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Ubiquitin C-Terminal Hydrolase-L1 as a Biomarker for Ischemic and Traumatic Brain Injury in Rats

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Abstract

Ubiquitin C-terminal hydrolase-L1 (UCH-L1), also called neuronal-specific protein gene product 9.5, is a highly abundant protein in neuronal cell body and has been identified as a possible biomarker based on recent proteomic study. In this study we examined whether UCH-L1 was significantly elevated in cerebrospinal fluid (CSF) following controlled cortical impact traumatic brain injury (CCI) and middle cerebral artery occlusion (MCAO; model of ischemic stroke) in rats. Quantitative immunoblots of rat CSF revealed a dramatic elevation of UCH-L1 protein 48 h after severe CCI and as early as 6 h after mild (30 min) and severe (2h) MCAO. A sandwich ELISA constructed to measure UCH-L1 sensitively and quantitatively showed CSF UCH-L1 levels were significantly elevated as early as 2h and out to 48h after CCI. Similarly, UCH-L1 levels were also significantly elevated in CSF from 6 h to 72 h after 30 min MCAO and from 6 to 120 h after 2 h MCAO. These data are comparable to the profile of the calpain-produced α II-spectrin breakdown product of 145 kDa (SBDP145) biomarker. Importantly, serum UCH-L1 biomarker levels were also significantly elevated after CCI. Similarly, serum UCH-L1 levels in the 2 h MCAO group were significantly higher than those in the 30 min group. Taken together, these data from two rat models of acute brain injury strongly suggest that UCH-L1 is a candidate brain injury biomarker detectable in biofluid compartments (CSF and serum).

Keywords

Biofluids; biomarker; cell death; diagnosis; ischemia; stroke; necrosis; traumatic brain injury

INTRODUCTION

Traumatic brain injury (TBI) is a significant civilian health problem representing a potentially catastrophic debilitating medical emergency with poor prognosis for long-term recovery. Each year in the United States at least 1.4 to 2 million people seek medical help for a TBI (Langlois *et al.* 2004). TBI also represents a major central nervous system disorder without any clinically proven therapy (Choi and Bullock 2001). It has been argued that diagnostic biofluid-based biomarkers would provide evidence for pathology progression and help guide therapy development (Choi 2002). Unfortunately there are currently no biomarkers with proven clinical utility for diagnosis of brain injury, whether caused by TBI, stroke, or other acute etiologies. The most well studied brain injury biomarkers are neuron-specific enolase (NSE) (Missler *et al.* 1997; Ross *et al.* 1996; Yamazaki *et al.* 1995), glial protein S-100 β (Missler *et al.* 1997; Raabe *et al.* 1999; Romner *et al.* 2000), glial fibrillary acidic protein (GFAP) (Pelinka *et al.* 2004a; 2004b; Vos *et al.* 2004; Lumpkins *et al.* 2008) and myelin basic protein (MBP) (Yamazaki *et al.* 1995; Wang *et al.* 2005). Some studies illustrated the diagnostic potential of these brain injury biomarkers, but other studies have provided conflicting results (Johnsson *et al.* 2000; Pelinka *et al.* 2003, 2004c; Berger *et al.* 2005, Berger *et al.* 2007; Ingebrigtsen and Romner 2003; Pelinka *et al.* 2005). NSE, for example, initially held promise as a brain injury biomarker since originally it was believed to be strictly neuronal. However, additional research found that NSE was also present in red blood cells and platelets decreasing its diagnostic utility as a marker due to possible cross contamination that could occur in blood samples (Johnsson *et al.* 2000). After multiple trauma, increase in NSE levels have been observed, but systemic NSE levels increased correspondingly with and without TBI, limiting its ability to be a discriminator of brain injury magnitude (Pelinka *et al.* 2005). Although assays of serum NSE together with S100 β have been valuable in prediction of TBI outcome (Berger *et al.* 2006; Berger *et al.* 2007).

More recently, our group and many others have characterized α II-spectrin breakdown products (SBDPs) as potential biomarkers for excitotoxic, traumatic and ischemic brain injury in rat and in human brain trauma (Siman *et al.* 1988, Siman *et al.*, 1989; Seubert 1988; Seubert 1989; Roberts-Lewis and Siman 1993; Pike *et al.* 2001; Pike *et al.* 2003; Siman *et al.*, 2004, Siman *et al.*, 2005; Wang *et al.* 2005; Pineda *et al.* 2007), while others proposed that the cleaved tau protein (c-tau) (Zemlan *et al.* 2002; Shaw *et al.* 2002; Wang *et al.* 2005; Siman *et al.*, 2005) and a fragmented form of the glutamate-N-methyl-D-aspartate (NMDA) receptor (NR2A/2B subtype) (Dambinova *et al.* 2003) might have similar utilities. In addition, studies by Petzold and of Shaw and their colleagues identifying neurofilament H as a promising axonal injury biomarker for various forms of acute brain damage (Petzold 2005; Petzold *et al.*, 2006; Petzold and Shaw 2007; Shaw *et al.*, 2005, Anderson *et al.*, 2008).

