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Characterization of Antibodies that Detect Human GFAP after Traumatic Brain Injury

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Abstract: After traumatic brain injury (TBI), glial fibrillary acidic protein (GFAP) and other brain-derived proteins and their breakdown products are released into biofluids such as CSF and blood. Recently, a sandwich ELISA was constructed that measured GFAP concentrations in CSF or serum from human mild-moderate TBI patients. The goals of the present study were to characterize the same two antibodies used in this ELISA, and to determine which GFAP bands are detected by this antibody combination. Here, both antibodies recognized GFAP specifically in human brain and post-TBI CSF in a cluster of bands ranging from 50–38 kDa, that resembled bands from calpain-cleaved GFAP. By immunoprecipitation, the anti-GFAP Capture antibody recovered full length GFAP and its breakdown products from human brain lysate and post-TBI CSF. These findings demonstrate that the anti-GFAP ELISA antibodies non-preferentially detect intact GFAP and GFAP breakdown products, underscoring their utility for detecting brain injury in human patients.

Keywords: GFAP, GFAP-BDP, traumatic brain injury, human

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Introduction

Traumatic brain injury (TBI) is a predominant cause of death and disability in the human population. Extensive reports have demonstrated that TBI pathology includes two primary components: the initial injury event, followed by a destructive cascade of secondary cell death within the injured core that spreads into adjacent brain tissue over time.¹⁻³ Secondary cell death mechanisms include upregulation and hyperactivation of proteases such as calpains and caspases within neurons and glia that then contribute to necrosis and apoptosis.⁴⁻⁷ The cell death occurring during the first day after TBI results in the release of brain proteins and their proteolytic fragments (biochemical markers, or biomarkers) into biofluids such as cerebrospinal fluid (CSF) and blood. Biomarkers that have been detected in human TBI biofluids previously include neuron specific enolase, S100B, myelin basic protein, glial fibrillary acidic protein (GFAP), ubiquitin carboxy hydrolase-like 1, neurofilament proteins, and α II-spectrin breakdown products.⁸⁻¹⁵ Currently, TBI diagnoses in human patients are made by administering neurological exams and neuroimaging tests such as MRI and CT scans.² The pattern and timing of biomarker appearance in TBI-associated biofluids represents a potential molecular signature capable of defining damage-linked parameters such as injury magnitude and outcome. Therefore, there is enormous diagnostic and prognostic promise in developing assays that measure TBI-associated biomarkers accurately and specifically, especially from blood samples. From a clinical perspective, TBI biomarkers have the potential to be as valuable for detecting brain injury as the troponin biomarker currently is for detecting heart damage.¹⁶

One means of quantitating protein-based biomarkers is the sandwich enzyme-linked immunosorbent assay (swELISA). In the swELISA, two antibodies directed against distinct regions of the biomarker protein are used. Briefly, the test sample (such as CSF or serum) is allowed to react sequentially with these antibodies, resulting in the biomarker protein being sandwiched between the primary (capture) and secondary (detection) antibodies. The use of two antibodies provides dramatically increased sensitivity and specificity over a single antibody-based

assay. Recently, a highly sensitive and specific swELISA for human GFAP was developed which was capable of measuring GFAP protein levels in serum samples from human mild-moderate TBI (mmTBI) patients.¹⁷ GFAP was detectable in serum within an hour of injury, with a lower limit of detection at 0.02 ng/mL. In serum samples drawn within 1–4 h post injury, GFAP levels distinguished patients with mmTBI (GCS 9-15) from uninjured controls, and discriminated between patients with CT lesions versus those without, at a cutoff of 0.035 ng/mL.¹⁷ In a separate study on human moderate TBI patients and using a distinct ELISA assay, GFAP levels in serum predicted mortality and unfavorable outcome at a cutoff of 1.5 ng/mL.¹⁸ Other studies support the utility of GFAP as a biomarker for human severe TBI, showing GFAP serum levels to be correlated with injury severity and outcome.^{17,19-25} Additionally, serum GFAP has also been useful for detecting brain damage in human ischemic stroke victims.²⁶

