Washington University School of Medicine
Digital Commons@Becker

Open Access Publications

2007

Clinical significance of αII-spectrin breakdown products in cerebrospinal fluid after severe traumatic brain injury

Jose A. Pineda
Washington University School of Medicine

Stephen B. Lewis
University of Florida

Alex B. Valadka
Baylor College of Medicine

Linda Papa
University of Florida

H. Julia Hannay
University of Houston

See next page for additional authors

Follow this and additional works at: http://digitalcommons.wustl.edu/open_access_pubs

Authors

Jose A. Pineda, Stephen B. Lewis, Alex B. Valadka, Linda Papa, H. Julia Hannay, Shelley C. Heaton, Jason A. Demery, Ming Cheng Liu, Jada M. Aikman, Veronica Akle, Gretchen M. Brophy, Joseph J. Tepas III, Kevin K. W. Wang, Claudia S. Robertson, and Ronald L. Hayes

Clinical Significance of αII-Spectrin Breakdown Products in Cerebrospinal Fluid after Severe Traumatic Brain Injury

JOSE A. PINEDA,[1,6,9] STEPHEN B. LEWIS,[1,3] ALEX B. VALADKA,[10] LINDA PAPA,[4]
H. JULIA HANNAY,[11] SHELLEY C. HEATON,[7] JASON A. DEMERY,[7]
MING CHENG LIU,[2] JADA M. AIKMAN,[2] VERONICA AKLE,[2]
GRETCHEN M. BROPHY,[12] JOSEPH J. TEPAS III,[5,6] KEVIN K.W. WANG,[1,2,8]
CLAUDIA S. ROBERTSON,[10] and RONALD L. HAYES[1,2,3,7,8]

ABSTRACT

Following traumatic brain injury (TBI), the cytoskeletal protein α-II-spectrin is proteolyzed by calpain and caspase-3 to signature breakdown products. To determine whether α-II-spectrin proteolysis is a potentially reliable biomarker for TBI in humans, the present study (1) examined levels of spectrin breakdown products (SBDPs) in cerebrospinal fluid (CSF) from adults with severe TBI and (2) examined the relationship between these levels, severity of injury, and clinical outcome. This prospective case control study enrolled 41 patients with severe TBI, defined by a Glasgow Coma Scale (GCS) score of ≤ 8, who underwent intraventricular intracranial pressure monitoring. Patients without TBI requiring CSF drainage for other medical reasons served as controls. Ventricular CSF was sampled from each patient at 6, 12, 24, 48, 72, 96, and 120 h following TBI and analyzed for SBDPs. Outcome was assessed using the Glasgow Outcome Score (GOS) 6 months after injury. Calpain and caspase-3 mediated SBDP levels in CSF were significantly increased in TBI patients at several time points after injury, compared to control subjects. The time course of calpain mediated SBDP150 and SBDP145 differed from that of caspase-3 mediated SBDP120 during the post-injury period examined. Mean SBDP densitometry values measured early after injury correlated with severity of injury, computed tomography (CT) scan findings, and outcome at 6 months post-injury. Taken together, these results support that α-II-spectrin breakdown products are potentially useful biomarker of severe TBI in humans. Our data further suggests that both necrotic/oncotic and apoptotic cell death mechanisms are activated in humans following severe TBI, but with a different time course after injury.

Key words: biomarker; CSF; spectrin; traumatic brain injury

[1]Center for Traumatic Brain Injury Studies, E.F and W.L. McKnight Brain Institute of the University of Florida, Gainesville, Florida.

Departments of [2]Neuroscience, [3]Neurosurgery, [4]Emergency Medicine, [5]Surgery, [6]Pediatrics, [7]Clinical & Health Psychology, and [8]Psychiatry University of Florida, Gainesville, Florida.

[9]Departments of Pediatrics and Neurology, Washington University, St. Louis, Missouri.

[10]Department of Neurosurgery, Baylor College of Medicine, Houston, Texas.

[11]Department of Psychology, University of Houston, Houston, Texas.

[12]Departments of Pharmacy and Neurosurgery, Virginia Commonwealth University, Richmond, Virginia.

INTRODUCTION

IN THE UNITED STATES, an estimated 1.4 million people sustain a traumatic brain injury (TBI) each year (Langlois et al., 2005). About 50,000 people die and at least 5.3 million live with long-term disabilities related to TBI (Binder et al., 2005). Currently available technologies to assess brain injury, such as computed tomography (CT) scanning and magnetic resonance imaging (MRI), are expensive and have limited ability to predict outcome, especially early after injury (Azouvi, 2000; Hanlon et al., 1999; Kurth et al., 1994; Wilson et al., 1995). Unlike other organ-based diseases where rapid diagnosis employing biomarkers prove invaluable to guide treatment of the disease, no such rapid, definitive diagnostic tests exist for TBI. Recent reviews of biomarkers of central nervous system (CNS) injury have highlighted the need for biomarker development (Choi, 2002; Ingebrigtsen and Romner, 2002; Pineda et al., 2004). Such diagnostic tests would provide physicians with quantifiable neurochemical markers to help determine the severity and anatomical and cellular pathology of the injury, and guide in the implementation of appropriate triage and medical management.

The most studied potential biochemical markers for TBI include creatine kinase (CK), glial fibrillary acidic protein (GFAP), lactate dehydrogenase (LDH), tau, and myelin basic protein (MBP), though the bulk of research in TBI has focused on neuron-specific enolase (NSE) and S-100β (Berger et al., 2002; Guan et al., 2003; Ingebrigtsen and Romner, 2002; Raabe et al., 2003). Neuron-specific enolase is rapidly detectible in cerebrospinal fluid (CSF) (Berger et al., 2002; Varma et al., 2003) and serum (Skogseid et al., 1992; Yamazaki et al., 1995) after TBI (Woertgen et al., 1997). However, studies comparing NSE serum or CSF levels to admission GCS in patients with TBI show conflicting results (Herrmann et al., 1999; Ross et al., 1996; Yamazaki et al., 1995). Conflicting data have also been reported for relationships between NSE levels and CT scan findings (Fridriksson et al., 2000) and long-term outcome (Raabe et al., 1998, 1999; Ross et al., 1996; Yamazaki et al., 1995). In mild TBI, NSE failed to separate patients from controls (Ross et al., 1996). NSE is also released in the blood by hemolysis, which could be a major source of error (Cooper, 1994; Johnsson, 1996).

Several studies have shown S-100β to be a potential biomarker for head injury (Herrmann et al., 1999; Raabe et al., 1998, 1999; Woertgen et al., 1997). S-100β was previously regarded as specific to astrocytes and Schwann cells, but has been found in several cell types including melanocytes, adipocytes, and chondrocytes (Michetti and Gazzolo, 2002) and is therefore not specific to the CNS. Questions have also been raised about the utility of S-100β due to reports of high serum levels in trauma patients not experiencing a head injury (Anderson et al., 2001; Pelinka et al., 2003). A supposedly cleaved form of tau, c-tau, has also been investigated as a potential biomarker of CNS injury. CSF levels of c-tau were significantly elevated in TBI patients compared to control patients and these levels correlated with clinical outcome (Shaw et al., 2002; Zemlan et al., 2002, 1999). Though levels of c-tau were also elevated in plasma from patients with severe TBI, there was no correlation between plasma levels and clinical outcome (Chatfield et al., 2002). A major limitation of all of these biomarkers is the lack of specificity for defining neuropathological cascades. Other potential biomarkers for TBI, such as F_2-isoprostane (a marker of lipid peroxidation), provide information on pathological events (Varma et al., 2003), but are not specific to the CNS.