Using differential proteomic methods, a systematic assessment was made to identify additional, previously unidentified protein biomarkers for TBI (Kobeissy *et al.* 2006). Forty-one protein candidates showed at least a two-fold increase in protein levels in the rat cortical tissue 48 h following experimental traumatic brain injury (TBI) (controlled cortical impact; CCI at a 1.6 mm depression depth, equivalent to severe TBI in humans) (Kobeissy *et al.* 2006). Of interest was that a previously described biomarker α II-spectrin breakdown product was identified in that study. As a follow-up, we then further applied a Systems Biology based approach to select down top candidate markers that represent distinct pathways and hot spots (Kobeissy *et al.*, 2008). In addition, we used three criteria to select candidate brain injury biomarkers with favorable attributes: (i) brain specificity; (ii) high abundance in brain; (iii) relatively small molecular weight (less than 30 kDa). Brain specificity was analyzed based on mRNA expression profile in human and rat using the BioGPS database (Genomics Institute of the Novartis Research Foundation; <http://biogps.gnf.org/#goto=welcome>). Abundance in brain was based on data-mining of

literature (PubMed source; <http://www.ncbi.nlm.nih.gov/pubmed/>), while calculated molecule weight was obtained via PubMed. We identified a handful of biomarker candidates that fulfill all three criteria. But due to the significant resource that is needed to develop a highly sensitive sandwich ELISA, in the current study, we focus on only one such candidate biomarker: ubiquitin C-terminal hydrolase-L1 (UCH-L1). UCH-L1, also known as neuronal-specific protein gene product (PGP 9.5), is a highly brain specific and highly abundant protein that resides in neurons (Jackson and Thompson 1981). UCH-L1 is present in almost all neurons and averages between 1 to 5% of the total soluble brain proteins (Jackson and Thompson 1981). It has also a small and compact molecular weight (24 kDa). There are 2 related enzymes in this class (UCH-L1, and UCH-L3), but only UCH-L1 is highly abundant in CNS (Wilkinson *et al.* 1992; Larsen *et al.* 1996; Wilkinson 1997; Larsen *et al.* 1998). These enzymes are involved in either the addition or removal of ubiquitin from proteins that are destined to be metabolized via the ATP-dependent proteasome pathway (Tongaonkar *et al.* 2000). It has been suggested that UCH-L1 plays a critical role in the removal of excessive, oxidized or misfolded proteins both during normal and neuropathological conditions. Based on this important neuronal function and its high specificity and abundance in the CNS, UCH-L1 was selected as a candidate biomarker for brain injury. We hypothesized that UCH-L1 would be released from injured neurons and would find its way into the cerebrospinal fluid (CSF) and eventually into circulating blood.

Materials and Methods

Materials

Sodium bicarbonate, (Sigma Cat #: C-3041, St. Louis, MO, USA), blocking buffer (Starting block T20-TBS, Pierce Cat#: 37543, Rockford, IL, USA), Tris buffered saline with Tween 20 (TBST; Sigma Cat #: T-9039, St. Louis, MO, USA). Phosphate buffered saline (PBS; Sigma Cat #: P-3813, St. Louis, MO, USA); Tween 20 (Sigma Cat #: P5927, St. Louis, MO, USA); Ultra TMB ELISA (Pierce Cat #: 34028, Rockford, IL, USA); and Nunc maxisorp ELISA plates (Fisher Scientific, Pittsburgh, PA, USA). Monoclonal and polyclonal UCH-L1 antibodies were made in-house.

In vivo model of TBI injury model

A controlled cortical impact (CCI) device was used to model TBI on rats as previously described (Pike *et al.* 1998). Adult male (280–300 g) Sprague-Dawley rats (Harlan: Indianapolis, IN, USA) were anesthetized with 4% isoflurane in a carrier gas of 1:1 O₂/N₂O (4 min.) and maintained in 2.5% isoflurane as anaesthesia in the same carrier gas. Core body temperature was monitored continuously and maintained at 37±1°C. Animals were mounted in a stereotactic frame in a prone position and secured by ear and incisor bars. Following a midline cranial incision and reflection of the soft tissues, a unilateral (ipsilateral to site of impact) craniotomy (7 mm diameter) was performed adjacent to the central suture, midway between bregma and lambda. The dura mater was kept intact over the cortex. Brain trauma was produced by impacting the right (ipsilateral) cortex with a 5 mm diameter aluminum impactor tip (housed in a pneumatic cylinder) at a velocity of 3.5 m/s with a 1.6 mm compression and 150 ms dwell time. Sham-injured control animals underwent identical surgical procedures but did not receive the impact injury. Appropriate pre- and post-injury management was performed to minimize pain and discomfort and to insure compliance with guidelines set forth by the University of Florida Institutional Animal Care and Use Committee and the National Institutes of Health guidelines detailed in the Guide for the Care and Use of Laboratory Animals. In addition, research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adhered to principles stated in the “Guide for the Care and Use of Laboratory Animals, NRC Publication, 1996 edition.”

Rat MCAO injury model

Since the prototypic brain injury biomarkers (α II-spectrin breakdown products) are elevated in CSF following middle cerebral artery occlusion (MCAO) (Pike *et al.* 2003) with a pattern similar to those observed following CCI (Pike *et al.* 2001; Ringger *et al.* 2005) the same MCAO model was used to examine UCH-L1 levels. MCAO was performed as follows: under 2.5% isoflurane anesthesia, the right common carotid artery (CCA) of the rat was exposed at the external and internal carotid artery (ECA and ICA) bifurcation level with a midline neck incision. The ICA was followed rostrally to the pterygopalatine branch and the ECA was ligated and cut at its lingual and maxillary branches. A 3-0 nylon suture was then introduced into the ICA *via* an incision on the ECA stump and advanced through the carotid canal until it became lodged in the narrowing of the anterior cerebral artery blocking the origin of the middle cerebral artery. The endovascular suture was left in place for either 30 minutes or 2 hours. Afterwards the rat was briefly reanesthetized and the suture filament was retracted to allow reperfusion. For sham MCAO surgeries, the same procedure was followed, but the filament was advanced only 10 mm beyond the internal-external carotid bifurcation and was left in place until the rat was sacrificed. During all surgical procedures, animals were maintained at $37\pm 1^\circ\text{C}$. It is important to note that at the conclusion of each experiment, if the rat brains showed pathologic evidence of subarachnoid hemorrhage upon necropsy they were excluded from the study. Appropriate pre- and post-injury management guidelines were maintained and these measures complied with all guidelines set forth by the University of Florida Institutional Animal Care and Use Committee and the National Institutes of Health guidelines detailed in the *Guide for the Care and Use of Laboratory Animals*.

