NEUROSYSTEMS

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 the neuronal cell body and has been identified as a possible biomarker on the basis of a recent proteomic study. In this study, we examined whether UCH-L1 was significantly elevated in cerebrospinal fluid (CSF) following controlled cortical impact (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 (2 h) MCAO. A sandwich enzyme-linked immunosorbent assay constructed to measure UCH-L1 sensitively and quantitatively showed that CSF UCH-L1 levels were significantly elevated as early as 2 h and up to 48 h after CCI. Similarly, UCH-L1 levels were also significantly elevated in CSF from 6 to 72 h after 30 min of MCAO and from 6 to 120 h after 2 h of MCAO. These data are comparable to the profile of the calpain-produced αII-spectrin breakdown product of 145 kDa 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).

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 USA, at least 1.4–2 million people seek medical help for a TBI (Langlois *et al.*, 2004). TBI also represents a major central nervous system (CNS) disorder without any clinically proven therapy (Choi & 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) (Yamazaki *et al.*, 1995; Ross *et al.*, 1996;

Missler *et al.*, 1997), glial protein S-100β (Missler *et al.*, 1997; Raabe *et al.*, 1999; Romner *et al.*, 2000), glial fibrillary acidic protein (Pelinka *et al.*, 2004a,b; Vos *et al.*, 2004; Lumpkins *et al.*, 2008), and myelin basic protein (Yamazaki *et al.*, 1995; Wang *et al.*, 2005). Some studies have illustrated the diagnostic potential of these brain injury biomarkers, but other studies have provided conflicting results (Johnsson *et al.*, 2000; Ingebrigtsen & Romner, 2003; Pelinka *et al.*, 2003, 2004c, 2005; Berger *et al.*, 2005, 2007). NSE, for example, initially held promise as a brain injury biomarker because it was originally believed to be strictly neuronal. However, additional research showed that NSE was also present in red blood cells and platelets, decreasing its diagnostic utility as a marker, owing to possible cross-contamination that could occur in blood samples (Johnsson *et al.*, 2000). After multiple trauma, increases 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 S-100β have been

valuable in the prediction of TBI outcome (Berger *et al.*, 2006, 2007).

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

Using differential neuroproteomic methods, a systematic assessment was performed 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 level in rat cortical tissue 48 h following experimental TBI [controlled cortical impact (CCI) at a depression depth of 1.6 mm, equivalent to severe TBI in humans] (Kobeissy *et al.*, 2006). It was of interest that a previously described biomarker, SBDP, was identified in that study. As a follow-up, we then further applied a systems biology based approach to select 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; and (iii) relatively small molecular mass (less than 30 kDa). Brain specificity was analysed on the basis of mRNA expression profiles 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 as source; http://www.ncbi.nlm.nih.gov/pubmed/), and calculated molecular masses were obtained via PubMed. We identified a handful of biomarker candidates that fulfill all three criteria but, because of the significant resources needed to develop a highly sensitive sandwich enzyme-linked immunosorbent assay (swELISA), in the current study we focused on only one such candidate biomarker: ubiquitin C-terminal hydrolase-L1 (UCH-L1). UCH-L1, also known as neuronal-specific protein gene product 9.5, is a highly brain-specific and highly abundant protein that resides in neurons (Jackson & Thompson, 1981). UCH-L1 is present in almost all neurons, and represents 1–5% of total soluble brain protein (Jackson & Thompson, 1981). It has also a small molecular mass (24 kDa) and a compact and almost globular shape. There are two related enzymes in this class (UCH-L1 and ubiquitin C-terminal hydrolase-L3), but only UCH-L1 is highly abundant in the CNS (Wilkinson *et al.*, 1992; Larsen *et al.*, 1996, 1998; Wilkinson, 1997). 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, during both normal and neuropathological conditions. Because of 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 was from Sigma (St Louis, MO, USA) (catalog no. C-3041), blocking buffer was from Pierce (Rockford, IL, USA) (Startingblock T20-TBS; catalog no. 37543), and Tris-buffered saline (TBS) with 0.05% Tween-20 (TBST) was from Sigma (catalog no. T-9039). Phosphate-buffered saline (PBS) was from Sigma (catalog no. P-3813), Tween-20 was from Sigma (catalog no. P5927), Ultra TMB ELISA was from Pierce (catalog no. 34028), and Nunc maxisorp ELISA plates were from Fisher Scientific (Pittsburgh, PA, USA). Monoclonal and polyclonal UCH-L1 antibodies were made in-house.

In vivo *model of TBI*

A CCI device was used to model TBI in 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 comprising 1 : 1 O_2/N_2O (4 min) and maintained in 2.5% isoflurane as anesthesia in the same carrier gas. The 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 in 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-injury and post-injury management was performed to minimize pain and discomfort and to ensure 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 middle cerebral artery occlusion (MCAO) injury model

As the prototypic brain injury biomarkers (SBDPs) are elevated in CSF following MCAO (Pike *et al.*, 2003) with a pattern similar to that 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 of the rat was exposed at the external carotid artery (ECA) and internal carotid artery (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 min or 2 h. Afterwards, the rat was briefly reanesthetized, and the suture filament was retracted to allow reperfusion. For sham MCAO surgery, 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 killed. During all surgical procedures, animals were maintained at 37 ± 1°C. It is important to note that, at the conclusion of each experiment, if the rat brains showed pathological evidence of subarachnoid hemorrhage upon necropsy, they were excluded from the study. Appropriate pre-injury and post-injury management guidelines were followed, 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 and 24 h, and 2, 3 and 5 days) after injury, animals were anesthetized and immediately killed by decapitation. Brains were quickly removed and rinsed with ice-cold PBS. The ipsilateral hemisphere (cerebrocortex around the impact area and hippocampus) was rapidly dissected, rinsed in ice-cold PBS, snap-frozen in liquid nitrogen, and stored at −80°C until use. For western blot analysis, the brain samples were pulverized over dry ice to a fine powder, which 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 dithiothreitol, and protease inhibitor cocktail (Roche Biochemicals, Basel, Switzerland). The brain lysates were then centrifuged at 15 000 *g* for 5 min at 4°C to clear and remove insoluble debris, snap-frozen, and stored at −80°C until use.