The GFAP protein itself is a component of intermediate filaments in mature astrocytes of the brain and spinal cord. As the most abundant cell type in the central nervous system (CNS), GFAP-expressing astrocytes exhibit many functions, including physical and metabolic support for neurons, formation and maintenance of the blood-brain barrier, and the production and release of neurotrophic factors and cytokines in response to stimuli.^{27,28} The present study represents a characterization of the same Capture and Detection antibodies used in the human anti-GFAP swELISA.¹⁷ GFAP is susceptible to calpain-mediated proteolysis, and human TBI biofluids are likely to contain GFAP-BDP; however as yet, these observations have been minimally characterized only.¹⁷ In the present study, GFAP protein is shown to be present in CSF from human severe TBI patients in a cluster of bands ranging from full length at 50 kDa, to 38 kDa. Additionally, this study defines which of the GFAP bands in the 50–38 kDa interval are likely to be collected and measured by the swELISA antibodies. Here, the anti-GFAP Capture antibody immunoprecipitated full length GFAP and GFAP-BDP from 50–38 kDa, from both human brain lysate and severe TBI CSF. These findings indicate that the anti-GFAP swELISA is likely to measure non-preferentially the full range of GFAP species present



in human biofluids. These data confirm and extend previous findings.¹⁷ Therefore, this report provides insight into the anti-GFAP biomarker assay,¹⁷ and will be of interest to clinicians and researchers who use it to measure GFAP in human biofluids.

Methods

Cell culture and transfections

Human embryonic kidney 293 (HEK293) cells were obtained from ATCC and maintained in high glucose DMEM (Invitrogen) supplemented with 10% FBS and 50 µg/mL gentamycin in 5% CO₂ at 37 °C. A cDNA for human GFAP isoform 1 (NM_002055) was obtained from Origene (SC118873). At 50% confluency, cells were transfected as per the manufacturer's instructions with a 1:5 ratio of DNA:Metafectene (Biontix). After 24 h, cells were rinsed thoroughly with PBS, and lysed as below.

Lysate preparation and quantitation

Human post-mortem brain tissue and other organs were purchased from Analytical Biological Services, Wilmington, DE. Brains were harvested freshly from rats or mice that were euthanized according to IACUC-approved protocols. For western blot analysis, organ tissues were pulverized to a fine powder with a mortar and pestle over dry ice. The pulverized tissue was then lysed for 4 hours at 4 °C in 20 mM Tris-HCl pH 7.4, 5 mM EDTA, 5 mM EGTA, 1% Triton X-100, 1 mM DTT, and complete protease inhibitor cocktail.²⁹ Brief sonication with a probe-type sonicator was included during the lysis period to increase protein recovery. For cell lines, after rinsing, cells were lysed directly on plates as above, except for only 1.5 h.²⁹ Suspensions were cleared by centrifugation at 10,000 g for 10 min at 4 °C, and supernatants were collected in fresh tubes to yield lysates. Protein concentrations were determined via Bio-Rad DC Protein Assay.

Recombinant human GFAP preparation

A construct encoding full length human GFAP with sequence optimized for expression in *E. coli*, and with an added amino-terminal His tag, was obtained (Qiagen). Recombinant human GFAP (rhGFAP) protein was purified from *E. coli* inclusion bodies via the His tag, at a yield of 2–50 mg/L.¹⁷

Antibodies

The anti-GFAP mouse monoclonal IgG antibody (Capture antibody) clone originated at Neoclone Biotechnology as previously described by Papa et al.¹⁷ The anti-GFAP rabbit IgG polyclonal antibody (Detection antibody) was made by immunizing rabbits with purified full length recombinant human GFAP protein (Banyan) at Pocono Rabbit Farm and Laboratory (Pocono) as previously described by Papa et al.¹⁷ Protein A purified anti-GFAP antibodies were used at 1–2 µg/mL to probe immunoblots unless otherwise noted. The capture antibody was biotinylated using a kit (Dojindo) according to the manufacturer's instructions. The mouse monoclonal antibody against α II-Spectrin (a.k.a. α -fodrin) was obtained from Enzo, previously Biomol.