CCI and MCAO tissue preparation

At the appropriate time points (2, 6, 24 hours and 2, 3, 5 days) after injury, animals were anesthetized and immediately sacrificed by decapitation. Brains were quickly removed, rinsed with ice cold PBS. The ipsilateral hemisphere (cerebrocortex around the impact area and hippocampus) were rapidly dissected, rinsed in ice cold PBS, snap-frozen in liquid nitrogen, and stored at -80°C until used. For Western blot analysis, the brain samples were pulverized over dry ice to a fine powder that was then lysed for 90 min at 4°C in a buffer of 50 mM Tris (pH 7.4), 5 mM EDTA, 1% (v/v) Triton X-100, 1 mM DTT, and protease inhibitor cocktail (Roche Biochemicals, Basel, Switzerland). The brain lysates were then centrifuged at $15,000 \times g$ for 5 min at 4°C to clear and remove insoluble debris, snap-frozen, and stored at -80°C until used.

CCI and MCAO rat CSF sample collection

The CSF was collected from animals as previously described (Pike *et al.* 2001). At the appropriate time points, injured, sham-injured and naïve animals were anesthetized as described above and secured in a stereotactic frame with the head allowed to move freely along the longitudinal axis. The head was flexed so that the external occipital protuberance in the neck was prominent and a dorsal midline incision was made over the cervical vertebrae and occiput. The atlanto-occipital membrane was exposed by blunt dissection and a 25 gauge needle attached to polyethylene tubing was carefully inserted into the cisterna magna. Approximately 0.1 to 0.15 ml of CSF was collected from each rat. After CSF collection, animals were removed from the stereotactic frame and immediately euthanized by decapitation. CSF samples were centrifuged at $4,000 \times g$ for 4 min. at 4°C to clear any contaminating erythrocytes. Cleared CSF samples were stored at -80°C until ready for use.

CCI and MCAO rat serum collection

At the end of each experimental period, rats were anesthetized by sodium pentobarbital and then euthanized by bilateral thoracotomy. Blood (3–4 ml) was collected directly from heart using syringe equipped with 21 gage needle placed in a polypropylene tube and allowed to stand for 45 min to 1 hour at room temperature to form clot. Tubes were centrifuged for 20 min at $3,000 \times g$, serum was removed, and aliquots were frozen and stored at -20°C until use. For long-term storage (>3 months), serum aliquots were placed in a -80°C freezer.

SDS-polyacrylamide gel electrophoresis and Immunoblotting analysis for UCH-L1

Cleared CSF samples (7 μL) were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Twenty micrograms (20 μg) of protein per lane were routinely resolved by SDS-PAGE on 10–20% Tris/glycine gels (Millipore, Billerica, MA, USA) at 130 V for 2 hours. Following electrophoresis, separated proteins were laterally transferred to polyvinylidene fluoride (PVDF) membranes in a transfer buffer at a constant voltage of 20V for 2 hours at ambient temperature in a semi-dry transfer unit (Bio-Rad, Hercules, CA, USA). After electro-transfer, the membranes were blocked for 1 hour at ambient temperature in 5% non-fat milk in TBS and 0.05% Tween-2 (TBST) then were incubated with the primary polyclonal UCH-L1 antibody (in-house) in TBST with 5% non-fat milk at 1:2000 dilution at 4°C overnight. This was followed by three washes with TBST, a 2 hour incubation at ambient temperature with a biotinylated linked secondary antibody (GE Healthcare, Piscataway, New Jersey, USA) and a 30 min incubation with Streptavidin-conjugated alkaline phosphatase (BCIP/NBT reagent: KPL, Rockville, MD, USA). Linear relationship between band intensity (densitometric units) of UCH-L1 and serial dilution of UCH-L1 standard protein was established. Semi-quantitative evaluation of intact UCH-L1 proteins levels was performed via computer-assisted densitometric scanning (Epson XL3500 scanner, Long Beach, CA, USA) and image analysis with ImageJ software (NIH, USA).

UCH-L1 and SBDP Sandwich ELISA

For UCH-L1 sandwich ELISA (swELISA), 96-well plates were coated with 100 μL /well capture antibody (500 ng/well purified mouse monoclonal anti-UCHL1, made in-house) in 0.1 M sodium bicarbonate, pH 9.2. Plates were then incubated overnight at 4°C , emptied and 300 μL /well blocking buffer (Starting block T20-TBS) was added and incubated for 30 min at ambient temperature with gentle shaking. This was followed by either the addition of the antigen standard (recombinant UCH-L1) for standard curve (0.05 – 50 ng/well) or samples (3–10 μL CSF) in sample diluent (total volume 100 μL /well). The plate was incubated for 2 hours at room temperature, then washed with automatic plate washer (5×300 μL /well with wash buffer, TBST). Detection rabbit polyclonal anti-UCH-L1-HRP conjugated (made in-house, 50 $\mu\text{g}/\text{mL}$) in blocking buffer was then added to wells at 100 μL /well and incubated for 1.5 h at room temperature, followed by washing. Lastly, the wells were developed with 100 μL /well chemiluminescent substrate solution (SuperSignal ELISA Femto, Company: Pierce, Cat#: 37075, Rockford, IL, USA) with incubation times of 1 minute. The signal was by a 96-well chemiluminescence microplate reader (GloRunner DXL Luminometer, Turner BioSystems Inc., Sunnyvale, CA, USA).