CCI and MCAO rat CSF sample collection

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 0.1–0.15 mL of CSF was collected from each rat. After CSF collection, animals were removed from the stereotactic frame and immediately killed by decapitation. CSF samples were centrifuged at 4000 *g* for 4 min at 4°C to clear any contaminating red blood cells. 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 with sodium pentobarbital and then killed by bilateral thoracotomy. Blood (3–4 mL) was collected directly from the heart, using a syringe equipped with a 21-gauge needle, placed in a polypropylene tube, and allowed to stand for 45 min to 1 h at room temperature to form clots. Tubes were centrifuged for 20 min at 3000 *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.

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting analysis for UCH-L1

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

UCH-L1 and SBDP swELISA

For UCH-L1 swELISA, 96-well plates were coated with 100 μL per well of capture antibody (500 ng per well purified mouse monoclonal anti-UCH-L1, made in-house) in 0.1 M sodium bicarbonate (pH 9.2). Plates were then incubated overnight at 4°C and emptied, and 300 μL per well of blocking buffer (Startingblock 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 the standard curve (0.05–50 ng per well) or samples (3–10 μL of CSF) in sample diluent (total volume, 100 μL per well). The plate was incubated for 2 h at room temperature, and then washed with an automatic plate-washer [5 × 300 μL per well with wash buffer (TBST)]. Detection antibody [horseradish peroxidase (HRP)-conjugated rabbit polyclonal anti-UCH-L1 (made in-house, 50 μg/mL)] in blocking buffer was then added to wells at 100 μL per well, and incubated for 1.5 h at room temperature; this was followed by washing. Finally, the wells were developed with 100 μL per well of chemiluminescent substrate solution [SuperSignal ELISA Femto Pierce (catalog no. 37075; Pierce)] with incubation times of 1 min. The signal was read by a 96-well chemiluminescence microplate reader (GloRunner DXL Luminometer; Turner BioSystems, Sunnyvale, CA, USA).

For SBDP of 145 kDa (SBDP145) and SBDP of 120 kDa (SBDP120), swELISAs were similar to that for UCH-L1. Briefly, a 96-well plate was coated with 100 μL per well of capture antibody (500 ng per well of purified rabbit polyclonal anti-SBDP145 or anti-SBDP120 fragment-specific antibody) (Nath *et al.*, 2000; McGinn *et al.*, 2009) overnight at 4°C. After the blocking buffer (Startingblock T20-TBS) step, antigen standard [recombinant glutathione-*S*-transferase–αII-spectrin (repeat 9–15) fusion protein cleaved with calpain-2 or with caspase-3 (1 : 50 ratio) for 30 min] was added, and serial dilutions were made to establish a standard curve. Stock solutions of 0.5–5000 ng/mL SBDP145 (0.005–50 ng in 10 μL) were diluted 1 : 10 with sample diluent to a final incubation volume of 100 μL per well. The standard curve range was therefore 0.05–500 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 per well. If amplification was needed, biotinyl-tyramide solution (Elast Amplification Kit; Perkin Elmer, Waltham, MA, USA) was added for 15 min at room temperature. After washing, 100 μL per well of

streptavidin–HRP (1 : 500) in PBS with 0.02% Tween-20 and 1% bovine serum albumin was added for 30 min, and this was followed by washing. Finally, the wells were developed with chemiluminescent substrate as above.

Statistical analysis

Semiquantitative evaluation of protein levels on immunoblots was performed with computer-assisted one-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 with the appropriate control group. Consequently, the magnitude of change from control in one model system was directly comparable to those in any other model system. ELISA data were also obtained and analysed. Data were assessed for distribution and variance. Comparisons for all groups between replicate immunoblots and between ELISA groups were performed using one-way ANOVA. For single comparisons, Student's t-test was applied. P-values equal to or less than 0.05 were considered to be statistically significant. All data were analysed with SIGMAPLOT 11 (SyStat Software, San Jose, CA, USA).

Results

Immunoblot evidence that UCH-L1 is a potential biomarker for acute brain injury in rats

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

Next, investigations were performed to determine whether increases in UCH-L1 level were also detectable in the CSF following ischemic brain injury. CSF UCH-L1 levels were found to be transiently elevated

(at 6 h, $P = 0.035$) after mild ischemia (MCAO of 30 min) followed by reperfusion, whereas they were sustained from 6 h to 3 days after more severe ischemia (MCAO of 2 h) (6 h, $P = 0.050$; 1 day, $P = 0.015$; 2 days, $P = 0.005$; and 3 days, $P = 0.048$)) (Fig. 2).

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

UCH-L1 expression was examined for brain specificity in a rat tissue panel by immunoblot. It was found to be highly expressed in the brain (Fig. 3A), with only minute amounts being found 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 (Fig. 3B), except for lower levels in the cerebellum and pons.

UCH-L1 antibody tools and swELISA configuration and validation

In order to quantitatively measure UCH-L1 levels in biofluids, a sensitive swELISA was developed. Two compatible antibody pairs (one mouse monoclonal anti-UCH-L1 antibody was used as capture antibody, and a rabbit polyclonal anti-UCH-L1 antibody was affinity purified, HRP-labeled, and used as detection antibody) and recombinant UCH-L1, expressed in *Escherichia coli* as the antigen standard, were used. First, we confirmed that these antibodies do indeed detect recombinant UCH-L1 target protein and a single UCH-L1 protein band from total rat brain and human brain lysate respectively (Fig. 4A). Second, the configured and optimized UCH-L1 swELISA with amplification and chemiluminescence detection has a linear dynamic range of at least three orders of magnitude and a detection limit of 0.01 ng/mL in CSF, and it is linear for three orders of magnitude and has a detection limit of 0.1 ng/mL in serum (standard sample volume, 100 μL) (Fig. 4B). However, as we diluted rat serum samples 1 : 4 (20 μL of serum plus 80 μL of TBS), the serum detection limit in actual serum samples was 0.025 ng/mL.

