm injury paralleling the significant difference in lesion size. We further showed that 24 hours after TBI, increased levels of CSF SBDP indicate the presence of a lesion. These studies strongly support the further study of CSF SBDP as a marker of CNS injury, and warrant evaluation of SBDP as a serum marker. Further examination may elucidate whether CSF or serum SBDP levels are predictors of outcome such as lesion size, GOS or neurological dysfunction. The contribution of this work is a foundation for future studies assessing the utility of this marker in human brain injury.

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RINGGER

AU1
Is “tau” really a keyword?

AU2
Update Ringger ref.