For SBDP145 and SBDP120 sandwich ELISAs are constructed similarly to that of UCH-L1. Briefly, 96-well plate was coated 100 μL /well capture antibody (500 ng/well purified rabbit polyclonal anti-SBDP145 or anti-SBDP120 fragment specific antibody)(McGinn et al., 2008; Nath et al., 2000)overnight at 4°C . After blocking buffer (Starting block T20-TBS) step, antigen standard (recombinant GST-fusion- αII -spectrin (repeat 9 to 15) cleaved with calpain-2 (1/50 ratio) or with cspase-3 for 30 min) to establish a standard curve. Stock solution of 0.5 ng/mL – 5000 ng/mL of prepared SBDP145 protein (0.005 ng-50 ng in 10 μL) were diluted 1/10 with sample diluent to a final incubation volume of 100 μL /well. Thus

the standard curve range is 0.05 ng/mL – 500 ng/mL in the wells. For rat CSF samples, 10 µL was typically used per well and diluted 1/10 with diluent to a final incubation volume of 100 µL/well. If amplification was needed, biotinyl-tyramide solution (Elast Amplification Kit, Perkin Elmer, Waltham, MA, USA) was added for 15 min at room temperature, washed and followed by 100 µL/well Streptavidin-HRP (1:500) in PBS with 0.02% Tween-20 and 1% BSA for 30 min followed by washing. Lastly, the wells were developed with chemiluminescent substrate as above.

Statistical analyses

Semi-quantitative evaluation of protein levels on immunoblots was performed via computer-assisted 1-dimensional densitometric scanning (Epson expression 8836XL high-resolution flatbed scanner and NIH ImageJ densitometry software). Data were acquired in arbitrary densitometric units and any changes in the outcome parameters were compared to the appropriate control group. Consequently, magnitude of change from control in one model system was directly comparable to those from any other model system. ELISA data were also obtained and analyzed. Data were assessed for distribution and variance. Comparisons for all groups between replicate immunoblots and between ELISA groups were performed using one-way analysis of variance (ANOVA). In the case of single comparisons the Student's t-test was applied. P-values equal to or less than 0.05 were considered statistically significant. All data were analyzed with SigmaPlot 11 (SyStat Software, Inc., San Jose, CA, USA) software.

Results

Immunoblot evidence that Ubiquitin C-terminal hydrolase-L1 (UCH-L1) is a potential biomarker for acute brain injury in rats

First, UCH-L1 was examined to see if it was detectable in rat CSF 48h following CCI by immunoblot analyses. UCH-L1 protein after injury was readily detectable in the samples and showed levels that were over and above the faint amounts of UCH-L1 observed in naïve and sham samples (Figure 1A). Quantitative immunoblot results show elevated UCH-L1 levels in the CSF 48h after CCI were statistically significant ($p=0.014$, $n=3$) when compared to both naïve and sham controls (Figure 1B). We further demonstrated that UCH-L1 band intensity (densitometric units) by immunoblotting with UCH-L1 antibody shows a strong relationship with serial dilution of UCH-L1 standard protein, thus validating this method of quantification (Figure 1C).

Next, examination was undertaken to determine whether UCH-L1 level increases were also detectable in the CSF following ischemic brain injury. CSF UCH-L1 levels were found to be transiently elevated (at 6h, $p=0.035$) after mild ischemia (MCAO-30 min) followed by reperfusion, while they were sustained from 6h to 3 days after a more severe ischemia (MCAO-2h: (6h, $p=0.050$; 1d, $p=0.015$; 2d, $p=0.005$, and 3d, $p=0.048$)) (Figure 2).

Tissue specificity and brain region distribution of UCH-L1 in uninjured rat brains

UCH-L1 protein expression was examined for brain specificity in a rat tissue panel by immunoblot. It was found to be highly expressed in the brain (Figure 3A) with the only minute amounts in some other organs (e.g. testis), thereby confirming and extending work by Saigoh *et al* (1999). In addition, it appeared to be uniformly expressed across all brain regions examined (Figure 3B) except for lower levels in the cerebellum and pons.

UCH-L1 antibody tools and sandwich ELISA configuration and validation

In order to quantitatively measure UCH-L1 levels in biofluids, a sensitive sandwich ELISA (swELISA) was developed. Two compatible antibody pairs (one mouse monoclonal anti-

UCH-L1 antibody was used as capture antibody, whereas a rabbit polyclonal anti-UCH-L1 antibody was affinity purified, HRP-labeled and used as detection antibody) and recombinant UCH-L1, expressed in *E. coli* as the antigen standard, were used. First, we confirmed that both of these antibodies indeed detect recombinant UCH-L1 target protein and single UCH-L1 protein band from total rat brain and human brain lysate respectively (Figure 4A). Secondly, the configured and optimized UCH-L1 sandwich ELISA with amplification and chemiluminescence detection has a linear dynamic range of at least 3 order of magnitude and detection limit of 0.01 ng/mL in CSF, and it is linear for 3 order of magnitude and a detection limit of 0.1 ng/mL for serum (standard sample volume 100 μ L) (Figure 4B). However, since we dilute rat serum samples 1 to 4 (20 μ L serum + 80 μ L Tris buffer saline (TBS)). Thus serum detection limit in actual serum samples is 0.025 ng/mL.

In addition, the data shown are based on 16 assays for serum (8 plates with duplicate wells); and 14 assays for CSF (7 plates with duplicate wells), performed over 2–3 days. The intra-assay and inter-assay precision, as measured by % CV (coefficient of variance) values are routinely below 10–12% within the detection range (Figure 4B).

UCH-L1 biomarker validation in rat CSF following TBI and MCAO

CSF samples from naïve (n=4), sham (n=5), and rats subjected to severe (CCI 1.6 mm: n=7) and moderate TBI (CCI 1.0 mm: n=5) were analyzed 2, 6, 24 and 48h after injury with the UCH-L1 swELISA (Figure 5A). Overall, UCH-L1 levels for both 1.6 mm and 1.0 mm TBI group, sham and naïve were all significantly different from each other ($p=0.01$). More specifically, the UCH-L1 signal for the CCI 1.0 mm for time points 2h and 6h were significantly higher than their counterparts in the sham group ($p=0.028$ and $p=0.020$, respectively) and for the CCI 1.6 mm time points 2h, 6h and 24h they were significantly higher than their sham counterparts ($p=0.018$; $p=0.016$; $p=0.019$, respectively). Additionally, the UCH-L1 signal for the CCI 1.0 mm for time points 2h, 6h, 24h, and 48h were significantly higher than their counterparts in the naïve group ($p=0.001$; $p=0.012$; $p=0.03$; $p=0.03$, respectively) and for the CCI 1.6 mm time points 2h, 6h and 24h they were significantly higher than their counterparts in the naïve group ($p=0.001$; $p=0.003$, respectively). The data show that UCH-L1 is elevated as early 2h after injury in the CSF and remains elevated out to 48h.