Immunoblotting

After lysate quantitation, equal amounts of protein were diluted to 2 µg/µL in NuPAGE 1x LDS buffer (Invitrogen) or Novex 1x Tris-glycine SDS-PAGE buffer (Invitrogen) including 25 mM DTT. Samples were heated for 2 min at 95 °C, then centrifuged for 1 min. Proteins were routinely resolved on 4%–12% NuPAGE Bis-Tris gels in MOPS buffer, or on 4%–20% Tris-glycine gels (Invitrogen) at 20 µg/lane. Samples were then transferred onto PVDF membranes using the 7 min iBlot method (Invitrogen). Membranes were blocked with 5% nonfat milk in TBST (50 mM Tris, 138 mM NaCl, 2.7 mM KCl, pH 8.0 + 0.05% Tween20; Sigma) for 1 h at room temperature and then incubated with primary antibody in 5% non-fat milk in TBST at 4 °C overnight. After washing in TBST, the membranes were incubated for one hour at room temperature with anti-mouse or anti-rabbit alkaline phosphatase-conjugated secondary antibodies at 1:5,000–1:10,000 (EMD Biosciences). Bands were visualized using BCIP/NBT phosphatase substrate (Kirkegaard and Perry Laboratories). Blots were scanned with a flatbed scanner to produce digital images, then figures were assembled using Adobe Photoshop and Powerpoint.

In vitro digestion

Calpain digestion was performed by incubating lysates with 1:200 or 1:50 (wt/wt) of recombinant rat calpain-2 (EMD Biosciences) in enzyme buffer

(100 mM Tris-HCl, pH 7.4 and 20 mM DTT) with 10 mM CaCl₂ at room temperature for 30 min.²⁹ For uncut controls, the enzyme was omitted, and 10 mM EGTA was substituted for CaCl₂. Digestions were terminated by flash freezing, and stored at -80 °C until gel analysis.

Immunoprecipitation

Human brain lysate or TBI CSF (pooled from 3 severe TBI patients) was diluted 1:3 with TBST. For human brain lysate, 200 µg total protein was diluted to 300 µL total volume, and for TBI CSF, 100 µL CSF was diluted to 300 µL total. Then 50 µL of each diluted sample was then removed and stored at -80 °C for gel analysis (pre, Fig. 4). Next, streptavidin beads (Pierce) were washed with TBST, and ~10 µL settled beads plus or minus biotinylated Capture antibody (0.5 µg) were added to 250 µL each diluted brain or CSF sample. Tubes were mixed at room temperature for 2 h. Samples were briefly microcentrifuged (Fisher Scientific Mini Centrifuge 05-090-128) to pellet beads, supernatants were removed and saved for gel analysis (post, Fig. 4), then beads were washed 5× with 1 mL TBST per wash at room temperature. Proteins bound to the beads were then eluted 3× sequentially with elution buffer (20 mM Tris pH 7.5 + 1.0% SDS), and eluates were pooled. To visualize GFAP, samples were blotted and probed with the anti-GFAP Detection antibody.

TBI patient population

CSF samples from adult severe TBI patients from Baylor College of Medicine (Houston, TX) were analyzed retrospectively in a fully de-identified manner. To be

considered a TBI patient, the following criteria had to be met: patients > 18 years of age with severe TBI defined as having a sum Glasgow coma score (GCS) of ≤8 on the post-resuscitation admission neurological examination, and requiring a ventriculostomy catheter for clinical management. Serial CSF samples were collected. The local ethics and hospital management committees and the IRB approved the protocol.

