P43/pro-EMAPII: A Potential Biomarker for Discriminating Traumatic Versus Ischemic Brain Injury

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Abstract

To gain additional insights into the pathogenic cellular and molecular mechanisms underlying different types of brain injury (e.g., trauma versus ischemia), recently attention has focused on the discovery and study of protein biomarkers. In previous studies, using a high-throughput immunoblotting (HTPI) technique, we reported changes in 29 out of 998 proteins following acute injuries to the rat brain (penetrating traumatic versus focal ischemic). Importantly, we discovered that one protein, endothelial monocyte-activating polypeptide II precursor (p43/pro-EMAPII), was differentially expressed between these two types of brain injury. Among other functions, p43/pro-EMAPII is a known pro-inflammatory cytokine involved in the progression of apoptotic cell death. Our current objective was to verify the changes in p43/pro-EMAPII expression, and to evaluate the potentially important implications that the differential regulation of this protein has on injury development. At multiple time points following either a penetrating ballistic-like brain injury (PBBI), or a transient middle cerebral artery occlusion (MCAo) brain injury, tissue samples (6–72 h), CSF samples (24 h), and blood samples (24 h) were collected from rats for analysis. Changes in protein expression were assessed by Western blot analysis and immunohistochemistry. Our results indicated that p43/pro-EMAPII was significantly increased in brain tissues, CSF, and plasma following PBBI, but decreased after MCAo injury compared to their respective sham control samples. This differential expression of p43/pro-EMAPII may be a useful injury-specific biomarker associated with the underlying pathologies of traumatic versus ischemic brain injury, and provide valuable information for directing injury-specific therapeutics.

Key words: brain injury biomarkers; diagnostic biomarker; middle cerebral artery occlusion; penetrating ballistic-like brain injury; protein expression

Introduction

ACUTE BRAIN INJURY, WHETHER FROM TRAUMATIC (i.e., blunt trauma and penetrating wounds) or ischemic (i.e., stroke) events, causes progressive tissue atrophy and related neurological dysfunction due to neuronal cell death. The etiology of brain trauma and ischemia is apparently different in that the former results primarily from the mechanical impact to the brain, which produces direct damage to neuronal cell bodies, white matter structures, and cerebral vasculature, whereas the latter is caused by severe reductions in cerebral blood flow that lead to immediate deprivation of oxygen and glucose and subsequent metabolic stress and energy failure. However, the ensuing development of pathological conse-

quences of these two types of injury is a complex cascade of cellular and molecular events, which in many cases are shared by both types of injury. In our model of penetrating ballistic-like brain injury (PBBI), the characteristic damage to the brain is the temporary cavity produced by the expansion of the probe, which causes intracerebral hemorrhage. However, significant reduction of regional cerebral blood flow has also been observed (Wei et al., 2008), indicating the participation of cerebral ischemia in the secondary injury mechanism of brain trauma. On the other hand, vascular perturbation also plays an important role in ischemic brain injury, which is manifested by the disruption of the blood–brain barrier and the associated vasogenic edema. Such intertwined pathogenesis of secondary injuries following brain trauma and ischemia

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often makes it difficult to differentiate the two at the cellular level, particularly in the absence of neuroimaging devices that are not readily available in a battlefield scenario. Therefore developing protein-based and injury-specific biomarkers are of particular interest for diagnostic and treatment purposes.

Differential changes in post-injury protein expression may be key components for assessing different types of brain injuries, and have the potential to serve both as specific diagnostic biomarkers, and to inform clinicians about potential therapeutic targets for neuroprotection. Toward this goal, in a previous study using high-throughput immunoblotting technology 24 h after an ischemic (middle cerebral artery occlusion, MCAo) or penetrating ballistic-like brain injury (PBBI) in rats (Yao et al., 2008), we attempted to elucidate differential patterns of protein expression between these two experimental brain injury models. The results of that study identified alterations in the expression levels of 29 (out of 998) proteins across the two injury models. Among the 29 altered proteins, the majority were up- or downregulated in both models, or in only one model. However, one protein of interest, endothelial monocyte-activating polypeptide II precursor (p43/pro-EMAPII), was the only protein that exhibited different changes in abundance after PBBI (increase) and MCAo (decrease).