Our laboratory has focused on αII-spectrin proteolysis as a biochemical marker of CNS injury (Pike et al., 2004, 2001; Ringger et al., 2004). Alpha-II-spectrin (or alpha-fodrin) is expressed in brain and other non-erythroid tissues. Studies in our laboratory have demonstrated that alpha-II-spectrin is primarily enriched in brain and comes from neurons rather than glia (Wang et al., unpublished data). Furthermore, alpha-II-spectrin appears to be localized to axons (Riederer, 1986; Hayes, 1997).

α-II-Spectrin (280 kDa), a structural component of the cortical membrane cytoskeleton (Goodman et al., 1995; Riederer et al., 1987), is a major substrate for the calpain and caspase-3 cysteine proteases (Roberts-Lewis and Siman, 1993; Seubert et al., 1988; Siman and Noszek, 1988; Siman et al., 1989; Wang, 2000; Wang et al., 1998), and our laboratory and others have provided considerable evidence that αII-spectrin is processed by the calpains and caspase-3 to signature cleavage products *in vivo* after experimental TBI in rats, both in brain tissue (Beer et al., 2000; Buki et al., 1999; Knoblach et al., 2002; Newcomb et al., 1997; Pike et al., 1998; Saatman et al., 1996; Yakovlev et al., 1997) and in CSF (Hall et al., 2005; Pike et al., 2001; Ringger et al., 2004; Siman et al., 2004). Understanding the contributions of these specific proteases to cell injury and death following TBI may have important diagnostic and therapeutic implications. A unique feature of this technique is the ability to concurrently detect calpain and caspase-3 proteolysis of α-II-spectrin, providing crucial information on the underlying injury mechanisms, and potentially the effects of therapy on prevention of calpain and caspase-3 proteolysis. While a large body of data from animal studies has demonstrated that proteolysis of αII-spectrin is a reliable measure of injury that is ultimately detectable in CSF, it is only recently that elevation of calpain and caspase-3 specific

αII-spectrin breakdown products (SBDPs) has been demonstrated in humans (Farkas et al., 2005). To our knowledge, no studies to date have reported data supporting SBDPs as a clinically useful biomarker in patients with severe TBI. Thus, the present study sought to determine whether signature proteolytic SBDPs were a reliable marker of CNS injury in humans.

METHODS

Study Sites

This study was approved by the Institutional Review Board (IRB) of the University of Florida and Baylor College of Medicine. For patients enrolled at the University of Florida, consent was obtained within 24 h of enrollment through an IRB-approved procedure. Adult patients presenting to the University of Florida Trauma System (Shands Hospital in Gainesville and Jacksonville) following a severe head injury defined by a GCS of ≤ 8 and requiring ventricular intracranial pressure (ICP) monitoring were enrolled consecutively for 16 months starting in April of 2003 and were followed for 6 months after study entry. All patient identifiers were kept confidential.

The clinical data and CSF samples contributed to the study from the Baylor College of Medicine site were collected from 23 patients admitted to Ben Taub General Hospital with a GCS ≤ 8 between August 29, 2003, and August 30, 2004, as a part of an IRB-approved protocol. The patients and/or their relatives gave informed consent for the CSF samples to be stored and for these samples as well as the clinical data to be used for IRB-approved research at a later time. De-identified CSF samples and data were transferred to the University of Florida for analysis. A confidentiality agreement with Baylor College of Medicine investigators was signed as required by the University of Florida IRB.

Samples

CSF control samples were obtained from either (1) hydrocephalic patients who had ventriculoperitoneal (VP) shunts placed and who had CSF samples taken intraoperatively or (2) unruptured subarachnoid hemorrhage patients who also had CSF samples taken intraoperatively. Control patients had a normal mental status at the time of enrolment, suggesting the absence of acute brain injury. The fact that control CSF samples were obtained intraoperatively would prevent meaningful increases in biomarker levels in control samples. The time course for SBDP levels in control patients was not established given the fact that control patients had no clinical evidence of acute brain injury and therefore changes in SBDP levels over time that could be attributed to brain pathology were not anticipated.

CSF samples from TBI patients were directly collected from the ventriculostomy catheter at 6, 12, 24, 48, 72, 96, and 120 h following TBI (ventriculostomy catheters are placed as routine medical care for patients with severe TBI at these institutions). Approximately 3–4 mL of CSF was collected from each subject. Samples were immediately centrifuged for 10 min at 4000 rpm to separate CSF from blood, and immediately frozen and stored at $-70°$ until the time of analysis.

Immunoblot Analyses

Human CSF samples (7 mcL) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) with twofold loading buffer containing 0.25 M Tris (pH 6.8), 0.2 M dithiothreitol (DTT), 8% SDS, 0.02% bromophenol blue, and 20% glycerol in distilled H_2O on 4–20% or 6% polyacrylamide gels (Invitrogen; catalog no. EC61352) in Tris-glycine running buffer at 130 V for 2 h. Following electrophoresis, separated proteins were laterally transferred to polyvinylidene fluoride (PVDF) membranes in a transfer buffer containing 39 mM glycine, 48 mM Tris-HCl (pH 8.3), and 5% methanol at a constant voltage of 20 V for 2 h at ambient temperature in a semi-dry transfer unit (Bio-Rad). After electrotransfer, blotting membranes were blocked for 1 h at ambient temperature in 5% non-fat milk in TBST (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.05% [w/v] Tween-20), then incubated in primary antibody (mouse anti-αII-spectrin; Affiniti, Cat#.FG6090; at 1/5,000 dilution) in TBST with 5% skim milk as recommended by the manufacturer at 4°C overnight. This was followed by three washes with TBST and a 2-h incubation at ambient temperature with a secondary antibody linked to horseradish peroxidase (for enhanced chemiluminescence [ECL] method; Amersham). After additional washes of the blots with TBST, ECL reagents (Amersham) were used to visualize the immunolabeling on x-ray film. Molecular weight of intact αII-spectrin and its breakdown products (BDPs) were assessed by running alongside rainbow-colored molecular weight standards (Amersham). Quantitative evaluations of αII-spectrin BDP levels were performed via computer-assisted densitometric scanning (Epson XL3500 high-resolution flatbed scanner) and image analysis with Image-J software (NIH).

Relationships between Biomarkers and Clinical Variables

To examine the relationship between accumulation of SBDPs and clinical variables, severity of injury was as-

sessed using the Glasgow Coma Scale (GCS) as previously described (Teasdale and Jennett, 1976) in the immediate post-resuscitation phase. Patients were classified according to a commonly used dichotomization of the GCS (GCS score 3–5 versus GCS score 6–8) (Narayan et al., 1981). A similar analysis was conducted for the best GCS score on day 1 post-injury. The best day 1 GCS was reported because it is less likely to be influenced by confounding factors that are not necessarily related to injury severity, and it has been found to be a significant predictor of outcome (Phuenpathom, 1993). We also studied the relationship between the presence of pre-hospital hypotension or pupillary abnormalities and SBDPs in the spinal fluid. Hypotension (systolic blood pressure <90 mm Hg) and pupillary abnormalities (one or both non-reactive or dilated pupils) were defined as recommended by the *Guidelines for Management and Prognosis of Severe Traumatic Brain Injury* (2000a,b). Hypoxia (oxygen saturation less than 90%) was documented in only three subjects and therefore not included in our analysis. Using the Marshall classification of CT findings after TBI (Marshall et al., 1992), we studied the relationship between CT findings on the day of injury and SBDPs. Patient outcome was assessed using the Glasgow Outcome Score (GOS) at 6 months after injury (Wilson et al., 1998). Information to assess GOS was obtained by direct patient contact in the context of a larger outcome assessment, or via telephone interview with the patient and/orˇa family member. The GOS score was determined using the following standard neurological parameters: *good recovery*: resumption of normal life despite minor deficit; *moderate disability*: person is disabled but independent, travels by public transportation, can work in sheltered setting (exceeds mere ability to perform activities of daily living); *severe disability*: the person is conscious but disabled, dependent for daily support (may or may not be institutionalized); *persistent vegetative state*: unresponsive and speechless; and *death* (Jennett and Bond, 1975). For the purpose of our analysis, outcome was further classified into two groups dichotomized GOS (good recovery/moderate disability versus severe disability/vegetative/dead) as previously described (Choi et al., 2002). GOS was compared to changes in levels of SBDPs from 6 through 120 h after admission.

