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Clinical Article

Spectrin breakdown products in the cerebrospinal fluid in severe head injury – preliminary observations

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Summary

Background. Calcium-induced proteolytic processes are considered key players in the progressive pathobiology of traumatic brain injury (TBI). Activation of calpain and caspases after TBI leads to the cleavage of cytoskeletal proteins such as non-erythroid alpha II-spectrin. Recent reports demonstrate that the levels of spectrin and spectrin breakdown products (SBDPs) are elevated *in vitro* after mechanical injury, in the cerebrospinal fluid (CSF) and brain tissue following experimental TBI, and in human brain tissue after TBI.

Methods. This study was initiated to detect spectrin and SBDP accumulation in the ventricular CSF of 12 severe TBI-patients with raised intracranial pressure (ICP). Nine patients with non-traumatically elevated ICP and 5 undergoing diagnostic lumbar puncture (LP) served as controls. Intact spectrin and calpain and caspase specific SBDPs in CSF collected once a day over a several day period were assessed via Western blot analysis. Parameters of severity and outcome such as ICP, Glasgow Coma Scale and Glasgow Outcome Scale were also monitored in order to reveal a potential correlation between these CSF markers and clinical parameters.

Results. In control patients undergone LP no immunoreactivity was detected. Non-erythroid alpha-II-spectrin and SBDP occurred more frequently and their level was significantly higher in the CSF of TBI patients than in other pathological conditions associated with raised ICP. Those TBI patients followed for several days post-injury revealed a consistent temporal pattern for protein accumulation with the highest level achieved on the 2^{nd}–3^{rd} days after TBI.

Conclusion. Elevation of calpain and caspase specific SBDPs is a significant finding in TBI patients indicating that intact brain spectrin- and SBDP-levels are closely associated with the specific neurochemical processes evoked by TBI. The results strongly support the potential utility of these surrogate markers in the clinical monitoring of patients with severe TBI and provide further evidence of the role of calcium-induced, calpain- and caspase-mediated structural proteolysis in TBI.

Keywords: Traumatic brain injury; calpain; caspase; spectrin; human; cerebrospinal fluid.

Introduction

Traumatic brain injury (TBI) is the primary cause of death in the population under the age of 40 representing an extreme challenge for individuals and families affected as well as the society at large [10]. Thus, understanding the pathobiochemical processes evoked by or operant in TBI is integral to developing new therapeutic strategies while exploring potential biochemical markers that can help assess the severity of the injury and predict outcome.

Traumatic brain injuries are typically classified on whether the primary damage evokes more localized brain damage and/or mass lesion formation (focal injury), or more scattered, non-focal injuries typically involving scattered axonal and/or microvascular damage (diffuse injury). Although focal and diffuse injuries can be evoked by different mechanism and their clinical manifestations can be different, several studies to date have shown that similar biochemical cascades take part in the pathobiology of these different injury types. These studies have demonstrated increased pathological activation of calpain and/or caspase-3 both after focal TBI [8, 9, 12, 16] and diffuse injuries [5, 6, 18]. Calpain and caspase-3, both members of cysteine protease family, have also been shown to play an important role in the proteolytic cascades associated with several other central nervous system disorders such as stroke, hypoxia-ischemia [3, 13], experimental hydrocephalus [7] and spinal cord injury [1]. Non-erythroid alpha II-spectrin, major component of the cytoskeleton, constitutes a

substrate for both calpain and caspase-3 [21]. Calpain-mediated cleavage of intact spectrin (280 kDa) results in 150 and 145 kDa-fragments specific for calpain, whereas the caspase-3-specific products are linked to 150 and 120-fragments [20]. Using antibodies targeting alpha II-spectrin breakdown products numerous recent reports have demonstrated that non-erythroid alpha II-spectrin and spectrin breakdown products (SBDPs) are elevated *in vitro* after mechanical injury [15, 4], also being found in the cerebrospinal fluid and brain following experimental TBI [16, 12, 8, 18], as well as human brain tissue post–TBI [11].

The goal of the current study was to further define the role of calcium-induced, calpain- and caspase-mediated structural proteolysis in the pathobiology of TBI, identifying potential biomarkers *specifically* associated with the pathological processes evoked by TBI and thereby purportedly capable of predicting the severity of the initial injury and outcome to help in the better clinical management of severely brain-injured patients.