p43/pro-EMAPII is normally associated with the macromolecular tRNA synthase complex, but under certain conditions it has been associated with other physiological functions. Its role in normal cells, as a component of the synthetase complex, may be related to its RNA binding capacity, while in certain disease and injury states, both p43/pro-EMAPII and its breakdown component EMAP-II play a pro-inflammatory role (Horssen et al., 2006). p43/pro-EMAPII can release mature EMAP-II (23 kDa) following enzymatic cleavage. The C-terminal domain of p43/pro-EMAPII is structurally and functionally equivalent to EMAP-II (Quevillon et al., 1997). Although the functional similarities and differences between p43/pro-EMAPII and mature EMAP-II remain undefined, it has been confirmed that conversion of the p43/pro-EMAPII protein to the mature EMAP-II form occurs coincidentally with apoptosis (Knies et al., 1998). EMAP-II was shown to induce apoptosis in endothelial cells, which leads to the possibility that it is a pro-inflammatory polypeptide with anti-angiogenic activity (Schluesener et al., 1997). The full length of p43 has shown higher cytokine activity than mature EMAP-II (Ko et al., 2001), and p43/pro-EMAPII acts on endothelial and immune cells to regulate angiogenesis and inflammation (Park et al., 2005). It has been shown that expression of EMAP-II can be induced by activated monocytes and microglial cells in the rat spinal cord after hemisection lesion, or in rat brain following traumatic injury (Mueller et al., 2003a, 2003b; Zhang et al., 2007), and that upregulation of EMAP-II by activated monocytes/microglial cells in damaged brain may serve as a sensitive marker of neurotoxic lesions in the rat brain (Brabeck et al., 2002). However, the altered expression of p43/pro-EMAPII in monocytes/microglial cells following brain injury has not been thoroughly explored.

The focus of the current study was to verify the differential changes in p43/pro-EMAPII levels in MCAo and PBBI injury models using Western blot and immunohistochemical techniques, and to further track the time course change in p43/pro-EMAPII protein levels during the acute phase of injury (6–72 h). We also investigated p43/pro-EMAPII expres-

sion levels in plasma and cerebrospinal fluid (CSF) to evaluate its potential as a candidate biomarker. Although the role of p43/pro-EMAPII in brain injury has not been fully explained due to the complexity of the injury mechanism, our findings of a differential expression of p43/pro-EMAPII in PBBI and MCAo suggests that p43/pro-EMAPII could be used as a valuable biomarker to differentiate the underlying pathologies associated with traumatic versus ischemic brain injuries.

Methods

Male Sprague-Dawley rats (250–300 g; Charles River Labs, Raleigh, VA) were used for this study. Anesthesia was induced by 5% isoflurane and maintained with 2% isoflurane delivered in oxygen during the surgery. All procedures were approved by the Walter Reed Army Institute of Research Animal Care and Use Committee. Research was conducted in compliance with the Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals (National Research Council), and other federal statutes involving animals. Animals were housed individually under a normal 12-h light/dark cycle (lights on at 6:00 AM) in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Middle cerebral artery occlusion model

Transient MCAo was induced according to the method described by Tortella and associates (1999). In brief, the right external carotid artery was exposed and its branches were coagulated. A 3-0 uncoated monofilament nylon suture was inserted into the external carotid artery and advanced into the internal carotid artery until it lodged in the proximal region of the anterior cerebral artery, thus occluding the origin of the MCA. The suture was secured with a piece of silk suture and allowed to remain in place for 2 h before it was retracted to allow reperfusion of blood into the MCA. Sham animals underwent the same surgical procedure without the insertion of the nylon filament into the MCA.

Penetrating ballistic-like brain injury model

Frontal PBBI was induced according to the method described by Williams and colleagues (2005). In brief, PBBI was produced by inserting a specially designed probe 1.2 cm into the right brain hemisphere through the frontal cortex and striatum. The probe was constructed from a 20-gauge stainless steel tube with perforations at one end. The perforations were sealed by an airtight section of elastic tubing (PBBI balloon) and arranged in a pattern to allow the PBBI balloon to inflate in an elliptical shape when an air pulse was delivered through the probe. The rapid air pulse was generated by Variable Pressure Waveform Generator model HPD-1700 (Dragonfly Inc., Ridgeley, WV), which induced a rapid expansion and contraction of the PBBI balloon while it was inserted into the brain (Williams et al., 2005). Sham animals underwent craniectomy without insertion of the PBBI probe.