Results

This study reports novel biochemical characterization of the same Capture and Detection antibodies used in the anti-GFAP swELISA (Banyan Biomarkers).¹⁷ These antibodies were examined here by western blot against human post-mortem brain lysate and the ELISA calibrator, recombinant human GFAP (rhGFAP; Fig. 1). When control (non head-injured) human post-mortem brain lysate was blotted and probed with the anti-GFAP antibodies, similar banding patterns were observed (Fig. 1A). Both antibodies detected full length GFAP protein at 50 kDa, as well as additional bands from 48–38 kDa (Fig. 1A). By contrast, when rhGFAP was blotted and probed with the two anti-GFAP antibodies, each antibody detected GFAP primarily in a single band at 50 kDa, with a few minor smaller bands (Fig. 1B). Since human brain tissue was obtained from normal individuals after their death, the banding pattern shown in Figure 1A suggested that GFAP might have been subjected to death-associated proteolysis. To test this, rhGFAP was digested with the cell death enzyme calpain-2. When calpain-cut rhGFAP was blotted and probed with the

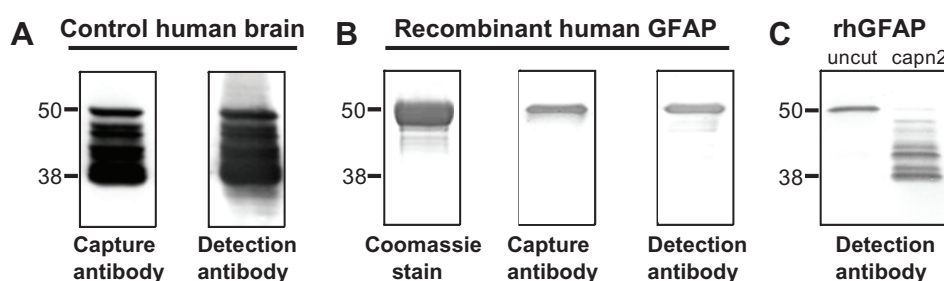


Figure 1. The anti-GFAP ELISA antibodies recognized full length human GFAP (50 kDa) and GFAP-BDP (48–38 kDa). (A) Control (normal, non head-injured) human post-mortem brain lysate (20 µg/lane) was separated by SDS-PAGE, blotted, and probed in parallel with the anti-GFAP Capture and Detection antibodies, which yielded similar banding patterns from 50–38 kDa. (B) Recombinant full length human GFAP (rhGFAP) was purified from *E. coli*, and then 4 µg was loaded and visualized with Coomassie stain. Also, 50 ng/lane was loaded, and blots were probed with the anti-GFAP Capture or Detection antibodies, which showed predominantly 50 kDa protein. (C) rhGFAP was cut or not with rat calpain-2 (capn2), then blotted at 50 ng/lane and visualized with the anti-GFAP Detection antibody. Capn2 digestion of rhGFAP yielded bands from 50–38 kDa.

Detection antibody, a range of rhGFAP bands was detected between 50 and 38 kDa (Fig. 1C), which resembled those in human post-mortem brain lysate (Fig. 1A). This result, not previously shown, indicated that calpain-2 was capable of digesting GFAP into multiple GFAP-BDPs, with the limit of digestion at 38 kDa. To examine the tissue specificity of the two anti-GFAP antibodies, proteins from a panel of human organs were examined by western blot (Fig. 2). Both antibodies detected GFAP from 50–38 kDa exclusively in the brain, and did not cross-react with proteins extracted from 9 other human organs.

The anti-GFAP swELISA was developed to measure GFAP in biofluids from human TBI patients, such as CSF or serum.¹⁷ Although our previous findings suggested that GFAP-BDPs were likely to be present in TBI-associated biofluids,¹⁷ this has not yet been characterized in the literature. In order to examine GFAP protein in TBI-associated samples, aliquots of CSF, drawn at one day after injury from 12 human severe TBI patients, were immunoblotted and compared to CSF from 5 healthy control individuals. Representative data from 8 TBI patients and one normal control are shown (Fig. 3). When blots were probed with the anti-GFAP Detection antibody, GFAP bands from 50–38 kDa were detected in most of the TBI patients examined (n = 12; 10/12 were GFAP positive; 8/12 positives are shown), but not in