In this study, the levels of non-erythroid alpha II-spectrin and SBDPs in the CSF of TBI patients sustaining severe injury were compared to two control groups: one with other disorders associated with elevated intracranial pressure (ICP) and the other with normal ICP. Clinical parameters of severity and outcome such as intracranial pressure, Glasgow Coma Scale and Glasgow Outcome Scale were monitored.

Materials and methods

The study included twelve patients with severe TBI (GCS < 9). The first control group consisted of patients with comparably raised ICP not associated with TBI and included 3 patients with subarachnoid haemorrhage (SAH), 3 with intraventricular haemorrhage (IVH), and 3 with brain tumours. The second control group consisted of 5 patients undergoing diagnostic LP that subsequently proved negative for SAH and/or meningitis (*clinical data of all patients are presented in Table 1*). All CSF harvesting was part of routine patient management, in accordance with institutional guidelines; the study protocol was approved by the Regional Ethics Committee (NIH-approved Institutional Review Board) of the Centre of Medical and Health Sciences of Pecs University. In the traumatically brain–injured group all patients were equipped with intraventricular catheters for the control of ICP. Via these catheters ventricular CSF samples were collected once a day, in some patients for several days following ventriculostomy. They were centrifuged at 6000 g for 6 min and stored at −80 °C. After defrosting samples were washed in fivefold PBS on Amicon ultrafiltration cell using polyethersulfone ultrafiltration membranes with 100 kDa nominal molecular weight limit. During this procedure the samples were purified from serum albumin and other proteins with less than 100 kDa molecular weight and concentrated to about five-fold. Protein concentrations were determined using Bio-Rad Protein Assay. Samples containing less than 1 mg/ml protein were dried by Heto dry Winner. The dehydrated substances were dissolved in distilled water to get a protein concentration of 1 mg/ml. Sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli using a mini-gel apparatus (Bio-Rad). Samples containing 20 micrograms of

Table 1.

Group	Age	Sex	Diagnosis	CT	GCS o.a.	ICP or OP	Date	GOS	Date
T-1	*41*	*M*	*SDH + Cont*	–	*4*	*35 Hgmm*	*D1-*	*2*	*28*
T-2	*79*	*FM*	*Cont*	*IVH*	*4*	*20 Hgmm*	*D1-*	*2*	*12*
T-3	28	M	Cont	IVH	5	20 Hgmm	D3	3	18
T-4	48	M	SDH	–	4	25 Hgmm	D3	3	25
T-5	51	M	EDH + Cont	–	6	10 Hgmm	D1	1	31
T-6	21	M	Cont	–	7	20 Hgmm	D1	4	9
T-7	51	M	SDH + Cont	–	5	40 Hgmm	D4	1	90
T-8	68	M	SDH	–	6	20 Hgmm	D4	2	14
T-9	51	FM	Cont	IVH	4	40 Hgmm	D1	1	3
T-10	18	M	EDH	–	4	35 Hgmm	D1	4	37
T-11	19	M	SDH + Cont	–	6	35 Hgmm	D3	3	17
T-12	72	FM	EDH + Cont	–	5	10 Hgmm	D2	2	16
C-1-1	69	FM	SAH	HC	9	20 Hgmm	D10	4	28
C-1-2	65	M	SAH	HC	8	15 Hgmm	D14	4	21
C-1-3	32	FM	SAH	OE	4	30 Hgmm	D2	2	22
C-1-4	46	FM	SAH	IVH	10	20 Hgmm	D3	5	23
C-1-5	43	M	SAH	IVH + HC	6	20 Hgmm	D10	3	43
C-1-6	30	M	IVH	HC	7	40 Hgmm	D2	1	7
C-1-7	66	FM	Tu. V-3	HC	9	25 Hgmm	D2	1	10
C-1-8	57	FM	Tu. V-4	HC	8	10 Hgmm	D1	5	30
C-1-9	28	M	Tu. V-4	HC	10	25 Hgmm	D2	3	16
C-2-1	18	M	MGITIS?	Neg.	15	6 Hgmm	NA	NA	NA
C-2-2	43	FM	SAH?	Neg.	15	8 Hgmm	NA	NA	NA
C-2-3	19	M	MGITIS?	Neg.	15	8 Hgmm	NA	NA	NA
C-2-4	59	M	MGITIS?	Neg.	15	5 Hgmm	NA	NA	NA
C-2-5	72	FM	SAH?	Neg.	15	10 Hgmm	NA	NA	NA