Tissue collection (brain, blood, and CSF)

All collection procedures were performed on animals deeply anesthetized with 70 mg/kg ketamine and 6 mg/kg xylazine. Samples of blood and CSF were collected at 24 h post-injury; brain tissues were collected at 6, 24, 48, and 72 h

post-injury. To collect CSF, a 4-cm midline incision was made in the occipital region from 0.5 cm anterior to the interauricular line. The atlanto-occipital dura mater was exposed by separating the nuchal muscles, and the CSF was collected through a small hole made by a 30-gauge syringe needle; 50–100 μ L of CSF was obtained if the injured brain was not severely swollen. Also, 1 mL of blood was collected from each rat by cardiac puncture. Both CSF and blood samples were collected in 1.5-mL heparin-coated tubes and centrifuged at $2000\times g$ for 10 min at 4°C. The supernatant (plasma or CSF) was collected and stored in a fresh tube. After collecting the biofluids, a 2-mm coronal section of injured brain tissue (~ 100 mg) was removed 5 mm from the frontal pole of the brain, and the injured and uninjured hemispheres were immediately separated. The remaining brain tissues were stained with 2,3,5-triphenyltetrazolium chloride (TTC) (Williams et al., 2005) for photographic assessment of the injury. Tissue samples from the heart, lung, liver, spleen, kidney, bone, skin, and testes, were collected from naïve (normal uninjured) rats. All collected samples were then stored at -80°C for further analysis.

Western blotting

Western blotting was performed on brain samples collected at 6, 24, 48, and 72 h post-injury ($n=6$ per group), and on plasma ($n=6$ per group) and CSF ($n=8$ –10 per group) collected at 24 h post-injury. The same p43/pro-EMAPII antibody that was used to identify changes in protein abundance between injury models using HTPI in our previous study (Yao et al., 2008) was also used in the current study. Protein concentration was determined by using a BCA protein assay kit (Pierce, Rockford, IL). From each sample, 20 μ g were separated by 4–20% SDS-polyacrylamide gradient gel electrophoresis and then transferred to an Immobilon-P membrane (Chen et al., 2003). The blots were then blocked for 1 h in PBST ($1\times$ PBS and 0.1% Tween 20) containing 5% nonfat dry milk, and incubated overnight at 4°C with primary antibody p43/pro-EMAPII (BD Bioscience Pharmingen, San Jose, CA) and anti- β -actin (Sigma, St. Louis, MO) in PBST containing 3% nonfat milk. The blots were washed four times in PBST (10 min each time) and incubated for 1 h with horseradish peroxidase-conjugated second antibody in PBST containing 3% nonfat dry milk. Immunoreactivity of the protein bands was detected by enhanced chemiluminescent autoradiography (ECL kit; Amersham Pharmacia Biotech, Arlington Heights, IL) per the manufacturer's instructions.

Immunohistochemistry

Immunohistochemistry was performed by FD Neurotechnologies (Baltimore, MD) on brain samples collected at 6, 24, 48, and 72 h post-injury ($n=4$ per group) using methods similar to those previously described (Williams et al., 2005). All steps were carried out at room temperature unless otherwise noted. At each of the indicated time points, the animals were intracardially exsanguinated with normal saline and perfused with ice-cold 4% paraformaldehyde. The skulls were then opened, and the brains were removed and rapidly frozen in -80°C isopentane pre-cooled with dry ice. Cryostat sections 30 μ m thick were cut coronally through the cerebral cortex containing the striatum from approximately 3.70 to -8.0 mm relative to the bregma. Every first and second sec-