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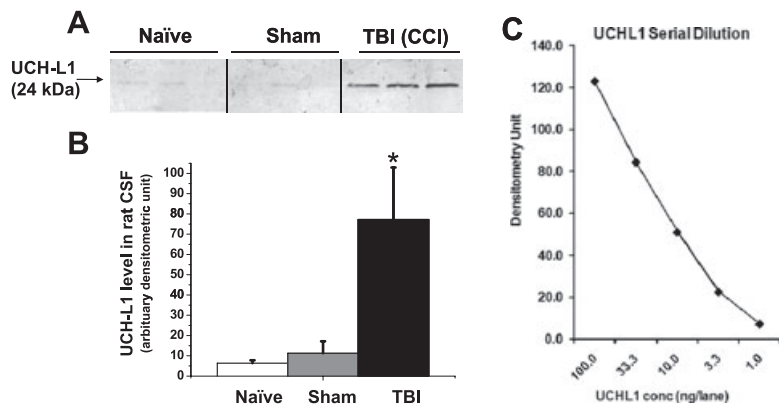

FIG. 1. Immunoblotting evidence of ubiquitin C-terminal hydrolase-L1 (UCH-L1) release into rat cerebrospinal fluid (CSF) 48 h following controlled cortical impact (CCI) traumatic brain injury (TBI). (A) Representative immunoblot results show the presence of UCH-L1 in rat CSF 48 h after TBI (CCI) as compared with sham (48 h) and naïve controls. (B) Quantification indicates statistically significant difference (*$P < 0.05$; $n = 3$) from sham. (C) Recombinant human UCH-L1 serial dilution and correlation with UCH-L1 band intensity (densitometric units) by immunoblot.

A

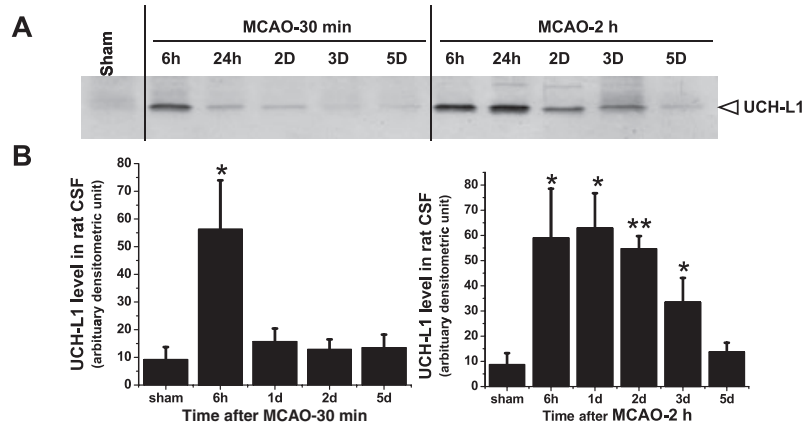| Sham	MCAO-30 min					MCAO-2 h				
	6h	24h	2D	3D	5D	6h	24h	2D	3D	5D

◁ UCH-L1

B

FIG. 2. Immunoblotting evidence of ubiquitin C-terminal hydrolase-L1 (UCH-L1) release into rat cerebrospinal fluid (CSF) following middle cerebral artery occlusion (MCAO). (A) Representative immunoblot results show significantly elevated levels of UCH-L1 in rat CSF 6 h after 30-min MCAO as compared with sham, and also at 6 h, 1 day, 2 days and 3 days after 2-h MCAO. (B) Quantification indicates statistically significant difference from sham ($n = 4$ per time point) for 30-min MCAO and 2-h MCAO (*$P < 0.05$, **$P < 0.01$).
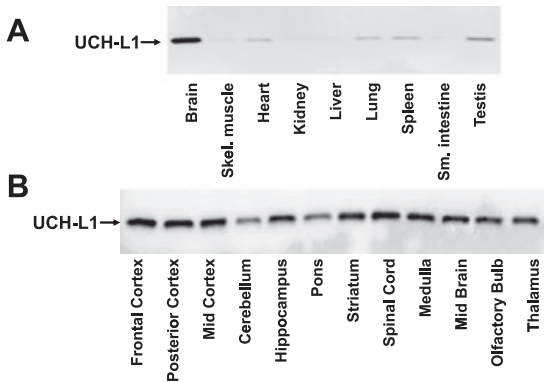

FIG. 3. Ubiquitin C-terminal hydrolase-L1 (UCH-L1) tissue specificity and brain region distribution. Immunoblot analyses of UCH-L1 levels in various rat tissues ($n = 3$ pooled) (A) and various rat brain regions ($n = 3$ pooled) (B); 15 μg of protein loaded per lane. conc, concentration. Skel., Skeletal; Sm., Small.

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

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

The CSF UCH-L1 elevation profile following CCI was also compared with that of SBDP145 produced by calpain during the acute neuronal necrosis stage (Fig. 5B). Overall, SBDP145 levels of the 1.6-mm and 1.0-mm CCI groups were significantly different from those of the sham and naïve groups ($P = 0.001$), and appeared to track closely the UCH-L1 elevation. In contrast, no significantly elevated levels of SBDP120 due to caspase-3 cleavage were detected in this model of TBI (data not shown). Tau and, to a lesser extent, S-100β elevations in CSF have previously been demonstrated in this CCI model of TBI (Ringger *et al.*, 2005).

In parallel, CSF time series samples from naïve ($n = 4$) and sham ($n = 5$), 30-min MCAO ($n = 8$) and 2-h MCAO ($n = 13$) rats at 6, 24, 48, 72 and 120 h post-injury were also assayed with UCH-L1 swELISA (Fig. 6A). For all groups (with all time points combined), there was a significant difference between each group pair ($P = 0.01$). It was also noted that the 2-h MCAO group UCH-L1 levels were two-fold to five-fold higher than those of the 30-min MCAO group. Individual comparisons show that the UCH-L1 levels for the 24-h time point for the 30-min MCAO group were significantly higher than those for the respective time point for the sham group ($P = 0.023$). Similarly, for the 30-min MCAO time points 24 and 120 h and the 2-h MCAO time point 6 h, the protein levels were significantly elevated above those of the naïve group (30-min MCAO, 24 h, $P = 0.010$, and 120 h, $P = 0.011$; 2-h MCAO, 6 h, $P = 0.050$). UCH-L1 protein levels for the sham group were almost indistinguishable from those for the naïve control group.