Statistical Analysis

For statistical analysis, biomarker levels were treated as continuous data, measured in arbitrary densitometric units (adu) and expressed as mean $\pm$ SEM. Data were assessed for equality of variance and distribution. Appropriate univariate techniques were chosen according to the type of data; these include Fisher's Exact test, *t*-tests with pooled or separate variances as appropriate, and the Mann-Whitney *U* test. Statistical significance was set at a *p*-value of <0.05. Number of subjects at each time point (n values) represent patients who had both clinical information and CSF samples available for analysis. All analyses were performed using the statistical software package SPSS version 10.0.5.

RESULTS

Demographic data from patients whose data were analyzed in this study are shown in Table 1. Control samples were obtained from 11 patients with normotensive hydrocephalus (NPH) in whom a ventricular catheter was placed for routine clinical care. Figure 1 demonstrates a representative immunoblot showing both intact α-II-spectrin and α-II-spectrin breakdown products. In TBI patients. Levels of the 150 kDa α-II-spectrin breakdown product (SBDP150) were predominantly elevated in the first 24 h post-injury, while the 145-kDa α-II-spectrin breakdown product (SBDP145) remained significantly elevated up to 72 h post-injury. The 120-kDa α-II-spectrin breakdown product (SBDP120) was significantly elevated at all time points except 24 h post-injury in TBI patients, compared to control patients (Fig. 2).

Sample analysis focused mainly on the first 24 h post-TBI. Comparisons of CSF levels of SBDPs and severity of injury revealed no significant differences between patients with a post-resuscitation GCS score of 3–5 versus patients with less severe injury (GCS score of 6–8) in the

TABLE 1. DEMOGRAPHIC AND CLINICAL DATA
FOR ALL SUBJECTS INCLUDED IN THE STUDY

Characteristics	Total (n = 41)
Mean age (years)	38
Range	(18–67)
Gender male/female	33/8
Median scene GCS	4
Median post-resuscitation GCS	5
Marshall classification	
Diffuse Injury class I	2
Diffuse Injury class II	16
Diffuse Injury class III	5
Diffuse Injury class IV	0
Evacuated mass lesion	15
Non-evacuated mass lesion	3
Pre-ICU hypoxia	3
Pre-ICU hypotension	11
Intoxicated (alcohol or drugs)	12/20

Data from patients enrolled at three Level I trauma centers.
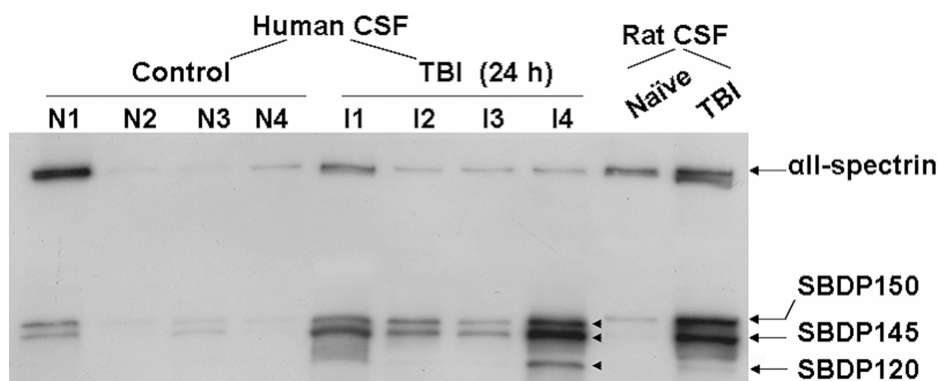
FIG. 1. Representative immunoblot of α-II-spectrin with individual control and TBI human CSF samples: Intact α-II-spectrin (280 kDa) and SBDP150, SBDP145 and SBDP120 are demonstrated. These are compared to rat CSF samples (control and TBI). Calpain produces two major α-II-spectrin breakdown products of 150-kDa and 145-kDa (SBDP150 and SBDP145) in a sequential manner. On the other hand, caspase-3 initially produces a 150-kDa SBDP that is further cleaved into a 120-kDa fragment (SBDP120). Immunoblots of α-II-spectrin degradation provide concurrent information on the activation of calpain and caspase-3.

first 24 h post-TBI (Table 2). Interestingly, patients whose GCS score did not improve (post-resuscitation GCS to best day 1) demonstrated significantly higher mean post-injury values of SBDP150 and SBDP145 SBDP ($p = 0.007$ and 0.01, respectively) but not SBDP120 ($p = 0.9$) in samples obtained within the first

12 h post-injury than patients who showed improvement. Patients with more severe injury on day 1 post TBI (best day 1 GCS score 3–5) had significantly higher mean levels of the SBDP150 and SBDP145 in the first 24 h post-injury ($p = 0.031$ and $p = 0.029$, respectively) as compared to patients with less severe injury (best day 1 GCS
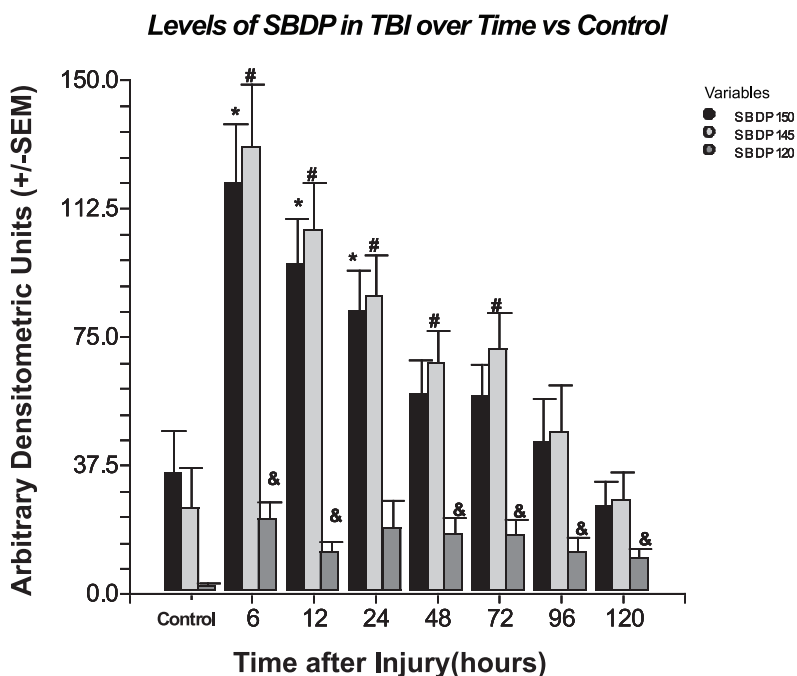
FIG. 2. Comparison of levels of SBDPs in patients with TBI versus control at all time points. Levels of the SBDP150 and SBDP145 were predominantly elevated in the first 24–72 h, post-injury, respectively, while the SBDP120 were significantly elevated at all time points except 24 h post-injury in TBI patients, compared to control patients ($n = 22$–47 for each time point; *,#,&$p < 0.05$ compared to control). Values represent arbitrary densitometry units (adu) means $\pm$ SEM.

score 6–8). This relationship was not seen for the SBDP120 (Fig. 3). Patients with more severe findings on admission CT scan (Marshall classification category I–II versus III–IV or presence of mass lesion) had similar mean 12- and 24-h levels of SBDPs post-injury (Table 2), but were noted to have significantly higher levels of the SBDP150 at the 48-h time point post-injury ($p = 0.02$). The difference was not significant for the SBDP145 or the SBDP120 ($p = 0.1$ and 0.1, respectively). Analysis was not conducted for mean values of samples obtained within 48 h after injury, given the number of samples available for each individual patient. We studied the relationship between pupillary abnormalities post-resuscitation and SBDPs in the first 24 h post-injury, but found no relationship between pupillary exam and SBDPs in CSF (Table 2). Mean SBDP150 and SBDP145 in the first 12 h post-injury were significantly elevated in patients with documented pre-hospital hypotension versus patients without hypotension (Fig. 4). This relationship was not significant for the SBDP120.