tions of each series of 12 sections were separately mounted on Superfrost Plus microscopic slides. The first series of sections was stained with hematoxylin and eosin (H&E), and the second series was processed for p43/pro-EMAPII immunohistochemistry. In both sections, the endogenous peroxidase activity was inactivated with hydrogen peroxidase, and then the sections were separately incubated with avidin followed by biotin solutions (Vector Laboratories, Burlingame, CA) to block nonspecific binding of endogenous biotin, biotin-binding protein, and lectins. This was followed by three 10-min washes in 0.1 M PBS. Four 10-min washes in 0.1 M PBS were also done following each of the remaining steps. The sections were incubated overnight at 4°C in 0.1 M PBS containing 1% normal goat serum, 4% BSA, 0.1% Triton X-100 (Sigma), and primary antibody p43/pro-EMAPII (BD Bioscience Pharmingen). The sections were then incubated for 1 h in goat anti-rabbit IgG secondary antibody in 0.1 M PBS containing 1% normal goat serum and 0.1% Triton X-100, followed by a 1-h incubation in 0.1 M PBS solution containing avidin-biotinylated horseradish peroxidase complex (Vectastain Elite ABC Peroxidase kit; Vector Laboratories). This was followed by another incubation for 10 min in 0.05 M Tris buffer (pH 7.2) containing 0.03% 3'-3'-diaminobenzidine (Sigma) and 0.0075% hydrogen peroxide. Finally, the sections were thoroughly rinsed in distilled water, dehydrated in ethanol, cleared in xylene, and cover-slipped with Permount® (Fisher Scientific, Fair Lawn, NJ).

Double immunofluorescent labeling with p43/pro-EMAPII and NeuN (neuronal nuclei-specific antibody) (Mullen et al., 1992) was performed on brain tissue collected 6 h post-injury to determine if p43/pro-EMAPII immunoreactivity occurred within neuronal cells. Brain sections (40 μ m) were incubated overnight at 4°C in 0.1 M PBS containing 1% normal donkey serum, 4% BSA, 0.3% Triton X-100 (Sigma), and mouse monoclonal anti-p43/pro-EMAPII antibody (1:200; BD Transduction Laboratories, San Diego, CA). This was followed by incubation in PBS/Triton X solution containing Alexa Fluor® 594 donkey anti-mouse IgG (Molecular Probes, Eugene, OR) for 1 h, and then in 10% normal mouse serum for another hour. The sections were then incubated in 0.1 M PBS containing 0.3% Triton X-100 and a biotin-conjugated mouse anti-NeuN (1:200; Chemicon, Temecula, CA) overnight at 4°C. Subsequently the sections were incubated with streptavidin conjugated Alexa Fluor 488 for 1 h. All the above steps were carried out at room temperature except as indicated, and each step was followed by washes in PBS. After the final wash in PBS, the sections were mounted on gelatin-coated slides and cover-slipped with Vectashield® (Vector Laboratories). Fluorescent signals were examined using a confocal laser-scanning microscope at a fixed laser power setting with 25 $\times$ and 40 $\times$ oil-immersion objectives. Separate optical images of the p43/pro-EMAPII and NeuN-immunoreactivity were captured from the same sections and were pseudo-colored red for p43/pro-EMAPII and green for NeuN. A digital overlay was generated by superimposing companion images, and areas of co-localization appeared in yellow.

Cell counting and optical density measurement

The total numbers of p43/pro-EMAPII immunoreactive cells were counted in the striatum and primary motor cortical regions from four fields (200 $\times$ 200 μ m per slide) in both

injured and uninjured hemispheres of each region (Yao et al., 2005). Mean cell counts were obtained from two slides per rat and four rats from each injury group. All cell counts were performed by the same investigator and verified by a second investigator blinded to the post-injury time point. All slides were analyzed on an Olympus AX70 microscope and images were captured with a DP controller digital camera (Olympus, Tokyo, Japan) using a 40 $\times$ objective at a fixed gain level for all slides. Image analysis software (Inquiry; Loats Associates, Westminster, MD) was used to quantify the degree of immunoreactivity by measuring the optical density (OD) of p43/pro-EMAPII-positive cells in each captured image. Background OD levels from each slide were subtracted from the OD of each cell and the mean values of the contralateral and ipsilateral hemispheres of each slide were calculated for comparison.