The profile of CSF UCH-L1 elevation following 2-h MCAO was comparable to those of calpain-generated SBDP145 (Fig. 6B) and caspase-3-generated SBDP120 (Fig. 6C). In these cases, both SBDP145 (2-h MCAO group) and SBDP120 (30-min MCAO and 2-h MCAO groups) were detectable and were found to be significantly elevated above the levels in their respective sham groups ($P = 0.001$). Note that CSF SBDP145 levels overall were higher than their SBDP120 counterparts (Fig. 6B vs. Fig. 6C), but were comparable with their UCH-L1 counterparts.

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

As UCH-L1 was robustly detected in the CSF after both experimental TBI (CCI) and ischemic stroke (MCAO), investigations were performed to determine whether UCH-L1 was able to cross the compromised
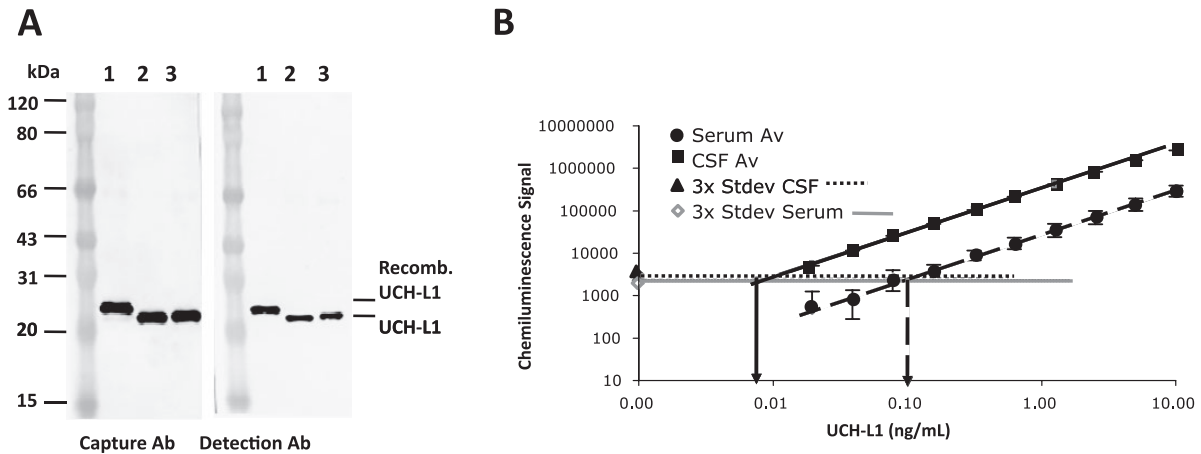
FIG. 4. Ubiquitin C-terminal hydrolase-L1 (UCH-L1) antibody (Ab) and sandwich enzyme-linked immunosorbent assay (ELISA) characterization. The sandwich ELISA set-up is described in Materials and methods. The capture anti-UCH-L1 antibody was mouse monoclonal antibody; the detection antibody was an affinity-purified horseradish peroxidase-labeled rabbit anti-UCH-L1 antibody; the antigen was recombinant human UCH-L1 (Boston Biochem). (A) Both the capture and detection antibodies detect recombinant UCH-L1 (lane 1) and single UCH-L1 protein bands 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 cerebrospinal fluid (CSF) (squares) and in serum (filled circles) [average + standard deviation (SD) shown]. Three times the SD of background (no antigen) in CSF (triangles) and in serum (diamonds) are also shown on the y-axis and plotted as dotted horizontal lines. The lowest detection limits are calculated on the basis of intercepts. Av, average; Stdev, SD.

blood–brain barrier (BBB) 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 (2–72 h post-injury) were analysed by UCH-L1 swELISA (Fig. 7A). Overall, for all groups (with all time points combined), there was a significant difference between each group pair ($P = 0.01$). For individual time points, UCH-L1 levels at 12 and 60 h were significantly different from those of sham at the same time points ($P = 0.006$ and $P = 0.02$, respectively). The data show that UCH-L1 is quite significantly elevated as early as 2 h after injury. Most interestingly, UCH-L1 levels in the sham group were statistically higher than those in naïve controls at various time points (2 h, $P = 0.050$; 6 h, $P = 0.001$; 12 h, $P = 0.001$; 36 h, $P = 0.032$), highlighting the sensitivity of the marker to injury.

Time series blood (serum) samples from naïve ($n = 5$), sham ($n = 5$), 30-min MCAO ($n = 9$) and 2-h MCAO ($n = 13$) rats were also assayed by UCH-L1 swELISA at 6, 24, 48, 72 and 120 h post-injury (Fig. 7B). The UCH-L1 levels for all groups were significantly different from each other (all time points combined: $P = 0.001$). In addition, for time points 6 h ($P = 0.001$) and 120 h ($P = 0.05$), the 30-min MCAO UCH-L1 levels were significantly higher than those of the sham group. Also, for time point 6 h, the 2-h MCAO UCH-L1 levels were significantly higher than those of the sham group ($P = 0.038$). Finally, for each of the time points 6 h through 48 h for both the 30-min MCAO and the 2-h MCAO groups, the UCH-L1 levels were significantly higher than those of the naïve group (30-min MCAO, 6 h, $P = 0.001$, 24 h, $P = 0.006$, and 48 h, $P = 0.030$; 2-h MCAO, 6 h, $P = 0.038$, 24 h, $P = 0.050$, and 48 h, $P = 0.050$).