When individual time points were analyzed, only the 12-h post-injury SBDP145 elevation reached statistical significance, although the SBDP150 trended towards significance at this same time point ($p = 0.04$ and $p = 0.06$, respectively).

To study the correlation between the change in CSF SBDP levels and clinical outcome in TBI patients, we dichotomized clinical outcome into two groups (good recovery/moderate disability vs. severe disability/vegetative/dead) as previously described (Choi et al., 2002). As shown in Figure 5 and Table 2, our analysis revealed a statistically significant association between clinical outcome and the SBDP150 and SBDP145 but not the SBDP120 at 12 h post-injury ($p = 0.01$, $p = 0.02$, and $p = 0.07$, respectively). This relationship remained significant for the SBDP150 and SBDP145 but not the SBDP120 when the mean values of samples all samples collected within 12 h post-injury were compared ($p = 0.01$, $p = 0.02$, and $p = 0.7$, respectively). Analysis comparing SBDPs and mortality revealed higher levels of the

TABLE 2. RELATIONSHIPS BETWEEN SBDP VALUES AND CLINICAL VARIABLES

| Clinical variable	SBDP150		SBDP145		SBDP120	
	12 h	24 h	12 h	24 h	12 h	24 h
Post-resuscitation GCS						
3–5	88.5 ± 18	88 ± 14	97.8 ± 18	92.8 ± 14	13.4 ± 4	16.3 ± 6
6–8	107.9 ± 15	84.7 ± 15	120.3 ± 17	86 ± 15	18.9 ± 7	13.5 ± 6
Best day–1GCS						
3–5	138.4 ± 25[a]	120.3 ± 18[a]	144.8 ± 27	124.8 ± 19[a]	17.6 ± 4	24.2 ± 12
6–8	82.6 ± 12	73 ± 11	94.3 ± 14	75.6 ± 11	15 ± 4	11.3 ± 4
Improvement in GCS						
No	121.9 ± 16[a]	103.8 ± 14[a]	130.4 ± 18[a]	104.7 ± 14	16.7 ± 4	16.2 ± 6
Yes	66.7 ± 14	60.5 ± 12	79.2 ± 15	67.1 ± 13	14.5 ± 6	13.2 ± 7
Admission CT scan						
I–II	94.3 ± 16	100.5 ± 13	104.5 ± 17	101.5 ± 13	10.9 ± 4	8.6 ± 2
III–IV mass lesion	98.5 ± 18	74.7 ± 14	109 ± 19	79.7 ± 16	19.7 ± 5	20.4 ± 8
Pupillary abnormalities						
No	108.7 ± 18	100 ± 18	119.4 ± 18	107.2 ± 18	16.2 ± 4	20.1 ± 9
Yes	87.9 ± 16	74.1 ± 12	98 ± 18	78.5 ± 13	15.3 ± 5	12.6 ± 4
Pre-hospital hypotension						
No	81.8 ± 14[b]	81.3 ± 12	91.5 ± 14[b]	84.1 ± 12	14.3 ± 4	13.8 ± 5
Yes	140.7 ± 15	104.1 ± 14	153.1 ± 17	108.3 ± 15	19.9 ± 8	19 ± 11
GOS 6 months post-injury						
Poor outcome	121.4 ± 15[c]	98.1 ± 13	131.2 ± 16[c]	99 ± 14	17.6 ± 4	19.2 ± 6
Good outcome	60 ± 16	69.8 ± 16	72.6 ± 19	77.6 ± 17	14.4 ± 7	9.4 ± 4

Mean (± SEM) for SBDP arbitrary densitometric units (mean value for samples obtained within the first 12 h and 24 h post-injury, respectively) after severe TBI. Admission CT scans were classified according to the Marshall Classification. Pupillary abnormalities represent post-resuscitation abnormal size and or abnormal reaction to light. GCS, Glasgow Coma Scale; GOS, Glasgow Outcome Scale; Hypotension is defined as systolic blood pressure of <90 mm Hg.

[a]$p < 0.05$ versus GCS 6–8.
[b]$p < 0.05$ versus no hypotension present.
[c]$p < 0.05$ versus Good Outcome.

Mean 24-Hour SBDP Levels and Day-1 GCS 3-5 vs GCS 6-8
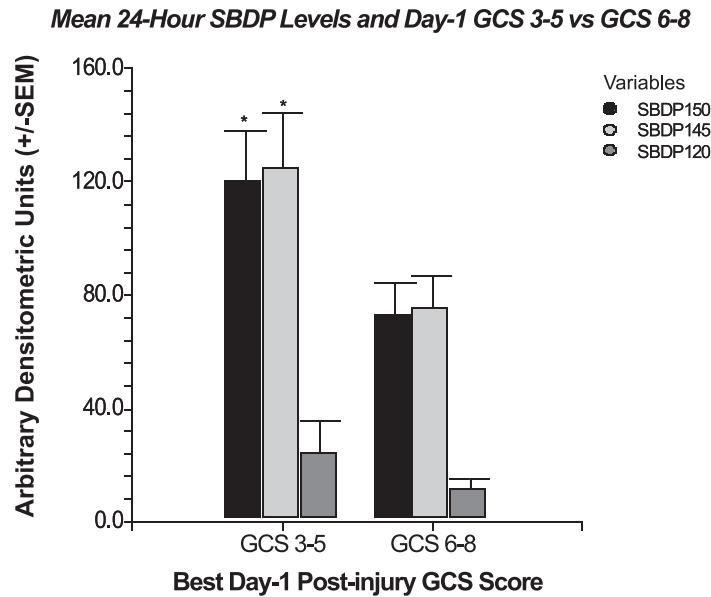
FIG. 3. Comparison of levels of SBDPs in patients with a GCS score of 3–5 and GCS score of 6–8 in the first 24 h post-injury. Mean (±SEM) arbitrary densitometric units (adu) for αII-spectrin 150-kDa, 145-kDa, and 120-kDa SBDPs. Mean 24-h levels of SBDP150 and SBSP145 are significantly elevated in patients with GCS 3–5 compared to those with a GCS of 6–8 ($n = 35$; $p = 0.031$ and $p = 0.029$, respectively). This relationship was not seen for the 120-kDa SBDP ($p = 0.182$).

SBDP150 and SBDP145 but not SBDP120 at 12 h post-injury in patients who died ($p = 0.04$, $p = 0.03$, and $p = 0.98$, respectively). This relationship remained unchanged when the mean values of all samples collected within 12 h post-injury were compared ($p = 0.01$, $p = 0.02$, and $p = 0.8$, respectively).