Data analysis

Densitometric quantification of the immunoblot bands from the Western blots was performed by Scion Image (National Institutes of Health, Bethesda, MD). Measurements of Western blot and immunohistochemistry data are presented as mean values $\pm$ standard error of the mean (SEM). Statistical comparisons were performed using analysis of variance

(ANOVA), followed by a post-hoc *t*-test between matching sham and injured samples, or between contralateral and ipsilateral brain regions. Values were considered significant at $p < 0.05$.

Results

Brain injury

All animals survived the PBBI or MCAo injuries to be sacrificed at 6, 24, 48, and 72 h post-injury. Brain lesions at 24 h post-MCAo and post-PBBI were approximately 20–80% of the total hemispheric cross-sectional area of the brain (Fig. 1). MCAo induced a non-hemorrhagic lesion with a large volume of infarcted tissue, whereas the PBBI-induced lesion included a distinct hemorrhagic component.

Western blotting

Quantitative measurement of Western blot protein density indicated that p43/pro-EMAPII was significantly down-regulated at 24 and 48 h following MCAo, but upregulated at all four time points following PBBI (Fig. 2). Similar changes in the expression of p43/pro-EMAPII between injury models was also detected in plasma and pooled CSF samples at 24 h post injury by Western blot (Fig. 3).

FIG. 1. Representative coronal sections of rat brains 24h following PBBI or MCAo injury. Infarction is indicated by the bright white regions of TTC staining or neuropil pallor in H&E slides. Regions with intracranial hemorrhage are denoted by bright red regions (H&E) throughout the core lesion of PBBI-injured rats.

FIG. 2. (A) Western blot (WB) analysis of p43/pro-EMAPII protein levels 6–72 h post-injury. (B) Quantitative measurement of p43/pro-EMAPII WB density normalized to β -actin levels ($n=6$ per group). Values are mean $\pm$ SEM (* $p < 0.05$ compared to corresponding matched sham controls [t -test]) (P, PBBI; PS, PBBI sham; M, MCAo; MS, MCAo sham).

p43/pro-EMAPII abundance levels were also measured in brain, heart, kidney, liver, lung, bone, spleen, skin, and testes of naïve rats by Western blot. The results indicated that the highest abundance of p43/pro-EMAPII was detected in brain, with minimal detection in the testes and a trace amount in the liver. p43/pro-EMAPII was not detected in heart, kidney, lung, bone, spleen, or skin samples (Fig. 4).

Immunohistochemistry

Further examination of post-injury p43/pro-EMAPII expression was accomplished using immunohistochemistry in samples from the same brain regions taken at the same post-injury time points as the samples used for the Western blots. In general, changes in p43/pro-EMAPII immunoreactivity were strictly limited to the PBBI- and MCAo-induced lesions and surrounding peri-lesional regions. From 6–72 h following

FIG. 4. Western blot analysis of p43/pro-EMAPII protein levels in different tissues of naïve (normal uninjured) rats. The highest amounts were found in brain tissue (M.W. = molecular weight markers).

FIG. 3. (A) Western blot (WB) analysis of p43/pro-EMAPII protein levels in plasma ($n=6$ per group) and pooled CSF samples ($n=8-10$ per group) at 24 h post-injury. (B) Quantitative measurement of p43/pro-EMAPII WB density normalized to β -actin levels. Values are mean $\pm$ SEM (* $p < 0.05$ compared to corresponding matched sham controls [t -test]) (P, PBBI; PS, PBBI sham; M, MCAo; MS, MCAo sham).

PBBI, p43/pro-EMAPII immunoreactivity was increased in the peri-lesional region surrounding the PBBI probe track in all brain samples examined. Conversely, a reduction or loss of p43/pro-EMAPII immunoreactivity was observed in peri-lesional and infarcted regions at 6–72 h post-MCAo (Fig. 5).