Discussion

This study 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). As UCH-L1 is a 24-kDa protein with no known active transport mechanism, it is likely that breakdown of the BBB 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: TBI (modeled with CCI in rats) and ischemic stroke (modeled with MCAO in rats). UCH-L1 was confirmed as being detectable in rat CSF, and its levels were significantly elevated after TBI and MCAO, according to western blot (Figs 1 and 2) and swELISA analyses (Figs 5 and 6). Furthermore, UCH-L1 was found to be present at elevated levels in blood (serum) within hours after both TBI and MCAO, as determined by swELISA (Fig. 7A and B).

There is a general correlation between CSF levels determined by western blot and by swELISA (compare Figs 5 and 6 with Figs 2 and 3). However, owing to the semiquantitative and less sensitive nature of western blot, it does not detect small increases of UCH-L1 in CSF as well as ELISA. 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 (Fig. 4B). We also performed 'same-sample' UCH-L1 detection by western blot and by ELISA (about 10 samples), and the range order correlation was very strong (data not shown) – we were only able to perform this comparison to a very limited extent, as only a very small amount of rat CSF (40–50 μL) can be obtained after brain injury in the rats.

It is noted that in all naïve animals, both CSF and serum compartments have zero levels of UCH-L1 (Figs 5 and 6). We did note that there were slightly elevated UCH-L1 levels in sham-operated animals. We attribute these increases to the fact that the sham craniotomy (for CCI) and sham surgery (for MCAO) can themselves cause mild and localized brain injury. For example, for the CCI model, we have previously given T2-weighed magnetic resonance imaging evidence of local lesion following sham craniotomy operation [Fig. 2 in Ringger et al. (2005)]. Thus, we contend that our UCH-L1 swELISA was sensitive enough to detect the release of even very low levels of UCH-L1 due to mild brain injury as a result of sham operations.

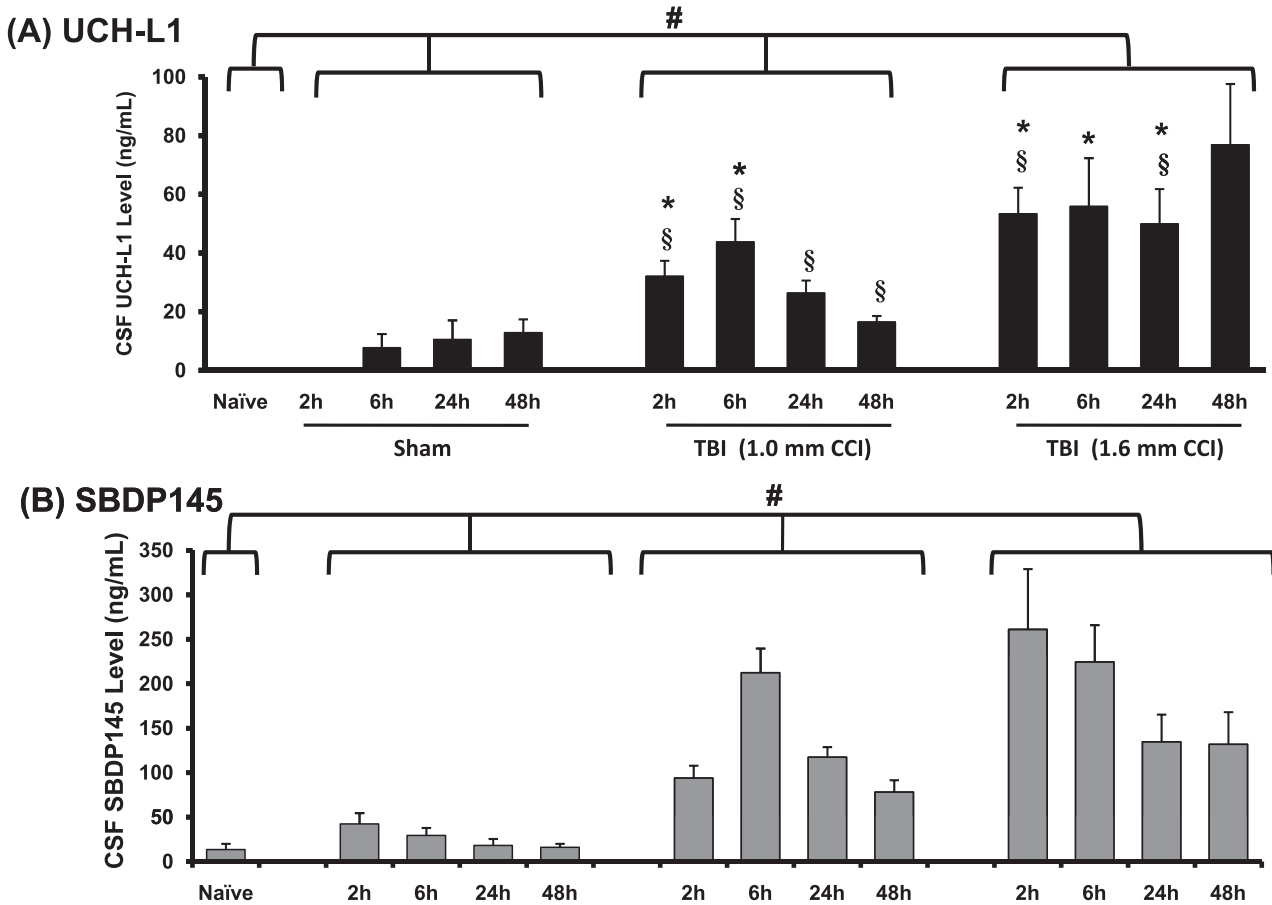## (A) UCH-L1

(B) SBDP145

FIG. 5. Sandwich enzyme-linked immunosorbent assay showing ubiquitin C-terminal hydrolase-L1 (UCH-L1) elevation in rat cerebrospinal fluid (CSF) following controlled cortical impact (CCI) traumatic brain injury (TBI) in comparison with αII-spectrin breakdown products (SBDPs). (A) UCH-L1 levels in rat CSF. For UCH-L1, group comparison indicates that all groups [naïve, sham, 1.0-mm CCI, and 1.6-mm CCI, all time points combined] are significantly different from each other ($^{\#}P = 0.01$). UCH-L1 levels were significantly above those in the sham group for 1.0-mm TBI and for 1.6-mm TBI ($^{*}P < 0.05$). Also, UCH-L1 levels were significantly above those in the naïve group for 1.0-mm TBI and 1.6-mm TBI ($^{\S}P < 0.05$). (B) Calpain-generated SBDP145 levels in the same CSF samples ($n = 7$) were also measured. For SBDP145, group comparison indicates that the 1.6-mm CCI (all time points combined), 1.0-mm CCI, sham and naïve groups are significantly different from each other (#$P = 0.001$).