DISCUSSION

Examination of α-II-spectrin proteolysis is a powerful technique that has been studied extensively in animal models to characterize the mechanisms of calpain and caspase-3 mediated proteolysis following both TBI (Beer et al., 2000; Buki et al., 1999; Knoblach et al., 2002; Newcomb et al., 1997; Pike et al., 1998; Ringger et al., 2004; Saatman et al., 1996; Siman et al., 2004; Yakovlev et al., 1997) and acute cerebral ischemia (Pike et al., 2004; Siman et al., 2005). This present work extends findings collected in animal models and humans following a severe head injury and supports the hypothesis that breakdown products of α-II-spectrin are a potentially reliable marker of severe TBI in humans. Importantly, this approach possesses the ability to distinguish between brain-injured patients and uninjured controls as levels of α-II-spectrin and SBDPs were significantly elevated in injured subjects across most time points examined. Moreover, SBDP 150 and SBDP145 peak at 6 h post-injury and re-

main significantly elevated for 24 and 72 h, respectively, while SBDP120 has a initial peak at 6 h, followed by a sustained elevation that persisted for at least 5 days post-injury (Fig. 2). The SBDP150, predominantly produced by calpain activity, exhibited a time course similar to that of the SBDP145, a signature product of calpain activity (Fig. 1). Elevations of calpain-related SBDPs correlated significantly with initial severity of injury (presence of pre-hospital hypotension, best day-1 GCS score, Marshall CT scan classification) and 6-month outcome. Samples obtained 12 h post-injury and the mean value of all samples obtained within the first 12 h post-injury appear to be the strongest predictors of severity of outcome. Analysis of the earliest sample collection time point (6 h) was limited by the number of samples available. In contrast to the best day-1 GCS score, the post-resuscitation GCS score did not correlate with SBDPs. This could be explained by the limitations of the GCS in assessing severity of injury (Stocchetti et al., 2004), for example, due to the presence of alcohol, sedative drugs, and neuromuscular blockade agents. Lower values of SBDPs in patients whose GCS score improved in the first 24 h post-injury support this conclusion.

Together, these findings suggest that the temporal profile of biomarker changes is an important predictor of clinical outcome. Equally important, these data indicate that there may be a critical period following injury during which these markers predict injury severity. We are

Mean 12-Hour SBDP Levels and Pre-ICU Hypotension
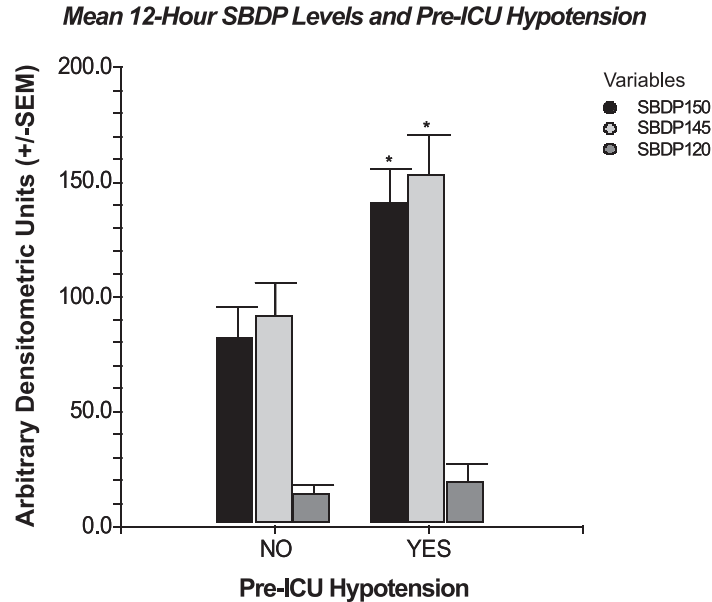
FIG. 4. Comparison of levels of SBDPs measured within 12 h post-injury in patients with pre-ICU hypotension versus no pre-ICU hypotension. Mean ($\pm$SEM) SBDP150 and SBDP145 in the first 12 h post-injury were significantly elevated in patients with documented pre-hospital hypotension versus patients without hypotension ($p = 0.03$ and $p = 0.034$; $n = 6$ and 14, respectively). This relationship was not significant for the 120-kDa SBDP ($p = 0.484$).

conducting kinetic studies of SBDPs in CSF to further define optimal frequency and timing of sampling after TBI in humans.

Caspase-3–related SBDP120 was consistently elevated after injury but did not correlate with the initial severity of injury or 6-month outcome. This study was limited to the early changes in the biomarkers, while the activity of the caspase-3 pathway may be more important at later times. Also the activity of the caspase-3 pathway over several days may be more related to outcome than the values on any given day. Current studies of biomarker kinetics will explore this possibility. Alternatively, caspase-3 may not be as important as calpain in determining injury magnitude and outcome in humans after severe TBI.

Our analysis revealed no correlation between post-resuscitation pupillary exam, an important clinical finding with a 70% positive predictive power to prognosticate outcome, and SBDPs (Narayan et al., 1981). This finding can be explained by both the limited ability of pupillary exam to predict outcome and the pathophysiology of pupillary changes after TBI (Meyer et al., 1993; Ritter et al., 1999).

The use of α-II-spectrin BDPs as a biomarker confers a number of advantages over other biomarkers currently being investigated. Because this protein is not found in erythrocytes, blood contamination is not a confound (Pike et al., 2001). Moreover, rapid appearance of a biomarker

in biological material is imperative, and SBDPs are detected in CSF within 2 h of experimental TBI (Ringger et al., 2004). Since we are examining CSF, contribution from other non-CNS tissue is probably minimal. However, these studies cannot unequivocally exclude the potential contribution to SBDP accumulation from injury to peripheral organs. Future studies will need to rigorously examine this issue with the appropriate poly-trauma control subjects.

Importantly, examination of α-II-spectrin proteolysis provides insight into the underlying proteolytic mechanisms at work after a head injury. Specifically, this technique allows concurrent examination of both calpain and caspase-3 mediated proteolysis following injury and in the present study, allowed confirmation of the role of cysteine proteases after TBI in humans and provides initial insight into the time course after injury of these two important brain injury pathways. There is not a simple, binary relationship between phenotypic expression of apoptosis and necrosis, and activation of caspase-3 and calpain. As reviewed in detail by Wang (2000), calpain is the primary mediator of necrotic/oncotic cell death with no measurable contribution of activation of caspase-3. However, calpain can also contribute in some cases to the phenotypic expression of apoptotic cell death (Wang, 2000). In fact, Newcomb-Fernandez et al. (2001) demonstrated that calpain and caspase-3 activation is reliably associated with expression of apoptotic cell death phe-

361
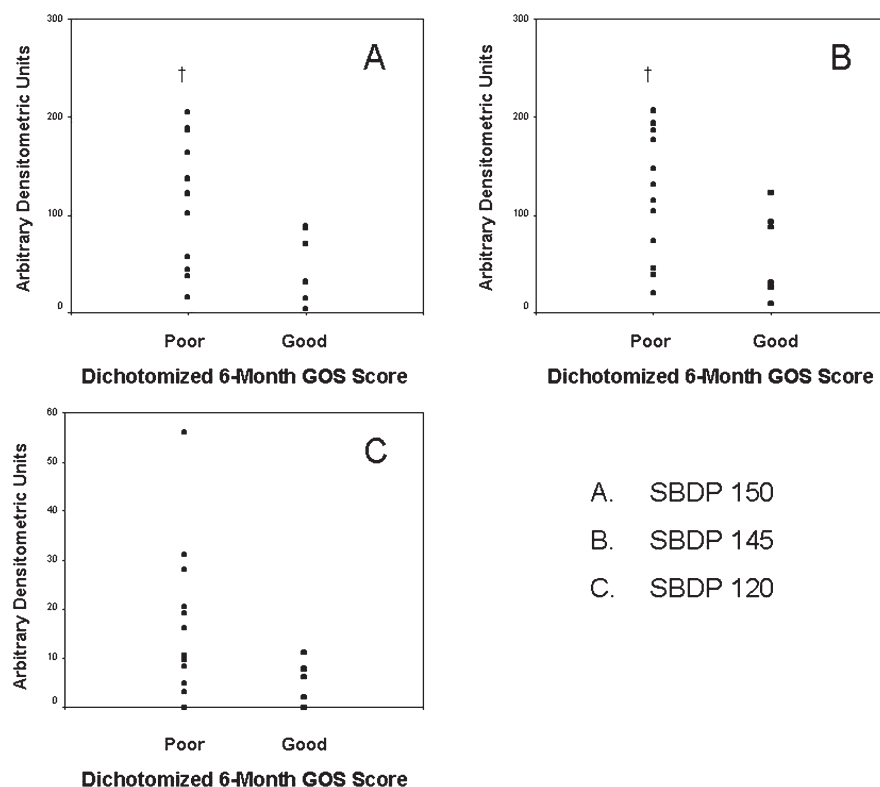
FIG. 5. Comparison of levels of SBDPs taken 12 h post-injury in patients with poor outcome versus good outcome at 6 months. Patients with poor outcome had significantly higher values of the SBDP150 and SBDP145 at 12 h post-injury when compared to patients with good outcome ($p = 0.01$ and $p = 0.02$). SBDP120 values were not significantly different between these two groups ($p = 0.07$). †$p < 0.05$ versus good outcome.