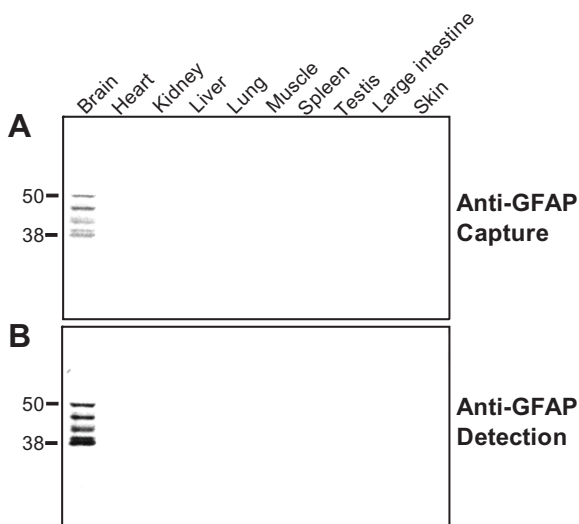


Figure 2. The anti-GFAP ELISA antibodies detected GFAP from 50–38 kDa specifically in human brain. Protein lysates from the indicated human organs (20 µg/lane) were blotted and probed with the indicated antibodies. Both the Capture (A) and Detection (B) antibodies recognized GFAP from 50–38 kDa exclusively in the brain.

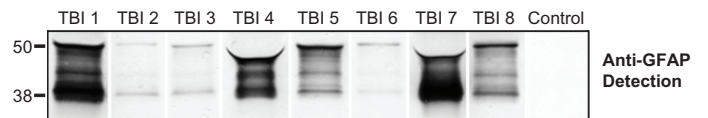


Figure 3. In human severe TBI CSF, GFAP was present in a range of bands from 50–38 kDa. Equal aliquots of CSF drawn at one day after injury from 8 severe human TBI patients were blotted and probed with the anti-GFAP Detection antibody. CSF from one representative control patient is also shown. Full length GFAP and GFAP-BDP were detected from 50–38 kDa. All samples were run together on the same gel/blot; intervening lanes were removed.

healthy control CSF (5/5 were GFAP negative; one representative control CSF sample is shown, Fig. 3). The Capture antibody showed similar results (not shown). The GFAP-positive CSF samples varied by patient in terms of overall GFAP levels and extent of GFAP breakdown (Fig. 3 and not shown). The GFAP bands in human TBI CSF were from 50–38 kDa and appeared similar to those in post-mortem human brain lysate, or calpain-cut rhGFAP (Figs. 1 and 2).

Next, experiments were performed to investigate which GFAP bands in the 50–38 kDa range were recognized by the anti-GFAP swELISA antibodies on immunoblots, and whether the antibodies showed a preference for GFAP-BDP over intact GFAP. To produce human GFAP-BDPs in a controlled manner, human HEK293 cells were transiently transfected with a construct encoding full length human GFAP, isoform 1. Lysates from these cells were then *in vitro* digested with low or high calpain-2. Digested and undigested lysates were then examined by immunoblotting (Fig. 4A). α II-spectrin (spectrin) was also examined in the same lysates, as a control for enzyme activity.^{30,31} In lysates from GFAP-transfected HEK293 cells, both GFAP and spectrin were cleaved by calpain. Spectrin was first cleaved to SBDP150, and then to a mixture of SBDP150 and SBDP145 with increasing enzyme concentration (Fig. 4A), as expected.^{30,31} When blots were probed with the two anti-GFAP antibodies, both antibodies detected full length GFAP at 50 kDa, calpain-cleaved GFAP at 38 kDa, and intermediate GFAP-BDPs (Fig. 4A). Both antibodies showed that GFAP was cleaved to bands migrating between 50 and 38 kDa with a low amount of calpain-2, and then mostly to 38 kDa with more enzyme (Fig. 4A).

In order to examine GFAP from healthy brain, fresh rat and mouse brain lysates were analyzed similarly by calpain-2 digestion. Rat brain was