UCH-L1 is attractive as a candidate biomarker for several reasons. First, UCH-L1 is expressed at a high level in neurons. It was, in fact, previously referred to as a neuronal-specific protein, neuronal-specific protein gene product 9.5 (Jackson & Thompson, 1981). Its tissue distribution is confined almost exclusively to the brain, with testis being the only other tissue with detectable levels (Fig. 3), and this is probably due to the involvement of UCH-L1 in spermatogenesis (Kwon *et al.*, 2005). The ovary was not examined for its presence. Interestingly, we also performed human tissue panel analysis, in which significant amounts were detected only in the brain, and not in the testis (data not shown).

Second, UCH-L1 is a small protein, with a molecular mass 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 of the injured cell membrane and compromised BBB after brain injury and help it to remain stable in circulating CSF and/or blood for detection.

Third, UCH-L1 has been implicated in neuronal cell pathologies, and appears to be a determinant of the means by which the cells degenerate. Gain-of-function mutations of UCH-L1 are confirmed factors in familial Parkinson's disease (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). Misfolded proteins, if not appropriately catabolized, often result in protein aggregations that are neurotoxic (e.g. α-synuclein, amyloid Aβ-peptides, tau, and huntingtin). 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 a surrogate but also 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's diseases, should be explored. We are currently undertaking such investigations.

This study showed that a swELISA 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 (Figs 5 and 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 early and rapid increases before declining. It should be mentioned that UCH-L1 shows some increase in CSF within the sham group for TBI. This is probably due to the mild injury caused by the craniotomy procedure triggering some release of UCH-L1. Taken
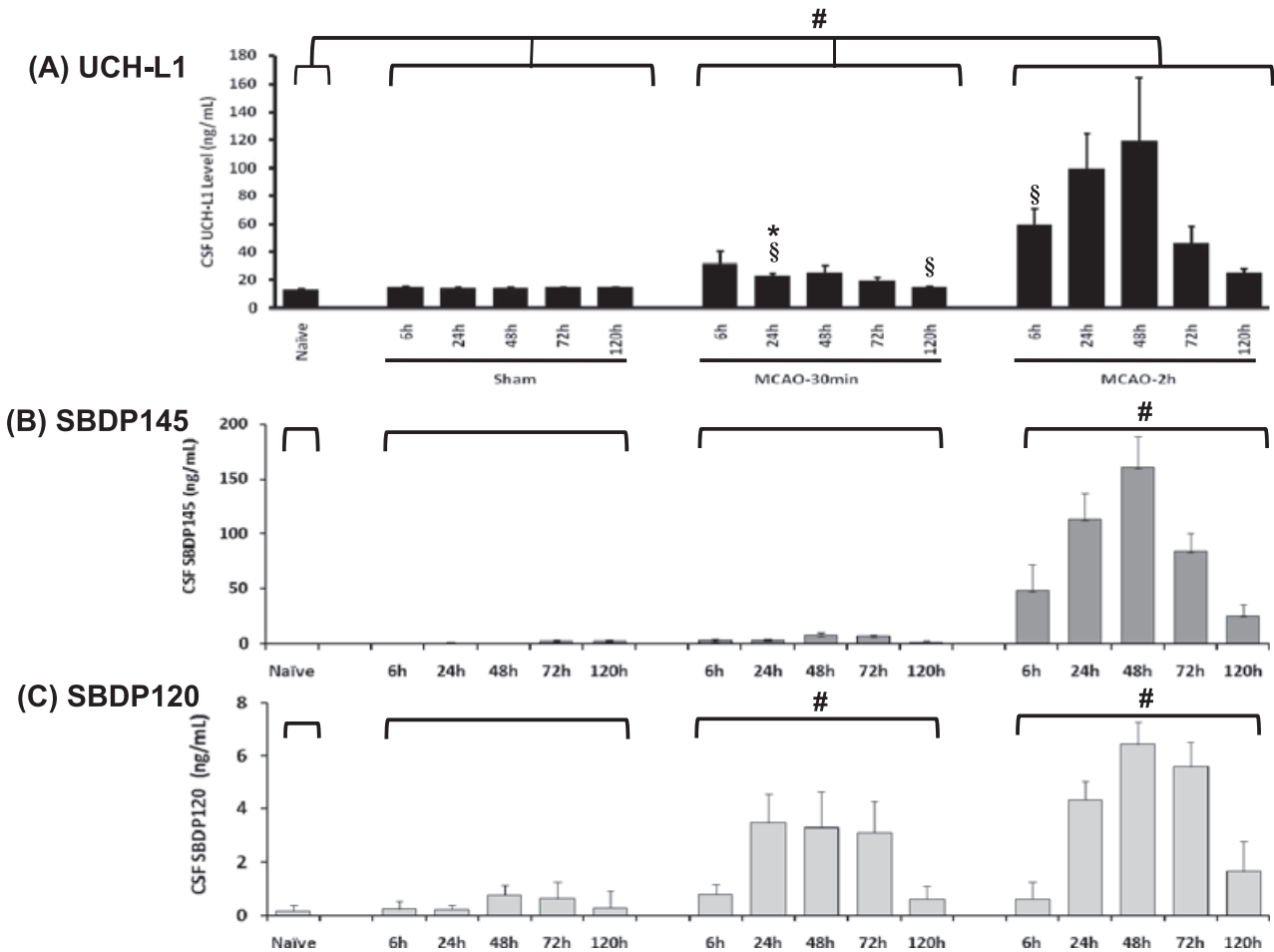
FIG. 6. Sandwich enzyme-linked immunosorbent assay showing ubiquitin C-terminal hydrolase-L1 (UCH-L1) elevation in rat cerebrospinal fluid (CSF) following middle cerebral artery occlusion (MCAO) in comparison with αII-spectrin breakdown products (SBDPs). (A) UCH-L1 levels in rat CSF in naïve, sham, 30-min MCAO and 2-h MCAO groups. For UCH-L1, group comparison indicates that all groups (all time points combined; naïve, sham, 30-min MCAO, and 2-h MCAO) are significantly different from each other ([#]$P = 0.01$). In comparison with counterparts such as sham and naïve, there are significant elevations 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 the 2-h MCAO group (all time points combined) is significantly different from the 30-min MCAO and sham groups ([#]$P = 0.001$). (C) Caspase-generated SBDP120 levels in the same CSF samples were also measured, and group comparison indicates that both the 2-h MCAO and 30-min MCAO groups (all time points combined) are significantly different from the sham group ([#]$P = 0.001$).