notypes after oxygen-glucose deprivation in primary septo-hippocampal cultures. Thus, although necrosis and apoptosis have traditionally been defined by phenotype, cell death processes may be more meaningfully described by biochemical mechanisms than by phenotypic analysis alone. In any case, our data suggests a significant contribution of calpain and caspase-3 mediated proteolysis to the pathobiology of human TBI. As shown by the significant increases in both calpain and caspase-3 mediated SBDPs, our preliminary data demonstrate that necrotic/oncotic and apoptotic cell death mechanisms overlap but appear to be activated at distinct time patterns in humans after a severe TBI. More detailed studies will be required to provide more definitive statements about the relevant contribution of calpain and caspase-3 to injury and outcome.

While other potential biomarkers distinguish between injured patients and control subjects (Berger et al., 2002; Ross et al., 1996; Varma et al., 2003; Zemlan et al., 2002), they provide limited insight into the specific mechanisms of brain damage following CNS injury. Though tau is specific to the CNS, it provides no information about spe-

cific neurochemical events that have occurred in the injured CNS since the specific protease potentially responsible for tau cleavage has not been identified. Moreover, many of these biomarkers have limited sensitivity and specificity for detecting damage, and questionable ability to predict clinical outcome after TBI, particularly NSE (Raabe et al., 1998, 1999; Ross et al., 1996). Serum assessments of c-tau have relatively low sensitivity in patients with TBI (Shaw et al., 2002). Elevated levels of S-100β have also been detected in trauma patients not experiencing a head injury (Anderson et al., 2001; Pelinka et al., 2003). Current studies of patients with multitrauma will help address the effect of extracranial injuries in SBDP values. A recent study in our laboratory demonstrated that severity of injury, lesion size, and behavioral deficits were positively correlated with levels of SBDPs in CSF from rats subjected to experimental TBI, suggesting that this biomarker can predict the magnitude of injury and resulting functional deficits. In contrast, levels of S-100β in the CSF did not correlate with lesion size in this model (Ringger et al., 2004).

While these data are encouraging, the authors rec-

ognize there are limitations to this study. αII-Spectrin can be cleaved to a 150-kDa SBDP by both calpain (SBDP150) and caspase-3 (SBDP150i), though these proteases act at different cleavage sites, producing fragments with different amino acid sequences (Wang, 2000). The techniques used in the current study were not capable of differentiating between the calpain-mediated and caspase-3–mediated SBDP150. However, as shown in Figure 1, the majority of SBDP150 was subsequently processed to SBDP145 by calpain. Our laboratory is now developing novel antibodies that specifically recognize calpain- and caspase-3–mediated SBDP150 fragments. In addition, the current study was performed in a limited cohort of patients with severe TBI, a disease that tends to be heterogeneous in nature. Nevertheless, this work lays the groundwork for future clinical studies examining the utility of αII-spectrin as a biomarker in larger sample sizes. Though increases in levels of the SBDP150 and SBDP145 were significantly correlated with initial severity of injury and 6-month outcome, no such correlation was demonstrated with the SBDP120 in this study. Future studies with a larger sample may uncover a relationship between levels of this SBDP and patient outcome. Such studies will also allow multivariate analysis including relevant clinical variables that can influence outcome.

Ongoing studies in our laboratories are focused on developing a serum-based assay sensitive enough to detect these SBDPs in blood. This minimally invasive assay will allow assessment of these biomarkers in injured patients not requiring ICP monitoring (e.g., patients with mild or moderate head injuries). Blood-based assays will also facilitate the development of appropriate normative data for calculations of sensitivity and specificity, and will help eliminate limitations posed by the nature of our control samples, such as the absence of multiple time point measurements in control patients. Control samples were obtained intraoperatively and therefore no meaningful increases in biomarker levels due to procedures such a ventriculostomy placement were anticipated. The effect of ventriculostomy placement on biomarker levels in TBI patients is not known. A blood based assay will avoid this potential limitation.

Because we do not have a solid cutoff for normal subjects, sensitivity and specificity analysis, as well as predictive power calculations were not done in this group of patients. Additionally, as shown by this work and others (Berger et al., 2002; Herrmann et al., 1999; Raabe et al., 1999; Zemlan et al., 2002), the temporal profile of changes in biomarker levels is an important factor in determining clinical outcome. Biomarker kinetic studies with more frequent sampling early after injury and logistic regression analysis in a larger sample size will fur-

ther define the clinical utility of SBDPs as a clinical biomarker. Larger subsequent studies will address clinical variables—such as gender and age—that are known to influence clinical outcome and the biochemical response to TBI (Roof, 2000; Hukkelhoven, 2003; Campbell, 2004). Such studies will be crucial to our understanding of the relationship between this biomarker and clinical outcome, particularly in patients experiencing different severities of head injury (spectrum of mild to severe trauma) and in deciphering how the temporal profile of biomarker changes relates to clinical outcome.

REFERENCES

ANDERSON, R.E., HANSSON, L.O., NILSSON, O., DIJLAI-MERZOUG, R., and SETTERGREN, G. (2001). High serum S100B levels for trauma patients without head injuries. Neurosurgery **48,** 1255–1260.

AZOUVI, P. (2000). Neuroimaging correlates of cognitive and functional outcome after traumatic brain injury. Curr. Opin. Neurol. **13,** 665–669.

BEER, R., FRANZ, G., SRINIVASAN, A., et al. (2000). Temporal profile and cell subtype distribution of activated caspase-3 following experimental traumatic brain injury. J. Neurochem. **75,** 1264–1273.

BERGER, R.P., PIERCE, M.C., WISNIEWSKI, S.R., et al. (2002). Neuron-specific enolase and S100B in cerebrospinal fluid after severe traumatic brain injury in infants and children. Pediatrics **109,** E31.

BINDER, S., CORRIGAN, J.D., and LANGLOIS, J.A. (2005). The public health approach to traumatic brain injury: an overview of CDC's research and programs. J. Head Trauma Rehabil. **20,** 189–195.

BRAIN TRAUMA FOUNDATION. American Association of Neurological Surgeons. Joint Section on Neurotrauma and Critical Care (2000a). Hypotension. J. Neurotrauma **17,** 591–595.

BRAIN TRAUMA FOUNDATION. American Association of Neurological Surgeons. Joint Section on Neurotrauma and Critical Care (2000b). Pupillary diameter and light reflex. J. Neurotrauma **17,** 583–590.