The CSF UCH-L1 elevation profile following CCI is also compared to that of the α II-spectrin breakdown product of 145 kDa (SBDP145) produced by calpain during the acute neuronal necrosis stage (Figure 5B). Overall, SBDP145 levels of the 1.6 mm and 1.0 mm CCI groups are significantly different from the sham and naïve groups ($p=0.001$) and appears to track closely with UCH-L1 elevation. In contrast, elevated levels of the α II-spectrin breakdown product of 120 kDa (SBDP120) produced by caspase-3 were not significantly detected in this model of TBI (data not shown). Tau, and to the lesser extent, S100 β elevations in CSF have previously been demonstrated before in this CCI model of traumatic brain injury (Ringger et al., 2005).

In parallel, CSF time series samples from naïve (n=4) and from sham (n=5), MCAO-30 min (n=8) and MCAO-2h (n=13) rats at 6, 24, 48, 72, and 120h post-injury were also assayed with UCH-L1 swELISA (Figure 6A). For all groups and all time points combined there was significant differences between each group ($p=0.01$). It was also noted that the more severe MCAO-2h group UCH-L1 protein levels were 2-to 5-fold higher than MCAO-30 min group. Individual comparisons show that the UCH-L1 levels for the 24h time point for the MCAO-30 min group were significantly higher than its respective time point for the sham ($p=0.023$). Similarly, for MCAO-30 min time points 24h and 120h and the 6h time point for the MCAO-2h, the protein levels were significantly elevated above the naïve group

(MCAO-30 min: 24h ($p=0.010$), 120h ($p=0.011$); MCAO-2h: 6h ($p=0.050$)). UCH-L1 protein levels for shams were virtually indistinguishable from naïve controls.

The profile of CSF UCH-L1 elevation following MCAO-2h is comparable to the calpain generated SBDP145 (Figure 6B) and the caspase-3 generated SBDP120 (Figure 6C). In these cases, both SBDP145 (MCAO-2h group) and SBDP120 (MCAO-30 min and -2h groups) are detectable and found to be significantly above their respective sham groups ($p=0.001$). Note that CSF SBDP145 levels overall were higher in amount than their SBDP120 counterparts (Figure 6B vs. Figure 6C) while on the other hand it was comparable to its UCH-L1 counterpart.

UCH-L1 as a blood-based candidate biomarker for acute brain injury

Since UCH-L1 was robustly detected in the CSF after both experimental TBI (CCI) and ischemic stroke (MCAO), examination was conducted to see if UCH-L1 was able to cross the compromised blood brain barrier following brain trauma or ischemic injury and subsequently be detected in blood (serum). Time series serum samples from naïve ($n=5$), sham ($n=8$) and TBI ($n=5$) rats (2 h to 72 h post injury) were analyzed by UCH-L1 swELISA (Figure 7A). Overall, UCH-L1 levels of the all the group all time points combined were significantly different from each other ($p=0.001$). For individual time points 12h and 60h tested as being significantly different, in respect to the same sham time points ($p=0.006$; $p=0.020$, respectively). The data show that UCH-L1 is quite significantly elevated after injury as early as 2h. Most interestingly the sham group was itself statistically higher than naïve controls at various time points (2h: $p=0.050$; 6h: $p=0.001$; 12h: $p=0.001$; 36h: $p=0.032$) notably highlighting the sensitivity of the marker to injury perturbation.

Time series blood (serum) samples from naïve ($n=5$), sham ($n=5$), MCAO-30 min ($n=9$) and MCAO-2h ($n=13$) rats were also assayed by UCH-L1 swELISA at 6, 24, 48, 72 and 120h post-injury (Figure 7B). The UCH-L1 protein levels for all groups were significantly different from each other (all time points combined: $p=0.001$). In addition, for time points (6h: $p=0.001$; 120h: $p=0.05$) the 30 min MCAO UCH-L1 protein levels were significantly elevated over their respective sham time points. For the MCAO-2h time point 6h the UCH-L1 protein levels were significantly elevated over its respective sham time point ($p=0.038$) as well. Finally, for each of the time points 6h through 48h for both the MCAO-30 min and the MCAO-2h the UCH-L1 protein levels were significantly elevated over its respective naïve time points (MCAO-30min: 6h ($p=0.001$); 24h ($p=0.006$); 48h ($p=0.030$); MCAO-2h: 6h ($p=0.038$); 24h ($p=0.050$); 48h ($p=0.050$)) as well.

Discussion

This report provides evidence supporting the use of UCH-L1 as a surrogate neurochemical marker of CNS injury. The study demonstrates that the rat models of TBI (CCI) and stroke (focal ischemic injury -MCAO) result in increased levels of UCH-L1 in post-injury CSF and serum. The results, provide the first evidence that detection of this specific protein can be used as an explicit biomarker for neural pathological events occurring in the injured brain in at least two preclinical models of brain injury (traumatic and ischemic). Since UCH-L1 is a 24 kilodalton protein with no known active transport mechanism, it is likely that breakdown of the blood-brain barrier following brain trauma or ischemia is responsible for its release into circulating blood.

In this study, the potential of UCH-L1 as a biofluid-based protein biomarker for two forms of brain injury was evaluated: traumatic brain injury (modeled with controlled cortical impact in rats) and ischemic stroke (modeled with MCAO in rats). UCH-L1 protein was confirmed as being detectable in rat CSF and its levels were significantly elevated after TBI

and MCAO, by Western blot (Figures 1, 2) and sandwich ELISA analyses (Figures 5, 6). Furthermore, UCH-L1 levels were found to be present at detectable and elevated levels in blood (serum) within hours after both TBI and MCAO as determined by sandwich ELISA analyses (Figures 7A, 7B).