protein (equal loading from all samples) were electrophoresed in 6.5% Tris/glycine gels. Next, separated proteins were transferred to nitrocellulose membranes. Blots were blocked for 1 hour in 1% non-fat milk in TBS with 0.1% Tween-20 (TBST). The presence of non-erythroid alpha II-spectrin and its breakdown products were examined with monoclonal mouse anti-spectrin antibody (Chemicon) capable of detecting intact non-erythroid alpha II-spectrin (280 kD) and 150, 145 and 120 kD cleavage fragments. (These proteins in the CSF detected by the antibody are exclusively derived from the central nervous system as erythroid spectrin is not recognized by the antibody applied. This premise excludes the possibility of false positive reactions due to intraventricular haemorrhages and focal lesions leading to the accumulation of erythroid spectrin in the CSF.)

Following 1 hour incubation at room temperature with this primary antibody (1:500 in 1% non-fat milk TBST) blots were incubated with biotinylated anti-mouse Ig (Amersham) in 1:400 dilution in 1% non-fat milk TBST for 1 hour at room temperature, next were incubated with Streptavidin-Biotinylated Horseradish Peroxidase Complex (Amersham, 1:1000) for half an hour at room temperature. Western Lighting™ Chemiluminescence reagents were used to visualize immunolabelling on Kodak films. Semi-quantitative evaluation of detected proteins was performed by computer-assisted densitometric scanning (Bio-Profile Bio-1D++, Vilber Lourmat). Data were normalized to background density as well as negative control reacted without the presence of the antigens. Densitometric data gained from CSF samples of TBI-patients and controls was compared with an independent samples t-test. Differences were assumed significant at a level of $p \leq 0.05$. Correlation between protein levels and clinical parameters was tested by linear regression.

Results

Thirty-four CSF samples from the 12 brain-injured patients in addition to 15 samples from 9 control patients were evaluated in the current investigation (Table 1).

Analysis of all protein bands examined demonstrated that intact (280 kDa) spectrin as well as the 120 kDa breakdown product was present in significantly higher percentage of TBI-patients than in patients with raised ICP of different etiologies (Table 2).

The CSF samples from patients undergoing routine, diagnostic LP (second control group) contained neither intact spectrin nor SBDP.

Selected patients (*on Table 1 these patients could be identified as T-1 and T-2*) with severe TBI were followed for several days to explore the time course of spectrin release in CSF. One patient (GCS = 4, GOS = 2, ICP = 35 post admission) revealed a peak of both intact spectrin and SBDPs on the third day (Table 3 and Fig. 1a and c). The density of all proteins decreased to the baseline levels by the day 7–8 followed by another increase in the density of 150 kDa spectrin at the 8th day post-injury. Similarly, another patient (GCS = 4, GOS = 2, ICP = 20 at admission) had a peak of SBDPs at the second day followed by a density decrease, however by the fourth day postinjury another increase was observed in the 150 kDa spectrin level as well as in the density of intact and 145 kDa spectrin (Table 3 and Fig. 1b and c).

Independent samples t-test was used to compare the density of protein bands taken from head injured patients versus those suffering from other central nervous system disorders (*clinical data of all patients are summarized in Table 1*). One sample from each patient, taken on the first

Table 2.

Band (kDa)	Occurrence in trauma	95% CI value	Occurrence in control	95% CI value	Significance
280	66%	±26.7%	11%	±20.4%	*
150	100%	–	88%	±21.2%	–
145	75%	±24.5%	44%	±32%	–
120	75%	±24.5%	22%	±27%	*

Table 3.

Date	Band							
	280 kDa patient 1	280 kDa patient 2	150 kDa patient 1	150 kDa patient 2	145 kDa patient 1	145 kDa patient 2	120 kDa patient 1	120 kDa patient 2
12 hrs	0	0	309	0	0	0	0	0
24 hrs	178	0	357	382	87	119	79	0
2 days	523	580	749	1383	749	1383	181	357
3 days	908	155	1406	240	1406	79	351	122
4 days	575	243	723	221	723	605	0	121
5 days	421	0	555	0	555	0	0	0
6 days	245	0	333	382	333	119	0	0
7 days	118	580	293	1383	0	1383	0	357
8 days	0	155	398	240	0	79	0	122
9 days	0	243	318	221	0	605	0	121