Cell count and OD levels for p43/pro-EMAPII-positive cells were evaluated in the peri-lesional region of the motor cortex to quantify the progressive increase or decrease in immunoreactivity following MCAo and PBBI. Results indicated that the number of p43/pro-EMAPII-positively-stained cells was significantly reduced over time in both models, with maximal reductions in cell count of 35% following PBBI (Fig. 6A), and 68% following MCAo (Fig. 6B) by 48–72 h post-injury. The OD measurements, however, indicated that the level of p43/pro-EMAPII abundance in p43/pro-EMAPII-positively-stained cells was significantly greater following PBBI at all four post-injury time points, but were significantly reduced at 24 and 48 h following MCAo compared to sham controls (Table 1).

The morphology of p43/pro-EMAPII-positive cells also changed during the course of injury, particularly following PBBI. Constitutive p43/pro-EMAPII staining was generally observed throughout the brain in large multipolar cells in sham animals, typical of neurons and glia, as well as the contralateral hemisphere throughout the course of the injury in both models (Fig. 5). Following PBBI the increase in p43/pro-EMAPII immunoreactivity appeared to occur in these same multipolar type cells, while at 24 h the appearance of dark triangular or round immunoreactive cells was apparent. In the later stages of injury (48–72 h) the increase in p43/pro-EMAPII immunoreactivity was isolated to small round cells or large phagocytic-type morphologies (likely infiltrating leukocytes or microglia). To verify the increase in

FIG. 5. Representative images of p43/pro-EMAPII immunoreactivity from the motor cortex (peri-injury region) 6–72 h post-injury as compared to sham controls ($n = 4$ per group). Sham brains were lightly immunoreactive for p43/pro-EMAPII. Following MCAo, p43/pro-EMAPII immunoreactivity was diminished. In contrast, p43/pro-EMAPII immunoreactivity increased following PBBI.

FIG. 6. Mean counts of p43/pro-EMAPII-immunoreactive cells in contralateral (open bars) and ipsilateral (black bars) hemispheres at 6–72 h post-PBBI (A), post-MCAo (B), and in sham control brains (24 h post-sham surgery). Counts were performed from four fields ($200 \times 200 \mu\text{m}$), two fields in the primary motor cortex and two in the dorsal striatum. Four animals at each time point were counted. Values are given as mean $\pm$ SEM (* $p < 0.05$ between contralateral and ipsilateral hemispheres [t -test]).

possible neuronal expression during the early course of injury, additional sections were collected at 6 h post-injury and double-labeled for p43/pro-EMAPII and NeuN (a neuronal marker). The results indicated that while neuronal morphology appeared generally intact at 6 h post-injury in the ipsilateral motor cortex of either injury model, p43/pro-EMAPII immunoreactivity was increased following PBBI, and was largely confined to the neuronal population, while p43/pro-EMAPII immunoreactivity was reduced following MCAo (Fig. 7).

Discussion

In a previous study, we found that p43/pro-EMAPII was the only protein exhibiting differing changes in abundance

level after PBBI and MCAo (Yao et al., 2008). In the present study, the Western blot time course of p43/pro-EMAPII expression confirmed that p43/pro-EMAPII expression was elevated in comparison to controls at all post-PBBI time points, yet was lower at 24 h and 48 h following MCAo. Immunohistochemical analysis verified that there was a significant accumulation of p43/pro-EMAPII signal in cells within the peri-lesional region surrounding the injury track following PBBI, and an overall reduction of p43/pro-EMAPII within the injured region following MCAo. Although EMAP-II is generally considered a marker for microglial infiltration (Horsen et al., 2006), it was confirmed that the precursor protein p43/pro-EMAPII is initially upregulated in neurons following PBBI, while expression is reduced in peri-lesional neurons following MCAo at 6 h from injury onset. However,

TABLE 1. CHANGES IN OPTICAL DENSITY (OD) VALUES OF p43/PRO-EMAPII-IMMUNOREACTIVE CELLS IN THE IPSILATERAL (INJURED) HEMISPHERE 6–72 h POST-INJURY COMPARED TO THEIR RESPECTIVE CONTRALATERAL VALUES

