Abstract. αII-spectrin breakdown products are regarded as potential biomarkers for traumatic brain injury (TBI). The aim of the present study was to further evaluate these biomarkers by assessing their clinical utility in predicting the severity of injury and clinical outcome of patients with TBI. Eligible patients with acute TBI (n=17), defined by a Glasgow Coma Scale (GCS) score of ≤8, were enrolled. Ventricular cerebrospinal fluid (CSF) was sampled from each patient at 24, 72 and 120 h following TBI. An immunoblot assay was used to determine the concentrations of SBDPs in the CSF samples. The concentrations of SBDPs combined with the GCS score at 24 h after injury and the Glasgow Outcome Score (GOS) at 30 days after injury were compared and analyzed. The levels of SBDPs in CSF were markedly increased following acute TBI in comparison with those in the control group. In the early period after TBI, the levels of SBDPs were closely associated with GCS score. Comparisons of the SBDP levels with the severity of injury revealed significant differences between patients with the most severe brain injury and patients with severe brain injury in the first 24 h post-injury (P<0.05). The levels and dynamic changes of SBDPs in CSF exhibited a close association with GOS at 30 days after injury. The levels of SBDPs differed significantly between patients grouped according to prognosis (P<0.05). These results suggest that in the early period after TBI, the levels and dynamic changes of SBDPs in CSF can be useful in the prediction of the severity of injury and clinical outcome of patients.

Introduction

At present, the severity of a traumatic brain injury (TBI) and the clinical outcome of acute TBI are predicted based on patient history, clinical manifestations, a clinical examination [Glasgow Coma Scale (GCS)] and cranial computed tomography findings (1). However, these commonly used techniques have significant limitations, including their ability to predict outcomes or detect subtle damage (2,3). Rapid, definitive diagnostic tests for TBI that enable physicians to determine the seriousness of an injury on the basis of quantifiable neurochemical markers, and to guide the implementation of the appropriate triage and medical management, are required.

Oncotic necrosis is characterized by cell and organelle swelling, leading to nuclear degradation, disruption of the cell membrane and cell lysis (4,5). Typically, when oncotic necrosis occurs, activation of members of the calpain family is upregulated (6). Conversely, apoptosis is characterized by chromatin condensation, internucleosomal DNA fragmentation, cell shrinkage and cell dismantlement into membrane-enclosed vesicles (7). In addition, it typically involves the activation of a specific family of proteases termed caspases (8). Generally, the oncotic necrosis of neurons is observed during the acute period following TBI, in particular within contused or hemorrhaging regions, whereas apoptotic neurons are detected a few days or weeks post-trauma (9,10). Importantly, the calpains and caspases produced during these processes may be evaluated as neurochemical markers of brain injury.

α-II-spectrin is a cytoskeletal protein that is a substrate for the calcium-activated cysteine proteases calpain and caspase-3. Calpain and caspase-3 cleave α-II-spectrin to generate α-II-spectrin breakdown products (SBDPs), including SBDP145, SBDP150 and SBDP120. Following acute neuronal injury, increased levels of SBDPs have been detected in several models of neuronal injury (11-13). In addition, following TBI, increased levels of calcium and calpain have been shown to correlate well with the magnitude of injury in rat models (14,15). Therefore, the present study aimed to investigate the potential use of SBDPs in the cerebrospinal fluid (CSF) as a biomarker of TBI.
Materials and methods

Patients. A total of 17 patients (5 women and 12 men) with an isolated head injury who had been admitted to the Emergency Department and Neurosurgical Intensive Care Unit of the Nanjing First Hospital (Nanjing, China), between June 2009 and December 2010, were included in this prospective study. The inclusion criteria were as follows: The patient had to have sustained a severe head injury with a GCS score of ≤8 and the patient required ventricular intracranial pressure monitoring. The patients were followed for 30 days following study entry. The age of the patients ranged from 24-68 years (mean age, 51.8±12.9 years). All patients were admitted to the hospital <6 h after sustaining the head injury. Patient consent was obtained within 24 h of study enrollment. The present study was approved by the Institutional Review Board of the Third Clinical Medical College of Nanjing Medical University.

Clinical variables that were potentially associated with outcome were recorded at 30 days post-injury, and included the GCS score on admission, as well as the patient's age, gender and Glasgow Outcome Score (GOS) (16). The GCS scores were assigned by a single neurosurgeon, who was blinded to a patient's SBP levels. Intubation and sedation procedures were performed following the examination. Patients were classified according to a commonly used dichotomization of the GCS score (17): Most severe brain injury, GCS score 3-5; severe brain injury, GCS score 6-8.