together, these findings indicate that UCH-L1 levels are elevated early, and that the levels appear to be injury severity-dependent.

Similar trends are also observed in serum for the CCI and the MCAO groups (Fig. 7). The ability to detect UCH-L1 as early as 2 h after TBI and 6 h after MCAO in serum suggests that 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 for 72–120 h for mild (30 min) and severe (2 h) MCAO, respectively. Thus, it might be possible to continuously monitor serum UCH-L1 levels to track the progression of brain injury or recovery. The fact that UCH-L1 is detectable in serum shortly after CCI and MCAO demonstrates that it is likely to cross the compromised BBB after injury. Our model of stroke is by MCAO, so we suspect that there might be some delay in biomarker release following reperfusion of the ischemic brain areas. However, a separate but smaller study ($n = 3–4$) identified biomarker (UCH-L1 and SBDP) release into CSF as early as 2 and 3 h after reperfusion (data not shown). Thus, the UCH-L1 release profile was very similar to that following experimental TBI.

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

There is, 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 part, 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 & Losy, 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 they are 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 suggests that it might be a promising new biomarker of CNS injury. To further improve the diagnostic accuracy for brain injury, it is also possible that UCH-L1 could be used in a panel of other existing biomarkers, such as S-100β (Nylén et al., 2008), NF-H
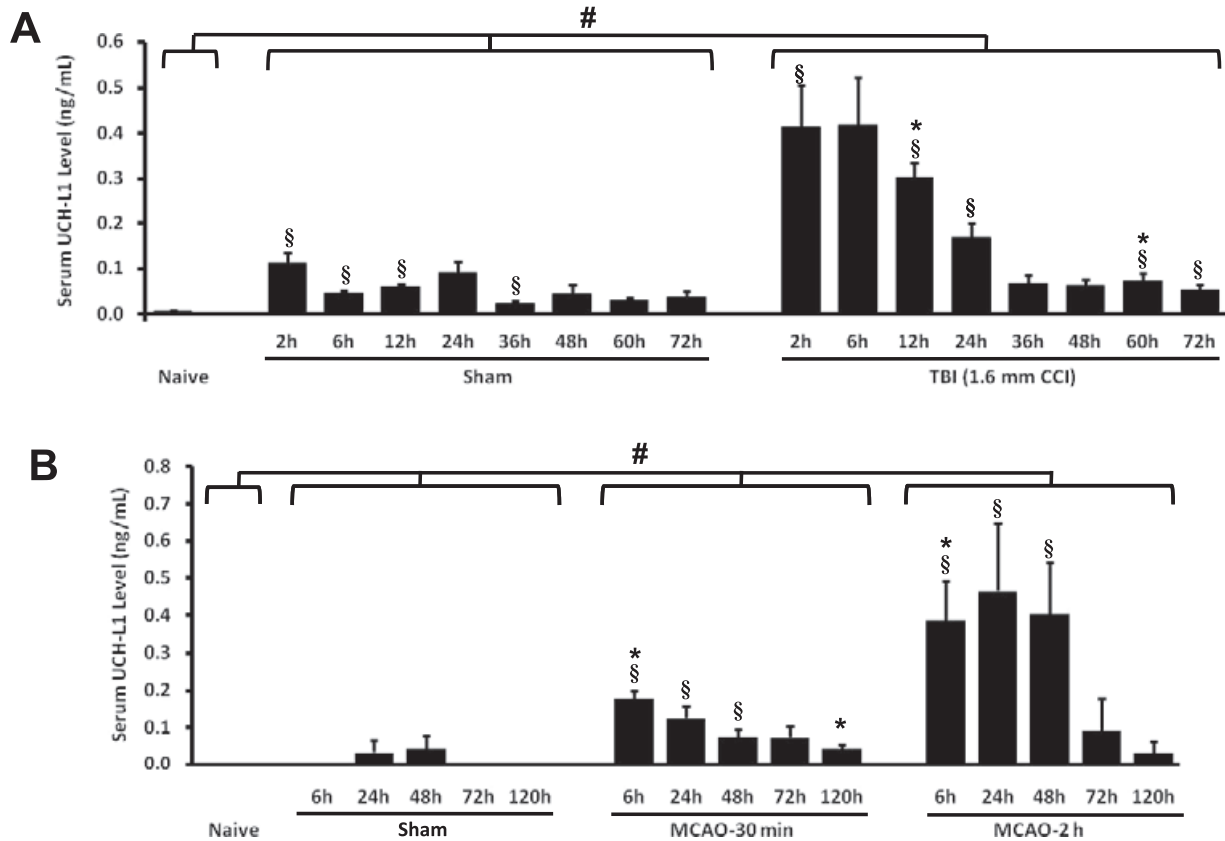
FIG. 7. Sandwich enzyme-linked immunosorbent assay shows ubiquitin C-terminal hydrolase-L1 (UCH-L1) elevation in rat serum following controlled cortical impact (CCI) traumatic brain injury (TBI) and middle cerebral artery occlusion (MCAO). (A) CCI model. Group comparison indicates that the CCI (1.6 mm) and sham groups (all time points combined) are significantly different ($^{\#}P = 0.001$). On comparison of individual time points, the UCH-L1 levels for selected time points are above those of their sham counterparts: 1.6-mm TBI, $^{*}P < 0.05$. The UCH-L1 serum levels, when compared to naïve time points, showed significant elevation for various TBI time points (1.66 mm; $^{\S}P < 0.05$), and for various sham time points ($^{\S}P < 0.05$), the latter highlighting the sensitivity of the marker to injury perturbation. (B) MCAO model. Group comparison indicates that all groups (all time points combined) are significantly different ($^{\#}P = 0.001$). On comparison of individual time points, UCH-L1 levels for selected time points were above those of their sham counterparts for 30-min MCAO ($^{*}P < 0.05$) and for 2-h MCAO ($^{*}P < 0.05$). Serum UCH-L1 levels for both 30-min MCAO and 2-h MCAO were significantly elevated above naïve ($^{\S}P < 0.05$).