There is a general correlation between CSF level by Western and by sandwich ELISA (compared Figure 5, 6 to Figure 2, 3). However, due to the semi-quantitative and less sensitive nature of Western blot, it does not detect low levels of UCH-L1 increase in CSF as well as the ELISA is capable. In fact, one of the objectives of the current study was to establish such a quantitative and sensitive method of UCH-L1 detection by ELISA (Figure 4B). We have also performed “same sample” UCH-L1 detection by Western and by ELISA (about 10 samples), the range order correlation is very strong (data not shown) – we only were able to perform this comparison very limitedly as only very small rat CSF volume (40–50 μ L) can be obtained after brain injury in the rats.

It is noted that in all native animals, both CSF and serum compartments have zero levels of UCH-L1 (Figure 5–6). We did note that there were slightly elevated UCH-L1 levels in sham-operated animals. We attribute these increases to the fact the sham craniotomy (for CCI) and sham surgery (for MCAO themselves can, in fact, cause mild and localized brain injury. For example, in the CCI model, we have previous shown T2 weighed MRI evidence of local lesion following craniotomy-sham operation (see Ringger et al., 2005 Figure 2). Thus, we contend that our UCH-L1 sandwich ELISA had a level of sensitivity it was able to detect even very low release of UCH-L1 due to mild brain injury as a result of sham operations.

UCH-L1 is attractive as a candidate biomarker for several reasons. First, UCH-L1 is highly expressed in neurons. It was, in fact, previously referred it as a neuro-specific protein PGP 9.5 (Jackson and Thompson, 1981). Its tissue distribution is almost exclusive to the brain with testis being the only other tissue with detectable levels (Figure 3) and this is likely due to the involvement of UCH-L1 in spermatogenesis (Kwon *et al*, 2005). The ovary was not examined for its presence. Interestingly, we have also performed human tissue panel analysis, in which UCH-L1 was only significantly detected in brain, but not in the testis (data not shown).

Secondly, UCH-L1 is a small protein with a molecular weight of about 24 kDa and has a compact and almost globular shape (Johnston *et al*, 1999). Western blots of CSF show that it remains as an intact protein with no detectable breakdown product. These features would facilitate its crossing the injured cell membrane and compromised brain blood barrier (BBB) after brain injury and remaining stable in circulating CSF and/or blood for detection.

Thirdly, UCH-L1 has been implicated in neuronal cell pathologies and upon analyses appears to be a determinate in the means by which the cells degenerate. Gain-of-function mutations of UCH-L1 are a confirmed factor in familial Parkinson's (Lincoln *et al*, 1999). The role of UCH-L1 in removing conjugated ubiquitin from degradation-targeted proteins further substantiates its importance in protein turnover in neurons (Saigoh *et al*, 1999). Often misfolded proteins, if not appropriately catabolized, may result in protein aggregations that are neurotoxic (e.g. α -synuclein, amyloid A β peptides, tau and Huntington's). Elevated protein aggregate formation has, in fact, been documented following cerebral ischemia (Hu *et al*, 2001; Ge *et al*, 2007). Thus, we propose that UCH-L1 might have the potential to be not only as a surrogate, but as a mechanistic biomarker for acute brain injury. In addition, its potential utility in diagnosing chronic neurodegenerative conditions, such as Alzheimer's, Parkinson's and Huntington diseases should be explored. We are currently undertaking such investigation.

This study showed that a sandwich ELISA could be developed that was sensitive enough to quantitatively detect UCH-L1 in the CSF. By measuring the UCH-L1 levels in rat CSF, it was possible to readily distinguish injured animals from controls as early as 6 h after TBI and 2 h after MCAO, respectively (Figures 5, 6) suggesting that it could be an early biomarker of injury. For the TBI studies, UCH-L1 levels in both CSF and serum showed both an early and rapid increase before declining. It should be mentioned that UCH-L1 shows some increase in CSF within the sham group for TBI. This is likely due to the mild injury caused by the craniotomy procedure thus triggering some release of UCH-L1. Taken together, UCH-L1 protein levels are elevated early and the levels appear to be injury-severity dependent.

Similar trends are also observed in serum for the CCI and the MCAO groups respectively (Figures 7). The ability to detect UCH-L1 as early as 2 hours in TBI and 6 hours after MCAO in serum suggests it may be a good early injury marker. Its elevated levels also appear to persist for at least 24 h in serum for TBI and 72 to 120 h for mild (30 min) and severe (2 h) MCAO, respectively. Thus, it might be possible to continuously monitoring serum UCH-L1 levels as a means for tracking progression of brain injury or recovery. The fact that UCH-L1 is detectable in serum shortly after CCI and MCAO demonstrates that either it is likely to cross the compromised BBB after injury. Our model of stroke is by middle cerebral artery occlusion, thus we suspect that there might be some delay in biomarker release following reperfusion of the ischemic brain areas. However, in a separate but smaller study (n=3–4), we indeed identified biomarker (UCH-L1 and SBDPs) release into CSF even as early as 2 and 3 h after reperfusion (data not shown). Thus, the UCH-L1 release profile is very similar to that following experimental TBI.

It should also be noted that the increase in the levels of UCH-L1 after TBI and MCAO in serum from the sham groups are likely due to mild injury caused by the preparatory procedures prior to the main injury. This again suggests that the UCH-L1 protein is a sensitive and injury-severity dependent marker.