The clinical outcome at 30 days after injury was considered the short-term outcome, which was assessed using the GOS. The GOS at 30 days after injury was determined by a designated member of the research team. Contact by telephone was made either to the patient (when appropriate) or to his/her relatives (who were identified at the time of admission and agreed to be contacted in the future for the purpose of outcome assessment) to assess the clinical outcome. Patients who continued to receive clinical care for rehabilitation at the time of the 30-days GOS were assessed by discussion with the patient's health care professional. For statistical analyses, outcome was divided into a good prognosis (GOS, 4-5) and a poor prognosis (GOS, 1-3) (18).

A total of 10 control patients without TBI, who required lumbar anesthesia for other medical reasons and exhibited normal pressure hydrocephalus, were enrolled from the Nanjing First Hospital. CSF samples were obtained from each patient prior to anesthesia. All patient identifiers remained confidential.

The exclusion criteria for the present study were as follows: Concomitant extracranial injuries such as pelvic or extremity fractures; intra-abdominal, intrapleural, or retroperitoneal hemorrhages; hepatic or splenic injuries; thorax or spinal cord damage; and major health problems such as diabetes mellitus; renal or cardiac failure; central nervous system diseases; bleeding disorders prior to the trauma, and alcohol intoxication. Patients with a history of neurological and neuropsychiatric disorders, history of alcohol, drug, or substance abuse, or a history of previous TBI were also excluded from this study.

CSF sample collection. Ventriculostomy catheters were placed under routine medical care for patients with severe TBI in the study institution. Therefore, CSF samples from patients with severe TBI were directly collected from the ventriculostomy catheter at 24, 48 and 72 h after injury. At each sampling point, 3-4 ml CSF was collected from each patient. The samples were immediately centrifuged for 10 min at 2,130 x g to separate the CSF from blood cells, and were then frozen and stored at -80˚C until examination.

Immunoblotting assay. The protein content of the CSF was assayed using a micro bicinchoninic acid assay kit (KGPBCA; KeyGen Biotech Co., Ltd., Nanjing, China) with albumin standards. For each sample, 40 µg CSF protein was added to 5X loading buffer (KGP101; KeyGen Biotech Co., Ltd.) and then heated at 100˚C for 5 min. Samples were loaded onto a 6.5% stacking acrylamide gel and electrophoresed in a vertical electrophoresis chamber for 100 min at 130 V. Separated proteins were horizontally transferred to Immobilon-P polyvinylidene fluoride membrane (KeyGen Biotech Co., Ltd.) and semi-dried at 15 V for 75 min at room temperature. All gels were stained with Coomassie blue (Bios, Beijing, China) to confirm equal loading of proteins on the gel. After three 10-min rinses in Tris-buffered saline (TBS), blots were blocked for 2 h in 5% non-fat dry milk in TBS with 1% Tween 20 (TBST). Following incubation overnight with primary anti-α-spectrin monoclonal antibody (1:5,000 dilution; MA1-91103; Thermo Fisher Scientific, Rockford, USA) and 5% non-fat milk/TBST at 4˚C, and three 10-min rinses in TBST, the blots were incubated with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:5,000 dilution; bs-0296G-HRP; Bios) for 1 h at room temperature. Blots were then rinsed again three times for 10 min in TBS at room temperature. Enhanced chemiluminescence reagents (ECL and ECL-Plus; KGP1125; KeyGen Biotech, Co., Ltd.) and Kodak BioMax Light Film (Eastman Kodak; Rochester, NY, USA) were used to visualize the immunolabeling. Semi-quantitative evaluation of protein levels was performed using computer-assisted one-dimensional densitometric scanning (Image J, Version 1.29x; National Institutes of Health, Bethesda, MD, USA). Data were acquired as integrated densitometric values from similarly exposed films.

Statistical analysis. Statistical analyses were performed using SPSS software, version 17.03 for Windows (SPSS, Inc., Chicago, IL, USA). Counting variables were analyzed using Fisher's exact test. Results are presented as the mean ± standard deviation (SD). Normally distributed variables were compared using Student's t-test, and non-normally distributed variables were analyzed using the Mann-Whiney U test. Analysis of variance was used to determine the statistical significance. A value of P<0.05 was considered statistically significant.

Results

General information. The present study included 17 patients with severe TBI (GCS score, 3-8) and 10 control patients with normal pressure hydrocephalus. Six patients had severe brain injury (GCS score, 6-8), and 11 patients had most severe brain injury (GCS score, 3-5). There were no significant differences in patient age and gender between the two groups (P>0.05;
A total of 12 patients were considered to have a poor outcome (GOS score, 1‑3), whereas the remaining five patients had a good outcome (GOS score, 4‑5).

Concentrations of CSF \(\alpha\)-II-SBDP over 5 days after injury in patients with severe TBI.

BSDPs were not detectable in the CSF of the patients from the control group. Therefore, the BSDP concentrations of the control group were considered to be ~0, and further data for the control group are not presented.