BUKI, A., SIMAN, R., TROJANOWSKI, J.Q., and POVLI-SHOCK, J.T. (1999). The role of calpain-mediated spectrin proteolysis in traumatically induced axonal injury. J. Neuropathol. Exp. Neurol. **58,** 365–375.

CAMPBELL, C.G., KUEHN, S.M., RICHARDS, P.M., VENTUREYRA, E., and HUTCHISON, J.S. (2004). Medical and cognitive outcome in children with traumatic brain injury. Can. J. Neurol. Sci. **31,** 213–219.

CHATFIELD, D.A., ZEMLAN, F.P., DAY, D.J. and MENON, D.K. (2002). Discordant temporal patterns of S100beta and cleaved tau protein elevation after head injury: a pilot study. Br. J. Neurosurg. **16,** 471–476.

CHOI, D.W. (2002). Exploratory clinical testing of neuroscience drugs. Nat. Neurosci. **5,** Suppl, 1023–1025.

CHOI, S.C., CLIFTON, G.L., MARMAROU, A., and MILLER, E.R. (2002). Misclassification and treatment effect on primary outcome measures in clinical trials of severe neurotrauma. J. Neurotrauma **19,** 17–22.

COOPER, E. (1994). Neuron-specific enolase. Int. J. Biol. Markers **9,** 205–210.

FARKAS, O., POLGAR, B., SZEKERES-BARTHO, J., DOCZI, T., POVLISHOCK, J.T., and BUKI, A. (2005). Spectrin breakdown products in the cerebrospinal fluid in severe head injury—preliminary observations. Acta Neurochir. (Wien) **147,** 855–861.

FRIDRIKSSON, T., KINI, N., WALSH-KELLY, C., and HENNES, H. (2000). Serum neuron-specific enolase as a predictor of intracranial lesions in children with head trauma: a pilot study. Acad. Emerg. Med. **7,** 816–820.

GOODMAN, S.R., ZIMMER, W.E., CLARK, M.B., ZAGON, I.S., BARKER, J.E., and BLOOM, M.L. (1995). Brain spectrin: of mice and men. Brain Res. Bull. **36,** 593–606.

GUAN, W., YANG, Y.L., XIA, W.M., LI, L., and GONG, D.S. (2003). Significance of serum neuron-specific enolase in patients with acute traumatic brain injury. Chin. J. Traumatol. **6,** 218–221.

HALL, E.D., SULLIVAN, P.G., GIBSON, T.R., PAVEL, K.M., THOMPSON, B.M., and SCHEFF, S.W. (2005). Spatial and temporal characteristics of neurodegeneration after controlled cortical impact in mice: more than a focal brain injury. J. Neurotrauma **22,** 252–265.

HANLON, R.E., DEMERY, J.A., MARTINOVICH, Z., and KELLY, J.P. (1999). Effects of acute injury characteristics on neurophysical status and vocational outcome following mild traumatic brain injury. Brain Inj. **13,** 873–887.

HAYES, N.V., PHILLIPS, G.W., CARDEN, M.J., and BARNES, A.J. (1997). Definition of a sequence unique in beta II spectrin required for its axon-specific interaction with fodaxin (A60). J. Neurochem. **68,** 1686–1695.

HERRMANN, M., CURIO, N., JOST, S., WUNDERLICH, M.T., SYNOWITZ, H., and WALLESCH, C.W. (1999). Protein S-100B and neuron-specific enolase as early neurobio-chemical markers of the severity of traumatic brain injury. Restor. Neurol. Neurosci. **14,** 109–114.

HUKKELHOVEN, C.W., STEYERBERG, E.W., RAMPEN, A.J. et al. (2003). Patient age and outcome following severe traumatic brain injury: an analysis of 5600 patients. J. Neurosurg. **99,** 666–673.

INGEBRIGTSEN, T., and ROMNER, B. (2002). Biochemical serum markers of TBI. J. Trauma **52,** 798–808.

JENNETT, B., and BOND, M. (1975). Assessment of outcome after severe brain damage. Lancet **1,** 480–484.

JOHNSSON, P. (1996). Markers of cerebral ischemia after cardiac surgery. J. Cardiothorac. Vasc. Anesthesia **10,** 120–126.

KNOBLACH, S.M., NIKOLAEVA, M., HUANG, X., et al. (2002). Multiple caspases are activated after traumatic brain injury: evidence for involvement in functional outcome. J. Neurotrauma **19,** 1155–1170.

KURTH, S.M., BIGLER, E.D., and BLATTER, D.D. (1994). Neuropsychological outcome and quantitative image analysis of acute haemorrhage in traumatic brain injury: preliminary findings. Brain Inj. **8,** 489–500.

LANGLOIS, J.A., MARR, A., MITCHKO, J., and JOHNSON, R.L. (2005). Tracking the silent epidemic and educating the public: CDC's traumatic brain injury–associated activities under the TBI Act of 1996 and the Children's Health Act of 2000. J. Head Trauma Rehabil. **20,** 196–204.

MARSHALL, L.F., MARSHALL, S.B., KLAUBER, M.R., et al. (1992). The diagnosis of head injury requires a classification based on computed axial tomography. J. Neurotrauma **9,** Suppl 1, S287–S292.

MEYER, S., GIBB, T., and JURKOVICH, G.J. (1993). Evaluation and significance of the pupillary light reflex in trauma patients. Ann. Emerg. Med. **22,** 1052–1057.

MICHETTI, F., and GAZZOLO, D. (2002). S100B protein in biological fluids: a tool for perinatal medicine. Clin. Chem. **48,** 2097–2104.

NARAYAN, R.K., GREENBERG, R.P., MILLER, J.D., et al. (1981). Improved confidence of outcome prediction in severe head injury. A comparative analysis of the clinical examination, multimodality evoked potentials, CT scanning, and intracranial pressure. J. Neurosurg. **54,** 751–762.

NEWCOMB, J.K., KAMPFL, A., POSMANTUR, R.M., et al. (1997). Immunohistochemical study of calpain-mediated breakdown products to alpha-spectrin following controlled cortical impact injury in the rat. J. Neurotrauma **14,** 369–383.

NEWCOMB-FERNANDEZ, J.K., ZHAO, Y., PIKE, B.R., et al. (2001). Concurrent assessement of calpain and caspase-3 activation after oxygen-glucose deprivation in primary septo-hippocampal cultures. J. Cereb. Blood Flow Metab. **21,** 1281–1294.

PELINKA, L.E., TOEGEL, E., MAURITZ, W., and REDL, H. (2003). Serum S 100 B: a marker of brain damage in trau-

matic brain injury with and without multiple trauma. Shock **19,** 195–200.

PHUENPATHOM, N., CHOOMUANG, M., and RATANALERT, S. (1993). Outcome and outcome prediction in acute subdural hematona. Surg. Neurol. **40,** 22–25.

PIKE, B.R., FLINT, J., DAVE, J.R., et al. (2004). Accumulation of calpain and caspase-3 proteolytic fragments of brain-derived alphaII-spectrin in cerebral spinal fluid after middle cerebral artery occlusion in rats. J. Cereb. Blood Flow Metab. **24,** 98–106.

PIKE, B.R., FLINT, J., DUTTA, S., JOHNSON, E., WANG, K.K., and HAYES, R.L. (2001). Accumulation of non-erythroid alpha II–spectrin and calpain-cleaved alpha II–spectrin breakdown products in cerebrospinal fluid after traumatic brain injury in rats. J. Neurochem. **78,** 1297–1306.

PIKE, B.R., ZHAO, X., NEWCOMB, J.K., POSMANTUR, R.M., WANG, K.K., and HAYES, R.L. (1998). Regional calpain and caspase-3 proteolysis of alpha-spectrin after traumatic brain injury. Neuroreport **9,** 2437–2442.