There are, as yet, no single ideal biomarker with proven clinical usefulness for TBI and cerebral ischemia. In the case of TBI, this may be due in measure to the difficulty in assessing brain injury. In the case of stroke, there has been a documented need for more objective outcome measurements such as biomarkers (Dirnagl *et al.*, 1999; Zaremba *et al.*, 2001). For example, stroke diagnosis is relatively straightforward when patients present with typical symptoms. However, these symptoms are often more subtle in nature delaying diagnosis by hours or even days (Elkind, 2003). Therefore, a rapid, reliable stroke biomarker would assist physicians in their diagnosis particularly when considering the administration of thrombolytic agents for treatment of acute ischemic stroke.

Our study of UCH-L1 as a potential biomarker for TBI and focal cerebral ischemia suggest that it might be a promising new biomarker of CNS injury. To further improve or achieve diagnostic accuracy of brain injury, it is also possible that UCH-L1 can be used as a panel of other existing biomarkers, such as S100 β (Nylén *et al.*, 2008), NF-H (Petzold and Shaw 2007) and SBDP150/SBDP145 and SBDP120 (see Figure 5–6). Future clinical studies will be needed to determine the validity of such a panel approach.

It is important to note that UCH-L1 is more variable in MCAO-2h than those found in MCAO-30min. This variability, as indicated by the error bars, can be observed in both CSF (Figure 5, 6) and serum (Figure 7). The reason in CSF for the larger variability in protein accumulation is unknown but may reflect differences in an individual rodent's CSF circulation after ischemia or its ability to repair the blood brain barrier. For example, differences in increased intracranial pressure after ischemia may restrict passage of CSF

through various foramina that may preclude detection of secreted proteins into the cisterna magna (this study's source of CSF). Future studies should examine differences in intraventricular *versus* intracisternal levels of UCH-L1.

There are additional considerations that suggest, in addition to stroke and TBI, UCH-L1 may have biomarker utility for neurodegenerative diseases including Alzheimer's disease (AD). It was recently noted that UCH-L1 levels were found to have increased in the hippocampus of AD patients. Previous studies reported the protein was oxidized with a resultant loss of activity. One of the major consequences was an impairment of the ubiquitination/deubiquitination machinery leading to increased protein aggregates, synaptic deterioration and degeneration in the hippocampus. These and other lines of evidence support a role for dysfunction of the ubiquitin-proteasome pathway in the pathogenesis of AD supporting the importance of the UCH-L1 protein (Sultana *et al*, 2007). It is important to remember that TBI is a risk factor for both Alzheimer's and Parkinson's disease.

In summary, our data suggest that UCH-L1 might join a growing list of candidate protein biomarkers that demonstrate potential clinical utility for traumatic brain injury and stroke management. Other markers included but not limited to cleaved tau, NSE and S100 β , neurofilament proteins, α II-spectrin breakdown products and GFAP. The time profile of UCH-L1 in biofluid is in fact similar to that of SBDP (Figure 5–6) and of S100b previously reported (Nylen *et al*. 2008). Thus there is a possibility that a panel of markers might emerge as the best diagnostic tool. But we caution that such hypothesis will need to be carefully examined in clinical studies.

We like to point out that the focus of the current study was not on correlation of UCH-L1 levels to histologic or functional deficit following brain injury. Thus, no attempt was made to compare on a case-by-case basis the magnitude of CSF and serum UCH-L1 levels with anatomical or behavioral measures of brain dysfunction. However, we noted that our rat TBI (CCI model) and the MCAO model show a well characterized histological and functional deficit respectively (Ringger *et al.*, 2005; Marin *et al.*, 2003). In fact, further extended studies should be directed to characterize if the elevated UCH-L1 levels in CSF and serum correlate with histological and functional deficits in these respective models. Based on the data we have collected so far in rats, we are not making any scientific claims on the "prognostic" values of UCH-L1 biomarker. We were simply describing our finding that UCH-L1 levels are elevated in two important biofluids (CSF and blood) in both TBI and stroke models using a quantitative sandwich ELISA method. What we are reporting here is that UCH-L1 levels are elevated in biofluids (CSF and serum) and are correlated to injury magnitude. This is a very significant advance for a candidate protein biomarker identified *de novo* and with significant effort put into building sensitive ELISA for the proof-of-principle confirmation of its elevation in biofluids. At the same time we submit that for the determination of the utility of UCH-L1 as a clinical diagnostic and prognostic tool, we strongly feel needs to be addressed in clinical studies not in animal models. In fact, we noted recently Siman *et al.* (2008) identified by Western blot that UCH-L1 was elevated in patients with mild central nervous system injury after surgically-induced circulation arrest. In addition, in a separate study, our group using sandwich ELISA showed that UCH-L1 levels were elevated in CSF in severe TBI patients and UCH-L1 levels were inversely correlated with 6-week mortality and with poor 6-month dichotomized Glasgow outcome score (Papa *et al.*, 2009, *in press*).

For comparison, Siman and his colleagues (2008) described that UCH-L1 (in addition to phospho-neurofilament-H, α II-spectrin breakdown product and 14-3-3 β , 14-3-3 ζ) is elevated in the CSF in humans following surgically-induced circulation arrest. The authors thus argue that these proteins could form a panel of biomarkers detecting mild brain injury

induced by circulation arrest. Their method of UCH-L1 detection was by Western blot, not by ELISA. Our recent study in human severe TBI (Papa et al., 2009, *in press*) also used a sandwich ELSIA for UCH-L1 and identified TBI (severe) diagnostic potential of UCH-L1 levels in CSF. However, in this rat study, we investigated severe and moderate TBI as well as stroke model (mild and severe) by sandwich ELISA with the use of both CSF and serum samples. Taken together, these human studies and our work in rats complement each other and further strengthen the notion that UCH-L1 might be a good stand-alone biomarker or part of a panel of candidate biomarkers for various forms of brain injury.

In summary there is much more work that needs to be done to validate the utility UCH-L1. It is obviously important to further confirm its elevation in human brain injury (CSF and blood) and to see how it compares to other candidate brain injury biomarkers. In addition, there is a need to address whether UCH-L1 is released from other organs in poly-trauma situations. Work is now in progress to determine if it has clinical utility and the required diagnostic specificity and selectivity for monitoring brain injury with minimal invasiveness in TBI and stroke patients.