The mean concentrations of BSDP150 and BSDP145 peaked at 24 h after injury and then showed a downward trend. No significant changes in the mean concentrations of BSDP120 were observed during the study (Fig. 1).

Association between the levels of BSDPs and GCS score.

Within the first 24 h after TBI, the BSDP145 and BSDP150 levels, but not the BSDP120 levels, were significantly lower in the patients with GCS scores of 6-8 than in patients with lower admission GCS scores of 3-5 (\(P<0.05\); Fig. 2).

Association between the levels of BSDPs and clinical outcome.

Higher levels of CSF BSDP145 and BSDP150 were found in patients who succumbed, remained vegetative, or were severely disabled (GOS score 1-3) than in patients who showed a good prognosis (GOS score 4-5) at all time points (\(P<0.05\)). The levels of CSF BSDP120 of the two outcome groups peaked at 24 h after injury and then showed a downward trend; it decreased more slowly in the poor prognosis group than in the good prognosis group. The levels of CSF BSDP120 at 24 h after trauma showed no significant differences between the two groups (\(P>0.05\)). However, the CSF BSDP120 levels were significantly higher at 72 h (\(P<0.05\)) and at 120 h (\(P<0.05\)) in the poor prognosis group (Table II).

Discussion

BSDPs are metabolic decomposition products of \(\alpha\)-II-spectrin, which is a major substrate for both calpain and caspase-3 cysteine proteases (12). \(\alpha\)-II-spectrin (280 kDa) is the major structural component of the cortical membrane cytoskeleton, and it is abundantly present in axons and presynaptic terminals (19). TBI results in altered Ca\(^{2+}\) homeostasis and activates several Ca\(^{2+}\)-dependent enzymes, including calpain and caspase-3 (20). Calpain proteolysis is primarily associated with oncotic necrosis, while caspase-3 proteolysis is primarily associated with apoptosis (6). Calpain cleaves \(\alpha\)-II-spectrin to generate BSDP145 and BSDP150. Caspase-3 cleaves \(\alpha\)-II-spectrin to generate BSDP120 and BSDP150. The BSDPs transfer from the damaged brain cells into the CSF.

Hence, the levels of BSDPs increase in the CSF following TBI. Zhang et al (21) used three chemical agents to induce necrosis and apoptosis in PC12 neuronal like cells, and demonstrated that the multiple \(\alpha\)-II-BSDPs could be used as biomarkers to distinguish between calpain- and caspase-dominant necrotic and apoptotic cell deaths. Using a mouse model of cortical injury, Pike et al (22) demonstrated that the levels of BSDP150 and BSDP145 were predominantly elevated in the first 24-72 h after injury.
post injury, with SBDP145 levels being greatly increased. In a study conducted by Cardali et al (23), the levels of SBDPs in CSF were measured for adult patients with severe TBI from 6 to 96 h after injury, and it was found that the SBDP levels in CSF were significantly increased, as compared with those in control patients at all time points examined. In patients with a better outcome, CSF SBDPs levels were significantly decreased from 6 to 96 h post injury. Patients whose SBDP levels remained elevated or failed to decline had a poor outcome (23). In the present study, the CSF levels of SBDP150, SBDP145 and SBDP120 were significantly elevated in patients with severe TBI, as compared with those in patients with hydrocephalus or a subarachnoid hemorrhage. Patients with the most severe injury (day 1 GCS score, 3-5) had significantly higher mean levels of SBDP150 and SBDP145 in the first 24 h post-injury, as compared with patients with a less severe injury (day 1 GCS score, 6-8); however, the difference was not significant for SBDP120. These results suggested that the levels of SBDPs in the CSF may be considered useful biomarkers for predicting the severity of injury in the early period of severe TBI.

In the present study, it was found that the CSF levels of SBDPs were significantly elevated in patients with severe TBI. However, SBDPs were not detected in the CSF of the control patients. SBDPs appeared rapidly in the CSF following severe TBI; SBDP150 and SBDP145 peaked at 6 h post-TBI and remained significantly elevated for 24 and 72 h post-injury; conversely, the levels of SBDP120 did not have a clear trend of variation post-TBI (24). Notably, a previous study found that SBDP150 and SBDP145 levels peaked at 48-72 h post-injury (25). In the present study, the levels of SBDPs in the CSF were predominantly elevated at 24 h post-TBI and then declined gradually over time. The CSF levels of SBDP145 and SBDP150 remained significantly elevated up to 72 h post-TBI and were significantly declined at 120 h post-injury, whereas the CSF levels of SBDP120 showed a slow gradual decline. Generally, oncotic necrosis of neurons occurs in the acute posttraumatic period, whereas apoptosis occurs a few days or weeks following the trauma (9). SBDP150 and SBDP145 are predominantly produced by calpain activity, which is primarily associated with oncotic necrosis, whereas SBDP120 is a signature product of caspase-3 activity, which is primarily associated with apoptosis (26). Therefore, it can be hypothesized that the levels of SBDP150 and SBDP145 in the CSF would likely peak in the acute post-traumatic period, whereas the levels of SBDP120 would peak at ≥1 week post-injury. In the present study, within the first 24 h after injury, the CSF levels of SBDP145 and SBDP150 were significantly higher in patients with hospital admission GCS scores of 3-5, as compared within patients with hospital admission GCS scores of 6-8. Although differences were observed in the CSF levels of SBDP120, the differences were not statistically significant. These results indicated that patients with severe brain injury had the highest levels of SBDP145 and SBDP150 in the first 24 h. The levels of CSF SBDP120 at 24 h post-trauma were not associated with hospital admission GCS scores, which may be due to the more delayed processes of apoptotic cell death.