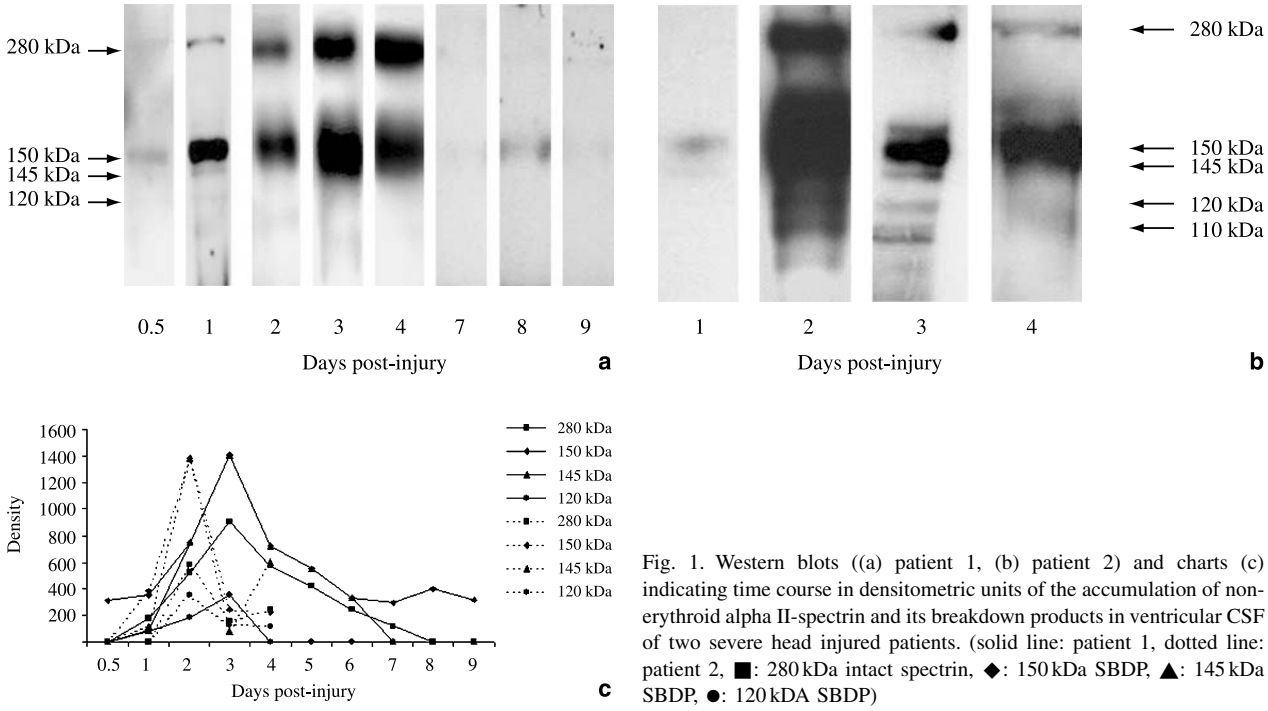

Fig. 1. Western blots ((a) patient 1, (b) patient 2) and charts (c) indicating time course in densitometric units of the accumulation of non-erythroid alpha II-spectrin and its breakdown products in ventricular CSF of two severe head injured patients. (solid line: patient 1, dotted line: patient 2, ■: 280 kDa intact spectrin, ◆: 150 kDa SBDP, ▲: 145 kDa SBDP, ●: 120 kDA SBDP)

to third day post-injury, was analyzed based upon the observations described above regarding the time course of SBDP levels in ventricular CSF. In those TBI patients, where multiple samples were available, maximal levels have been considered. In the first control group, where ventriculostomy was performed to decrease ICP primarily evoked by occlusive or malresorptive hydrocephalus and oedema, maximal levels detected at the insertion of the ventricular drainage were considered.

The result of the densitometric analysis and statistical comparison is summarized in detail in Table 4. In sum,

Table 4.

Band	Diagnosis	Mean density	SEM	P	Significance
280 kDa	TBI	251.083	±42.41	0.031	*
	Control-I	33.111	±16.55		
150 kDa	TBI	622.5	±64.40	0.023	*
	Control-I	268.55	±28.25		
145 kDa	TBI	414.33	±80.65	0.055	–
	Control-I	68	±13.75		
120 kDa	TBI	179.91	±19.57	0.012	*
	Control-I	44.66	±14.84		