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Abbreviations used

UCH-L1	Ubiquitin C-terminal hydrolase-L1
BBB	blood brain barrier
CCI	controlled cortical impact
CCA	common carotid artery
CSF	cerebrospinal fluid
ECA	external carotid artery
gad	gracile axonal dystrophy
ICA	internal carotid artery
MBP	myelin basic protein
MCAO	middle cerebral artery occlusion
PGP9.5	neuronal-specific protein gene product 9.5
swELISA	sandwich enzyme linked immunosorbent assay
TBI	traumatic brain injury

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Fig. 1. Immunoblotting evidence of UCH-L1 release into rat CSF 48 h following controlled cortical impact TBI

A) Representative immunoblot results show presence of UCH-L1 protein in rat CSF 48h after TBI (control cortical impact) as compared to sham (48h) and naïve controls. **B)** Quantification indicates statistical significance (* $p < 0.05$; $n = 3$) from sham. **C)** Recombinant human UCH-L1 serial dilution and correlation to UCH-L1 band intensity (densitometric unit) by immunoblot.

Fig. 2. Immunoblotting evidence of UCH-L1 release into rat CSF following middle cerebral artery occlusion

A) Representative immunoblot results show significant elevated levels of UCH-L1 protein in rat CSF 6h after MCAO-30 min as compared to sham and 6h, 1D, 2D, and 3D after MCAO-2h. **B)** Quantification indicates statistical significance from sham (n=4 per time point) for MCAO-30 min and MCAO-2h (* $p < 0.05$, ** $p < 0.01$).

Fig. 3. UCH-L1 tissue specificity and brain region distribution

A) Immunoblot analyses of UCH-L1 protein levels in various rat tissues (n=3 pooled) and **B)** various rat brain regions (n=3 pooled); 15 μ g protein loaded per lane.

Fig. 4. UCH-L1 antibody and sandwich ELISA characterization

Sandwich ELISA set-up was described in Methods. The capture anti-UCH-L1 antibody was mouse monoclonal antibody; the detection antibody was an affinity purified HRP-labeled rabbit anti-UCH-L1 antibody; the antigen was recombinant human UCH-L1 (Boston Biochem). **A)** Both of the capture and detection antibodies detect recombinant UCH-L1 (lane 1) and single UCH-L1 protein band from total rat brain (lane 2) and human brain lysate (lane 3) respectively. **B)** Configured and optimized UCH-L1 sandwich ELISA standard curves (with UCH-L1 antigen) were performed in CSF (squares) and in serum (filled circles) (average + standard deviation shown). Three times standard deviation of background (no antigen) in CSF (triangle) and in serum (diamond) were also shown on Y-axis and plotted as dotted horizontal lines. Lowest detection limits calculated based on intercepts.

Fig. 5. Sandwich ELISA showing UCH-L1 elevation in rat CSF following controlled cortical impact TBI in comparison to α II-spectrin breakdown products(SBDP)

A) UCH-L1 levels in rat CSF. For UCH-L1, group comparison indicates that all groups (naïve, sham, CCI (1.0 mm), CCI (1.6 mm) all time points combined) are significantly different from each other (# $p=0.01$). UCH-L1 levels were significantly above sham for TBI (1.0 mm) and for TBI (1.6 mm) * $p<0.05$. Also, UCH-L1 levels were significantly above naïve for TBI (1.0 mm) and TBI (1.6 mm) § $p<0.05$. **B) Calpain-generated SBDP145 levels** in the same CSF samples ($n=7$) were also measured. For SBDP145, group comparison indicates that CCI (1.6 mm) (all time points combined), CCI (1.0 mm), sham and naïve groups are significantly different from each other(# $p=0.001$).

Fig. 6. Sandwich ELISA showing UCH-L1 elevation in rat CSF following MCAO in comparison to α II-spectrin breakdown products (SBDP)

A) UCH-L1 levels in rat CSF in naïve, sham, MCAO-30 min, and MCAO-2h. For UCH-L1, group comparison indicates that all groups (all time points combined; naïve, sham, MCAO-30 min and MCAO-2h) are significantly different from each other (# $p=0.01$). In comparisons with the counterparts such as sham and naïve there was significant evaluations of UCH-L1 (* $p<0.05$ and § $p<0.05$, respectively). **B)** Calpain-generated SBDP145 levels in the same CSF samples were also measured and group comparison indicates that MCAO-2h group (all time points combined) is significantly different from MCAO-30 min and sham groups (# $p=0.001$). **C)** Caspase-generated SBDP120 levels in the same CSF samples were similarly measured and group comparison indicates that both MCAO group-2h and MCAO-30 min groups (all time points combined) are significantly different from the sham group (# $p=0.001$).

Fig. 7. Sandwich ELISA shows UCH-L1 elevation in rat serum following controlled cortical impact TBI and MCAO

A) Control cortical Impact model (CCI). Group comparison indicates that CCI (1.6 mm) and sham groups (all time points combined) are significantly different (# $p=0.001$). By comparison of individual time points the UCH-L1 levels for select time points were above their sham counterparts: TBI (1.6 mm): * $p<0.05$). And in the comparison of UCH-L1 levels to naïve there were significance for sham (§ $p<0.05$) highlighting the sensitivity of the marker to injury perturbation, and for TBI (1.6 mm: § $p<0.05$). **B)** MCAO model. Group comparison indicates that all groups (all time points combined) are significantly different (# $p=0.001$). By comparison of individual time points UCH-L1 levels for select time points were above their sham counterparts for MCAO-30 min (* $p<0.05$) and for MCAO-2h (* $p<0.05$). For UCH-L1 levels for serum for both MCAO-30 min and -2h showed significance above naïve (§ $p<0.05$).