In the present study, patients were categorized into two groups based on their clinical outcomes. The outcome analysis revealed a statistically significant association between the clinical outcome and the SBDP150 and SBDP145 levels at all time points. This indicates that the higher the levels of CSF SBDP145 and SBDP150, the greater the probability of a poor prognosis. Moreover, a slower decline of CSF SBDP145 and SBDP150 levels may also predict a poor prognosis. Comparative analysis of SBDP120 and clinical outcome did not reveal a statistically significant association at 24 h post injury. The levels of CSF SBDP120 were higher in patients who showed a poor prognosis (GOS score, 1-3) than in patients who showed a good prognosis (GOS score, 4-5) at 72 h and 120 h after injury. Previous study results showed that CSF SBDP120 levels exhibited no significant differences between poor and good prognosis groups at 24 h after severe TBI (27). However, Pike et al (22) demonstrated that the levels of CSF SBDP120 at 24 h and 7 days after severe TBI were significantly correlated with GOS scores determined 3 months after injury. On consideration of these results, it can be concluded that there is some uncertainty concerning the trend in the changes of the levels of CSF SBDP120, and further studies are required for elucidation. The results of the present study provide evidence that the CSF SBDPs can be a reliable marker of central nervous system injury. CSF SBDP145 and SBDP150 are potentially useful biomarkers of severe TBI in humans. The change tendency of these biomarkers measured early after injury can be used in

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<th>Biomarkers</th>
<th>Prognosis on the basis of GOS</th>
<th>CSF SBDP levels</th>
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<tr>
<td></td>
<td>24 h</td>
<td>72 h</td>
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<tr>
<td>SBDP150</td>
<td>Good</td>
<td>52.69±10.01</td>
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<tr>
<td>SBDP145</td>
<td>Poor</td>
<td>76.59±18.37*</td>
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<tr>
<td>SBDP120</td>
<td>Good</td>
<td>62.25±13.9</td>
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<tr>
<td>SBDP145</td>
<td>Poor</td>
<td>83.42±17.21*</td>
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Values presented are mean ± standard deviation in arbitrary densitometric units. Good prognosis, GOS score 4-5; poor prognosis, GOS score 1-3. *P<0.05 vs. the good prognosis group. CSF, cerebrospinal fluid; SBDP, α-II-spectrin breakdown product; GOS, Glasgow Outcome Score.
the evaluation of the severity of injury and clinical outcome in patients with severe TBI. The association between the CSF SBDP120 and the severity of injury and clinical outcome after TBI still requires further research.

Since α-II-spectrin is not found in erythrocytes, the influence of hemolysis can be avoided (28). The present study has several limitations. Firstly, rapidly changing clinical conditions of patients with severe TBI may lead to the CSF sample collection at all time points and long-term monitoring becoming very difficult. Thus, all available samples were included in the analyses in the present study to maximize the sample size. Secondly, the biomarkers were only assessed in patients experiencing severe TBI. Assessment of these biomarkers in patients experiencing different severities of head injury (for example, patients with mild or moderate head injuries) in future studies is suggested. Thirdly, the levels of SBDPs were determined using a semi-quantitative western blot analysis. More advanced detection methods are required in future studies. The low number of enrolled patients also impacted the credibility of the results. However, the present study data provide an initial assessment of relationships between CSF SBDPs and the severity and clinical outcome of severe TBI. Many unaddressed problems remain that require investigation in further studies, for example, by detecting SBDPs in the serum of injured experimental animals and humans and investigating the secondary injury effect on CSF SBDP levels.

In conclusion, the present pilot study indicates that the trends in CSF SBDP levels measured early after injury can be used in the evaluation of the severity of injury and clinical outcome of patients with severe TBI. They are potentially useful biomarkers of severe TBI. However, a series of uncertainties remain that require further investigation.

Acknowledgements

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