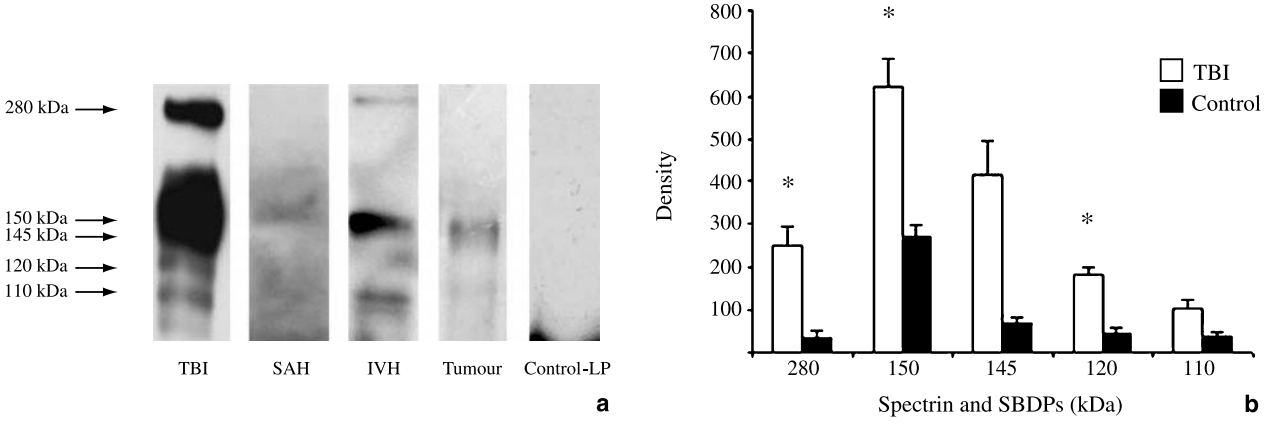

Fig. 2. Characteristic Western blots (a) and bar charts (b) indicating ventricular CSF level of non-erythroid alpha II-spectrin and its breakdown products in TBI and non-injured control patients with other pathological conditions associated with raised ICP. (Blots from patients underwent diagnostic lumbar punction are not shown.) Asterisks indicate significance of difference, error bars represent standard error of means, data derive from TBI and non-injured control patients, 34 versus 15 samples, respectively). $P < 0.05$

non-erythroid alpha II-spectrin and SBDP protein band densities were significantly higher in CSF of TBI patients than non-TBI controls (Fig. 2a, b).

Association between Glasgow Coma Scale (GSC), Glasgow Outcome Scale (GOS), ICP and protein levels was also examined to evaluate the potential correlation between clinical and neurobiochemical parameters.

Most probably due to the relatively low number of patients linear regression analysis did not reveal a significant correlation either between the accumulation of various SBDPs and injury severity (defined by GCS), outcome (defined by GOS) or ICP.

However, it is of note that the negative association between injury severity (defined by GCS) was close to the level of significance (280 kDa: $r^2 = 0.318$ $P = 0.07$; 150 kDa: $r^2 = 0.245$ $P = 0.121$; 145 kDa: $r^2 = 0.209$ $P = 0.156$).

Discussion

This is the first study to demonstrate the accumulation of non-erythroid alpha II-spectrin and its breakdown products in the ventricular CSF of patients with severe TBI.

It is of particular importance that CSF-samples from control patients, undergone diagnostic lumbar puncture without raised ICP/opening pressure/were proved negative for intact, non-erythroid alpha II-spectrin as well as its breakdown products, a finding clearly indicating that the accumulation of these substances is associated with brain injury and/or raised ICP.

Interestingly, non-erythroid alpha II-spectrin and SBDPs reached significantly higher levels in TBI than in other pathological conditions associated with comparably elevated ICP, indicating that these changes were in direct response to the injury and not a secondary response to intracranial hypertension. The fact that only two patients were followed for several days in this study clearly reflects the preliminary nature of our observation however it is of note that the peak values as well as the time course of those markers (280 kDa and 120 kDa) which displayed significant alterations in some aspects of our analysis were rather consistent in both cases.

Recent studies in animal models of head injury provide further credit to this observation in terms of describing similar, reliable time course of SBPD accumulation in head injured rodents [17].

To date, experimental studies have demonstrated that CSF levels of non-erythroid alpha II-spectrin and SBDPs are elevated following ischemic stroke [2, 12]. In these ischemic studies it was suggested by Bartus and colleagues that calpain-mediated structural proteolysis was responsible for brain damage associated with cerebral ischemia [2]. These observations reported in cerebral ischemia suggested that traumatically induced brain tissue destruction could lead to the CSF accumulation of these substances following TBI, a premise consistent with our current study.