PINEDA, J.A., WANG, K.K., and HAYES, R.L. (2004). Biomarkers of proteolytic damage following traumatic brain injury. Brain Pathol. **14,** 202–209.

RAABE, A., GROLMS, C., KELLER, M., DOHNERT, J., SORGE, O., and SEIFERT, V. (1998). Correlation of computed tomography findings and serum brain damage markers following severe head injury. Acta Neurochir. (Wien) **140,** 787–792.

RAABE, A., GROLMS, C., and SEIFERT, V. (1999). Serum markers of brain damage and outcome prediction in patients after severe head injury. Br. J. Neurosurg. **13,** 56–59.

RAABE, A., KOPETSCH, O., WOSZCZYK, A., et al. (2003). Serum S-100B protein as a molecular marker in severe traumatic brain injury. Restor. Neurol. Neurosci. **21,** 159–169.

RIEDERER, B.M., ZAGON, I.S., and GOODMAN, S.R. (1986). Brain spectrin (240/235) and brain spectrin (240/235E): two distinct spectrin subtypes with different locations within mammalian nueral cells. J. Cell Biol. **102,** 2088–2097.

RIEDERER, B.M., ZAGON, I.S., and GOODMAN, S.R. (1987). Brain spectrin(240/235) and brain spectrin(240/235E): differential expression during mouse brain development. J. Neurosci. **7,** 864–874.

RINGGER, N.C., O'STEEN, B.E., BRABHAM, J.G., et al. (2004). A novel marker for traumatic brain injury: CSF alphaII-spectrin breakdown product levels. J. Neurotrauma **21,** 1443–1456.

RITTER, A.M., MUIZELAAR, J.P., BARNES, T., et al. (1999). Brain stem blood flow, pupillary response, and outcome in patients with severe head injuries. Neurosurgery **44,** 941–948.

ROBERTS-LEWIS, J.M., and SIMAN, R. (1993). Spectrin proteolysis in the hippocampus: a biochemical marker for neuronal injury and neuroprotection. Ann. N.Y. Acad. Sci. **679,** 78–86.

ROOF, R.L., and HALL, E.D. (2000). Gender differences in acute CNS trauma and stroke: neuroprotective effects of estrogen and progesterone. J. Neurotrauma **17,** 367–388.

ROSS, S.A., CUNNINGHAM, R.T., JOHNSTON, C.F., and ROWLANDS, B.J. (1996). Neuron-specific enolase as an aid to outcome prediction in head injury. Br. J. Neurosurg. **10,** 471–476.

SAATMAN, K.E., BOZYCZKO-COYNE, D., MARCY, V., SIMAN, R., and McINTOSH, T.K. (1996). Prolonged calpain-mediated spectrin breakdown occurs regionally following experimental brain injury in the rat. J. Neuropathol. Exp. Neurol. **55,** 850–860.

SEUBERT, P., LARSON, J., OLIVER, M., JUNG, M.W., BAUDRY, M., and LYNCH, G. (1988). Stimulation of NMDA receptors induces proteolysis of spectrin in hippocampus. Brain Res. **460,** 189–194.

SHAW, G.J., JAUCH, E.C., and ZEMLAN, F.P. (2002). Serum cleaved tau protein levels and clinical outcome in adult patients with closed head injury. Ann. Emerg. Med. **39,** 254–257.

SIMAN, R., and NOSZEK, J.C. (1988). Excitatory amino acids activate calpain I and induce structural protein breakdown *in vivo*. Neuron **1,** 279–287.

SIMAN, R., NOSZEK, J.C., and KEGERISE, C. (1989). Calpain I activation is specifically related to excitatory amino acid induction of hippocampal damage. J. Neurosci. **9,** 1579–1590.

SIMAN, R., McINTOSH, T.K., SOLTESZ, K.M., CHEN, Z., NEUMAR, R.W., and ROBERTS, V.L. (2004). Proteins released from degenerating neurons are surrogate markers for acute brain damage. Neurobiol. Dis. **16,** 311–320.

SIMAN, R., ZHANG, C., ROBERTS, V.L., PITTS-KIEFER, A., and NEUMAR, R.W. (2005). Novel surrogate markers for acute brain damage: cerebrospinal fluid levels corrrelate with severity of ischemic neurodegeneration in the rat. J. Cereb. Blood Flow Metab. **25,** 1433–1444.

SKOGSEID, I.M., NORDBY, H.K., URDAL, P., PAUS, E., and LILLEAAS, F. (1992). Increased serum creatine kinase BB and neuron specific enolase following head injury indicates brain damage. Acta Neurochir. (Wien) **115,** 106–111.

STOCCHETTI, N., PAGAN, F., CALAPPI, E., et al. (2004). Inaccurate early assessment of neurological severity in head injury. J. Neurotrauma **21,** 1131–1140.

TEASDALE, G., and JENNETT, B. (1976). Assessment and prognosis of coma after head injury. Acta Neurochir. (Wien) **34,** 45–55.

VARMA, S., JANESKO, K.L., WISNIEWSKI, S.R., et al. (2003). F2-isoprostane and neuron-specific enolase in cerebrospinal fluid after severe traumatic brain injury in infants and children. J. Neurotrauma **20,** 781–786.

WANG, K.K. (2000). Calpain and caspase: can you tell the difference? Trends Neurosci. **23,** 20–26.

WANG, K.K., POSMANTUR, R., NATH, R., et al. (1998). Simultaneous degradation of alphaII- and betaII-spectrin by caspase 3 (CPP32) in apoptotic cells. J. Biol. Chem. **273,** 22490–22497.

WILSON, J.T., HADLEY, D.M., WIEDMANN, K.D., and TEASDALE, G.M. (1995). Neuropsychological consequences of two patterns of brain damage shown by MRI in survivors of severe head injury. J. Neurol. Neurosurg. Psychiatry **59,** 328–331.

WILSON, J.T., PETTIGREW, L.E., and TEASDALE, G.M. (1998). Structured interviews for the Glasgow Outcome Scale and the extended Glasgow Outcome Scale: guidelines for their use. J. Neurotrauma **15,** 573–585.

WOERTGEN, C., ROTHOERL, R.D., HOLZSCHUH, M., METZ, C., and BRAWANSKI, A. (1997). Comparison of serial S-100 and NSE serum measurements after severe head injury. Acta Neurochir. (Wien) **139,** 1161–1165.

YAKOVLEV, A.G., KNOBLACH, S.M., FAN, L., FOX, G.B., GOODNIGHT, R., and FADEN, A.I. (1997). Activation of CPP32-like caspases contributes to neuronal apoptosis and neurological dysfunction after traumatic brain injury. J. Neurosci. **17,** 7415–7424.

YAMAZAKI, Y., YADA, K., MORII, S., KITAHARA, T., and OHWADA, T. (1995). Diagnostic significance of serum neuron-specific enolase and myelin basic protein assay in patients with acute head injury. Surg. Neurol. **43,** 267–271.

ZEMLAN, F.P., JAUCH, E.C., MULCHAHEY, J.J., et al. (2002). C-tau biomarker of neuronal damage in severe brain injured patients: association with elevated intracranial pressure and clinical outcome. Brain Res. **947,** 131–139.

ZEMLAN, F.P., ROSENBERG, W.S., LUEBBE, P.A., et al. (1999). Quantification of axonal damage in traumatic brain injury: affinity purification and characterization of cerebrospinal fluid tau proteins. J. Neurochem. **72,** 741–750.

Address reprint requests to:
Jose A. Pineda, M.D.
Washington University School of Medicine
Division of Critical Care Medicine
Campus Box 8116
One Children's Place, Suite 5S20
St. Louis, MO 63110

E-mail: Pineda_J@kids.wustl.edu