(Petzold & Shaw, 2007), SBDP of 150 kDa/SBDP145, and SBDP120 (see Figs 5 and 6). Future clinical studies will be needed to determine the validity of such a panel approach.

It is important to note that the UCH-L1 level was more variable in the 2-h MCAO group than in the 30-min MCAO group. This variability, as indicated by the error bars, was observed in both CSF (Figs 5 and 6) and serum (Fig. 7). The reason for the larger variability in protein accumulation in the CSF is unknown, but may reflect differences between individual rodents in CSF circulation after ischemia or ability to repair the BBB. For example, differences in increased intracranial pressure after ischemia may restrict the passage of CSF through various foramina, precluding detection of proteins secreted into the cisterna magna (this study's source of CSF). Future studies should examine differences between intraventricular and intracisternal levels of UCH-L1.

There are additional considerations suggesting that, in addition to stroke and TBI, UCH-L1 may have biomarker utility for neurodegenerative diseases, including Alzheimer's disease (AD). It was recently found that UCH-L1 levels had increased in the hippocampus of AD patients. Previous studies reported that 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 aggregation, synaptic deterioration and degeneration in the hippocampus. These and other

lines of evidence indicate a role for dysfunction of the ubiquitin–proteosome pathway in the pathogenesis of AD, supporting the importance of UCH-L1 (Sultana *et al.*, 2007). It is important to remember that TBI is a risk factor for both AD 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 TBI and stroke management. Other markers include, but are not limited to, cleaved tau protein, NSE, S-100β, neurofilament proteins, SBDPs, and glial fibrillary acidic protein. The time profile of UCH-L1 in biofluid is, in fact, similar to that of SBDP (Figs 5 and 6) and of S-100β (Nylén *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 a hypothesis will need to be carefully examined in clinical studies.

We would like to point out that the focus of the current study was not on correlation of UCH-L1 levels with histological or functional deficits 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 well-characterized histological and functional deficits, respectively (Marin *et al.*, 2003; Ringger *et al.*, 2005). In fact, further extended studies should be performed to determine whether the

elevated UCH-L1 levels in CSF and serum correlate with histological and functional deficits in these respective models. From the data collected so far in rats, we are not able to make any scientific claims about the 'prognostic' value of the UCH-L1 biomarker. We are simply reporting our finding, obtained using a quantitative swELISA method, that UCH-L1 levels are elevated in two important biofluids (CSF and blood) in both a TBI model and a stroke model, and that these elevations correlate with injury magnitude. This is a very significant advance for a candidate protein biomarker: identification *de novo*, and the development of a sensitive ELISA for the proof-of-principle confirmation of its elevation in biofluids. At the same time, we strongly feel that the utility of UCH-L1 as a clinical diagnostic and prognostic tool needs to be addressed in clinical studies, rather than animal models. In fact, Siman *et al.* (2008) recently showed, by western blot, that UCH-L1 was elevated in patients with mild CNS injury after surgically induced circulation arrest. In addition, in a separate study, our group, using swELISA, showed that UCH-L1 levels were elevated in CSF in severe TBI patients and that UCH-L1 levels were inversely correlated with 6-week mortality and with poor 6-month dichotomized Glasgow outcome score (Papa *et al.*, 2010).

For comparison, Siman *et al.* (2008) reported that UCH-L1 (in addition to phospho-neurofilament-H, SBDP, 14-3-3beta, and 14-3-3zeta) is elevated in the CSF in humans following surgically induced circulation arrest. The authors therefore argue that these proteins could form a panel of biomarkers for the detection of mild brain injury induced by circulation arrest. Their method of UCH-L1 detection was western blot, not ELISA. Our recent study in severe human TBI (Papa *et al.*, 2010) also used swELISA for UCH-L1, and identified a diagnostic potential of UCH-L1 levels in CSF for severe TBI. However, in this rat study, we investigated severe and moderate TBI as well as stroke (mild and severe) by swELISA with the use of both CSF and serum samples. The human studies and our work in rats complement each other, and provide further support for 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, much more work needs to be done to validate the utility of UCH-L1. It is obviously important to further confirm its elevation in human brain injury (CSF and blood) and to determine how it compares with other candidate brain injury biomarkers. In addition, there is a need to address whether UCH-L1 is released from other organs in polytrauma situations. Work is now in progress to determine whether it has clinical utility and the required diagnostic specificity and selectivity for monitoring brain injury with minimal invasiveness in TBI and stroke patients.

Acknowledgements

Abbreviations

AD, Alzheimer's disease; BBB, blood–brain barrier; CCI, controlled cortical impact; CNS, central nervous system; CSF, cerebrospinal fluid; ECA, external carotid artery; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; ICA, internal carotid artery; MCAO, middle cerebral artery occlusion; NSE, neuron-specific enolase; PBS, phosphate-buffered saline; SBDP, αII-spectrin breakdown product; SBDP120, αII-spectrin breakdown product of 120 kDa; SBDP145, αII-spectrin breakdown product of 145 kDa; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; swELISA, sandwich enzyme-linked immunosorbent assay; TBI, traumatic brain injury; TBS, Tris-buffered saline; TBST, Tris-buffered saline with 0.05% Tween-20; UCH-L1, ubiquitin C-terminal hydrolase-L1.

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