Recent studies provided further evidence that calpain- and caspase-mediated proteolytic cleavage fragments should be considered future surrogate markers in central nervous system injury of various origin [19].

Moreover, based upon our previous observations regarding the role of calpain- and caspase-mediated structural proteolysis in the pathogenesis of experimental TBI [4, 5] we anticipated that the magnitude of spectrin breakdown in TBI would exceed those levels observed in other CNS-disorders associated with raised ICP.

Again, these assumptions were supported by our findings that revealed significant elevation of calpain and caspase-3 specific SBDPs in TBI patients versus patients suffering from other CNS disorders. These observations clearly indicate that intact brain spectrin – and SBDP-levels are closely associated with those specific neurochemical processes evoked by and/or operant in TBI.

What remains to be determined in larger population of TBI patients is whether or not differing types of TBI are associated with specific patterns and degrees of cysteine-protease mediated damage. For example would a patient sustaining primary focal lesion and/or mass lesion formation show a different response than a patient demonstrating primary diffuse injury. This issue will be considered in future studies in a larger population of brain – injured patients who have sustained different types of primary injury.

Most probably due to the relatively small number of patients enrolled to this study we were not able to establish statistically significant association between injury-severity, ICP values and outcome and the accumulation of SBDPs, thus potential correlations between SBDPs and clinical parameters require further study in a larger patient population. Nevertheless, the observations summarized in the above passages as well as some of the results of the analysis of the correlation between SBDP-levels in CSF and injury severity indicate that non-erythroid alpha II-spectrin and SBDPs should be considered as candidate biomarkers in TBI.

The current observations, however, do provide evidence for the contribution of calcium-induced calpain- and caspase-mediated proteolysis in the pathogenesis of

human TBI, as previously suggested in multiple animal studies [13, 9, 18].

In sum, the current, preliminary studies indicate the utility of cerebrospinal fluid monitoring for cysteine-protease-mediated proteolytic damage as a potential marker for evaluating TBI patients. Obviously, these studies require expansion to address some of the questions alluded to above. Further, they might require the use of alternative approaches including but not limited to ELISA analyses, to further enhance the usefulness of these measurements in the routine clinical setting.

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Comments

Traumatic brain injury is a very complex pathology due to the variability of tissue damage encountered in the individual patient, and also for the multitude of biochemical cascades activated generating a variety of secondary insults. It is for these reasons, that the identification of biomarkers specific to selective types of brain damage (focal vs diffuse) is of great importance to 1) discriminate the biochemistry involved, 2) be applied for diagnostic purposes, 3) for monitoring the onset of secondary damage, and 4) as predictors of outcome. Most studies published recently have shown a direct correlation between the levels of S-100 and the presence and size of focal brain damage, however to date there are no reliable markers to estimate the degree of axonal damage.

Previous studies by Farkas and collaborators based on an animal model of axonal injury in the rat, showed an association with foci of axonal damage with mitochondrial dysfunction, calpain and caspase-3 activation and calpain-mediated spectrin proteolysis, suggesting their participation not only in neuronal cell death but also in axonal disconnection (Buki *et al*, J Neuropathol Exp Neurol 58: 365, 1999 & J Neurosci: 20: 2825, 2000). Furthermore, the same group showed a

decreased number of injured axons when rats were treated with a calpain inhibitor (Buki *et al* J Neurotrauma 20: 261, 2003). The present study, reports for the first time that spectrin breakdown products are elevated in human CSF in response to different neuropathologies including trauma as well as focal brain injury. It remains to be established whether these levels are related to axonal injury possibly coexistent with focal lesions. The patient population analysed is undersized to establish any association; therefore, further investigation on larger groups of patients with either type of brain damage is warranted before drawing any conclusions.

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This manuscript reports on analysis of cerebrospinal fluid (CSF) samples collected from a small group of patients who sustained a TBI compared to two control groups. The levels of non-erythroid spectrin and spectrin breakdown products (SBDPs) were measured by western blotting. This is essentially a confirmatory study, using human samples, of a body of evidence from animal studies implicating calpain- and caspase-mediated spectrin breakdown in the pathophysiology of TBI. However, it is essential that such human studies are performed to assess the relevance of measures made in animal models and therefore the study is of clinical significance. The data indicate that after human TBI, levels of spectrin and SBDPs in CSF are elevated compared to a group which had raised ICP due to causes other than TBI and samples taken for diagnostic lumbar puncture.

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