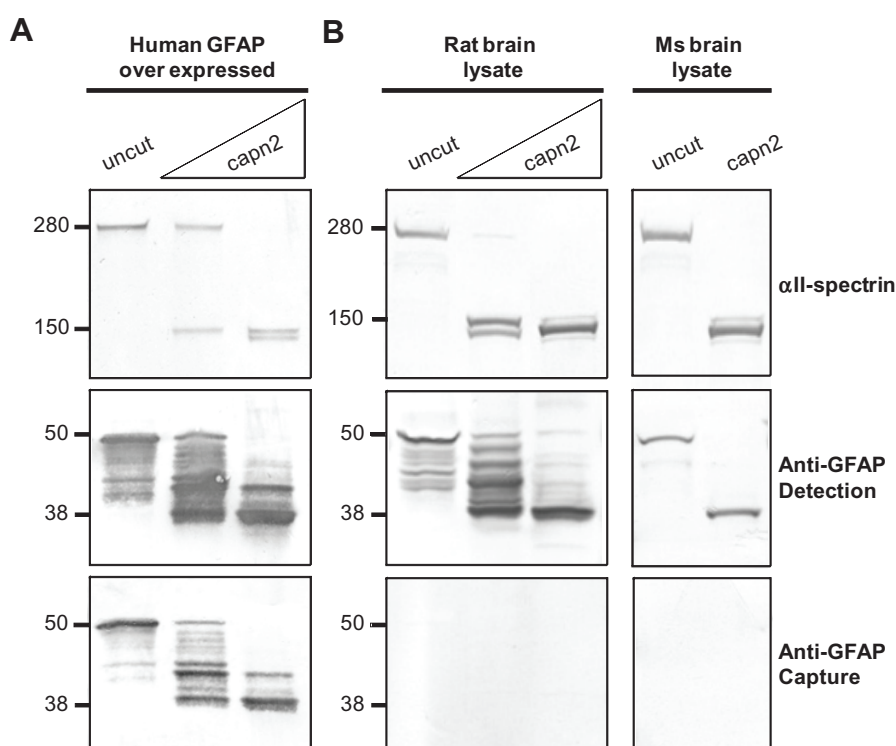


Figure 4. The anti-GFAP Capture antibody detected human GFAP and GFAP-BDP, but had poor affinity for rodent GFAP. Lysates from the indicated sources were digested with increasing amounts of rat capn2 (triangles) and probed with the antibodies labeled on the right. Spectrin served as a positive control for digestion efficiency, and was cleaved to SBDP150 and SBDP145 in a capn2 concentration-dependent manner. (A) In lysates from human HEK293 cells over-expressing human GFAP, both antibodies detected GFAP from 50–38 kDa, with capn2 cleaving GFAP to a 38 kDa limit fragment. (B) The anti-GFAP Detection antibody recognized full length endogenous GFAP and capn2-generated GFAP-BDPs from rat and mouse brain, while the Capture antibody did not.

digested with two levels of calpain-2, low and high, and mouse brain was digested with a high level of calpain-2 only. Calpain-2 cleaved spectrin as expected in both rat and mouse brain lysates (Fig. 4B). The anti-GFAP Detection antibody recognized rat GFAP from 50–38 kDa, and mouse GFAP at 50 and 38 kDa (Fig. 4B). This analysis revealed that calpain was able to cleave rodent GFAP, and that the Detection antibody recognized intact GFAP and GFAP-BDP from healthy brain. The Capture antibody did not detect GFAP from rat or mouse brain (Fig. 4B). Under some conditions such as increased loading and/or increased anti-GFAP Capture antibody concentration, the Capture antibody did detect some rat GFAP bands, but the bands detected varied from blot to blot (not shown). The anti-GFAP Capture antibody did not detect mouse brain GFAP even when blots were probed with high concentration of antibody (up to 11 $\mu\text{g}/\text{mL}$; not shown).

Antigen binding by antibodies in western blot and ELISA formats is different. On SDS-PAGE immunoblots, the antigen is denatured and reduced,

while in biofluids, the native antigen is folded. To provide insight into the ability of the anti-GFAP Capture antibody to bind native GFAP, immunoprecipitations (IPs) were performed. CSF samples were selected and pooled from 3 human TBI patients that exhibited robust GFAP breakdown, displaying together a greater quantity of 38 kDa GFAP-BDP compared to full length 50 kDa GFAP by immunoblot (Fig. 5, lane 1). In the IP experiments, conditions were designed to mimic those used in the swELISA assay. IPs were performed with biotinylated anti-GFAP Capture antibody and streptavidin-coupled beads. Post-mortem normal human brain lysate served as the positive control. As a negative control, streptavidin beads only were used (no antibody). Following incubation, washing, and elution, immunoprecipitates (eluates) were blotted and probed with the anti-GFAP Detection antibody. Samples of diluted brain lysate or CSF before (pre) and after (post) IP were also run on the same blots, for comparison (Fig. 5). From human brain lysate, the biotinylated Capture antibody pulled down GFAP bands from 50–38 kDa (Fig. 5A, lane 3). The pattern