		<i>Ipsilateral</i>	<i>Contralateral</i>	% Change of OD	<i>p</i> Value
Post-PBBI	Sham	148 ± 15	148 ± 15	0.00	0.91
	6 h	157 ± 18	142 ± 17	9.55	0.01 ^a
	24 h	173 ± 15	136 ± 10	21.39	0.01 ^a
	48 h	187 ± 13	131 ± 15	29.95	0.01 ^a
	72 h	192 ± 13	137 ± 15	28.65	0.01 ^a
Post-MCAo	Sham	141 ± 11	142 ± 9	−0.71	0.50
	6 h	143 ± 9	149 ± 14	−4.20	0.06
	24 h	142 ± 12	151 ± 14	−5.74	0.04*
	48 h	142 ± 17	150 ± 12	−5.33	0.04*
	72 h	141 ± 16	147 ± 13	−4.26	0.08

^a*p* < 0.05 between contralateral and ipsilateral hemispheres (*t*-test). Values are given as mean ± SD. Counts were from four fields (200 × 200 μm) of four animals at each time point.

phagocytic cellular morphologies associated with p43/pro-EMAPII reactivity were observed in the later stages of PBBI injury (24–72 h). Similar to observations in brain tissue, p43/pro-EMAPII also showed an opposing trend in expression level in plasma and pooled CSF samples analyzed by Western blot. We further confirmed that p43/pro-EMAPII expression was most prominent in brain than in 8 other types of tissue samples examined in naïve (normal uninjured) rats. These data confirmed that (1) decreased p43/pro-EMAPII expression after MCAo was primarily due to the down-regulation of p43/pro-EMAPII in neurons, as opposed to neuronal cell loss or related protein degradation, and (2) increased p43/pro-EMAPII expression observed after PBBI was a manifestation of neuronal p43/pro-EMAPII expression, indicating that at least in the initial stages of injury, the up-regulation of p43/pro-EMAPII levels is initiated by peri-lesional neurons. Taken together, these results indicate that p43/pro-EMAPII may be useful as a brain-specific biomarker.

The pathobiology of traumatic and ischemic brain injury is complex, inducing the expression of a variety of factors that affect the formation of new axons and blood vessels in the damaged region that may relieve or aggravate the injury. PBBI causes progressive oncotic and apoptotic cell death with consequential neurological dysfunction (Williams et al., 2005, 2007), while hypoxic-ischemic neuronal injury involves excitotoxicity, calcium overload, nitric oxide, oxidative stress, apoptosis, and necrosis that all contribute to neuronal loss (Won et al., 2002). It has been shown that EMAP-II plays a role in apoptotic cell death following experimental traumatic brain injury and spinal cord injury, and virus-induced inflammation of the nervous system (Schluesener et al., 1997; Mueller et al., 2003a, 2003b, 2003c; Horssen et al., 2006), and is generally considered to be a marker for microglial cells in these lesions. Studies related to the role of p43/pro-EMAPII in brain injury and CNS disorders are few, and the factors that influence its expression level and the molecular mechanisms by which p43/pro-EMAPII functions to regulate endothelial cell behavior under different pathological conditions are still unclear.

In the current study one of the main pathological differences between PBBI and MCAo was hemorrhage. Therefore, the measured increase in p43/pro-EMAPII expression following PBBI could be a direct influence of the hemorrhagic component of the injury. The expression of p43/pro-EMAPII after hemorrhagic injury may be increased in response to factors such as leukocyte or monocyte infiltration known to surround the PBBI site (Williams et al., 2006a, 2006b). These responses may also affect angiogenesis and inflammation in the injured brain. Interestingly, according to Park and associates (2002), p43/pro-EMAPII plays a dose-dependent biphasic role in angiogenesis. Low concentrations of p43/pro-EMAPII are pro-angiogenic, and activate extracellular signal-regulating kinase, resulting in the induction and activation of matrix metalloproteinase 9, while high concentrations p43/pro-EMAPII are anti-angiogenic, activate Jun N-terminal kinase, and mediate apoptosis of endothelial cells. Indeed, p43/pro-EMAPII has been reported to be induced and released by apoptosis and cellular stress (Knies et al., 1998; Murray et al., 2004). These findings are consistent with our results, and reveal a possible mechanism for the differing expression of p43/pro-EMAPII for penetrating trauma and for ischemic brain injury.