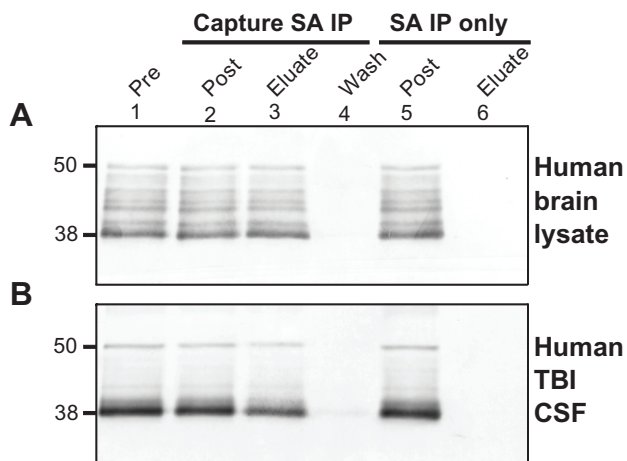


Figure 5. The Capture antibody pulled down full length human GFAP and GFAP-BDP. Immunoprecipitations (IP) were performed with biotinylated anti-GFAP Capture antibody and streptavidin (SA)-coupled beads (Capture SA IP) or with SA beads only as a negative control (SA IP only). GFAP was immunoprecipitated separately from two sources, human post-mortem brain lysate (A) or CSF pooled from 3 severe TBI patients (B). Samples of brain lysate or CSF were resolved prior to IP (pre lane 1), and after IP (post lanes 2, 5). Proteins were eluted from the SA beads (eluate lanes 3, 6), and then the beads were washed with SDS-PAGE buffer (wash lane 4). Blots were probed with anti-GFAP Detection antibody. (A and B). Biotinylated anti-GFAP Capture antibody recovered full length GFAP and GFAP-BDP from human brain lysate and TBI CSF, without appearing to enrich for any particular GFAP band.

of GFAP bands that were pulled down (Fig. 5A, lane 3) appeared identical to those in the original lysate (Fig. 5A, lane 1). The wash lane (lane 4) was clean indicating good elution. By contrast, the streptavidin beads alone (without Capture antibody) did not recover GFAP from brain lysate (Fig. 5A, lane 6). From human TBI CSF, the biotinylated Capture antibody pulled down full length human GFAP at 50 kDa, GFAP-BDP at 38 kDa, and minor intermediate bands (Fig. 5B, lane 3). Again, the pattern of GFAP bands that were eluted was very similar to that in the starting pooled CSF (Fig. 5B, lane 1). In the CSF after IP (Fig. 5B, lane 2), there was a slight reduction in GFAP signal compared to CSF before IP (Fig. 5B, lane 1). Again the wash lane was relatively clean (Fig. 5B, lane 4), and the streptavidin beads alone did not precipitate GFAP (Fig. 5B, lane 6). These data reveal that the anti-GFAP Capture antibody was able to bind to full length GFAP and GFAP-BDP from 50–38 kDa, without appearing to enrich for any particular band.

Discussion

This study demonstrates that the anti-GFAP swELISA antibodies¹⁷ non-preferentially recognized full length human GFAP (50 kDa) and GFAP-BDP (48–38 kDa)

on western blots. GFAP was cleaved by calpain into GFAP-BDP ranging in size from 48–38 kDa, with the 38 kDa band appearing as the limit of digestion. Both antibodies detected GFAP and GFAP-BDP specifically in the brain, and not in the 9 other human organs examined. In human severe TBI CSF from multiple patients, GFAP was present in a range of bands from 50–38 kDa that resembled calpain-cleaved GFAP bands. Banding patterns and GFAP quantities varied among patients, with some patients showing more GFAP breakdown than others. Also, the anti-GFAP Capture antibody detected human GFAP and human GFAP-BDP on immunoblots quite well, but showed low affinity for endogenous GFAP from healthy rat and mouse brain, demonstrating specificity for human GFAP. Finally, the Capture antibody immunoprecipitated full length human GFAP and GFAP-BDP from two sources, human brain lysate and pooled severe TBI CSF.

Previously, the Banyan anti-GFAP swELISA measured GFAP in human post-TBI serum samples, and distinguished head-injured subjects from controls.¹⁷ The same assay does not measure GFAP sensitively in samples from rodent animal models (not shown). Epitope mapping has revealed that there are two epitopes for the Capture antibody within human GFAP (not shown). Neither of the two epitopes was entirely conserved between rat and human GFAP, and the epitopes diverged further in mouse GFAP. The lack of epitope conservation between species likely explains why the Capture antibody did not detect rodent GFAP efficiently by immunoblotting. The remarkable specificity of the assay for human GFAP therefore originates with the anti-GFAP Capture antibody.

IP of GFAP from TBI serum is challenging due to much lower levels compared to TBI CSF.²⁴ The larger volume of TBI serum required for such an experiment was prohibitive. In the CSF IP experiment, the specificity of the Capture antibody for intact GFAP vs. GFAP-BDP was examined (Fig. 4). The Capture antibody pulled down full length GFAP at 50 kDa and GFAP-BDP at 38 kDa. Moreover, the same GFAP bands were detected in the starting samples (Fig. 4, lane 1) and in the eluates (Fig. 4, lane 3) in both human brain lysate and CSF, suggesting no bias in collection of GFAP species. The present results confirm and extend previous findings,¹⁷ indicating that Banyan's GFAP swELISA is likely to capture intact



GFAP and GFAP-BDP from human biofluids. The state of GFAP protein in human TBI serum samples is not presently known, and may be different from TBI CSF. However since GFAP bands from 50–38 kDa were seen in 10/12 human severe TBI CSF samples examined (Fig. 3 and not shown), it seems likely that GFAP-BDP are also present in TBI sera. GFAP has been detected in blood samples from normal and non-head injured humans, but at dramatically lower levels than in TBI patients.^{17,22,24}

Serum-based biomarkers that can be measured rapidly and quantitatively are invaluable in clinical settings for guiding diagnosis and treatment decisions.¹⁶ The existence of GFAP-BDPs, of the size range produced by calpain digestion *in vitro*, in TBI biofluids suggests calpain action, and thus provides insight into the mechanism of cell death. From a diagnostic perspective, it is valuable to know that the anti-GFAP Capture antibody is likely to collect the full range of GFAP species present in human biofluids.

Author Contributions

Conceived and designed the experiments: JSZ, LF. Made critical reagents: JSZ, DS, AC, BY, KN, LF. Performed experiments: JSZ, BY, AC. Analysed the data: JSZ. Wrote the first draft of the manuscript: JSZ. Contributed to the writing of the manuscript: JSZ. Agree with manuscript results and conclusions: JSZ, KN. Made critical revisions: JSZ, DS, KN, LF. All authors reviewed and approved of the final manuscript: JSZ, DS, AC, BY, KN, LF.

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Stefania Mondello provided advice. Sincere thanks are extended to all contributors.

Disclosures and Ethics

The authors are employees of Banyan Biomarkers, Inc., but do not receive royalties from the company and will not benefit financially from this publication. This work was supported in part by Department of Defense grant W81XWH-06-1-0517 to Ronald Hayes, and Department of Defense contract W81XWH-10-C-0251 to Ronald Hayes. This work was also partially supported by NINDS-NIH grant R01-NS052831-01 to Steve Robicsek. The NINDS-NIH grant provided the method for collecting TBI patient samples described in the Methods section. The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting true views of the Department of Defense or the NINDS-NIH. None of the funding sources were involved in the design of the study reported here, writing of the manuscript, or choice of journal. Experiments involving human subjects were done ethically and in accordance with all necessary guidelines as stated in the Methods section.

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