p43/pro-EMAPII has been shown to be cleaved *in vitro* by enzymes such as cathepsin L and MMP-9 (Liu and Schwarz, 2006). Cathepsin L and MMP-9 were found to influence the ability of endothelial cells to generate angiogenesis inhibitors (Urbich et al., 2005; Stetler-Stevenson, 1999). Cathepsin L is essential for matrix degradation and invasion of endothelial progenitor cells during the revascularization process following tissue injury (Urbich et al., 2005). MMP-9, a member of the matrix metalloproteinase (MMP) family of extracellular endopeptidases that selectively degrade components of the extracellular matrix, also plays a role in angiogenesis (Stetler-Stevenson, 1999). Although speculative at this stage, the degradation of p43/pro-EMAPII by cathepsins or MMP-9 may reduce levels of p43/pro-EMAPII in injured brain tissue following ischemia, and therefore it is likely that p43/pro-EMAPII expression is downregulated during the revascularization process after ischemic injury. This improves the prospects of using p43/pro-EMAPII as a biomarker to distinguish between hemorrhagic and ischemic strokes, and makes it even more useful as a potential tool for injury-specific drug development. Further research is needed to elucidate the mechanisms by which ischemic and traumatic injuries result in differential expression of p43/pro-EMAPII, to determine the molecular mechanisms of p43/pro-EMAPII itself, and to explore the potential uses for angiogenic growth factor regulators as therapeutic agents for use after brain injury.

Changes in p43/pro-EMAPII expression may also be triggered by factors expressed in response to hypoxic and ischemic changes seen after middle cerebral artery occlusion. Hypoxia is one of the major signals that induce angiogenesis, initiating revascularization to enhance blood supply to the periphery of the ischemic lesion. However, the literature detailing the role of p43/pro-EMAPII during hypoxia and ischemia are limited, and the mechanisms whereby p43/pro-EMAPII is released from injured brain cells remain uncertain. It was found by Matschurat and colleagues (2003) that p43/pro-EMAPII was subsequently cleaved to the mature EMAP-II protein by enzymes activated either during apoptosis or under hypoxic conditions in tumors, but caspase

FIG. 7. Double immunofluorescent-labeling with p43/pro-EMAPII and NeuN from the primary motor cortex region 6 h following MCAo or PBBI. p43/pro-EMAPII-labeled cells (red); NeuN labeled cells (green); NeuN plus p43/pro-EMAPII labeling (yellow).

activation was not involved in the cleavage of p43/pro-EMAPII in hypoxia. Therefore, they believe that apoptosis and hypoxia are independent mechanisms for the formation of mature EMAP-II protein in tumor cells.

EMAP-II has also been reported as a novel cytokine with pro-inflammatory properties that is accumulated by activated monocytes/microglial cells in the injured rat brain (Brabeck et al., 2002; Mueller et al., 2003a; Zhang et al., 2007). Microglia/macrophages are commonly seen during the repair process to remove inhibitory tissue debris and secrete growth-promoting factors for regeneration, but expression of p43/pro-EMAPII in microglia/macrophages following brain injury has not been reported. In the present study we observed an increase in p43/pro-EMAPII that was initiated predominantly in neurons during the early stages post-injury (6h). It was not until the ensuing post-injury days that p43/pro-EMAPII-positive cells appeared in phagocytic cellular morphologies. Thus, following injury p43/pro-EMAPII may take on different roles during the progression of the injury.

To our knowledge, this is the first study that systematically analyzed the expression of a putative protein biomarker in two distinct brain injury models. Our findings demonstrate that p43/pro-EMAPII increased after PBBI and decreased following MCAo in brain tissue, CSF, and plasma. These differing changes in p43/pro-EMAPII expression and its predominance in rat brain support its value as a potential biomarker for differentiating between traumatic and ischemic brain injuries. These findings also provide insight into the complex mechanics of brain injuries, and may lead to novel therapies for neural repair.

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Author Disclosure Statement

This material has been reviewed by the Walter Reed Army Institute of Research, and it had no objection to its publication. The opinions or assertions contained herein are the private views of the authors, and are not to be construed as official or to reflect the views of the Department of the Army or the Department of Defense.

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