Relative Deficiency of Plasma A Disintegrin and Metalloprotease with Thrombospondin Type 1 Repeats 13 Activity and Elevation of Human Neutrophil Peptides in Patients with Traumatic Brain Injury

Monisha A. Kumar,^{1–3,*} Wenjing Cao,^{4,*} Huy P. Pham,⁵ Dheeraj Raju,⁶ Kelsey Nawalinski,²
Eileen Maloney-Wilensky,² James Schuster,² and X. Long Zheng⁴

Abstract

Traumatic microvascular injury (tMVI) is a universal endophenotype of traumatic brain injury (TBI) that is responsible for significant neurological morbidity and mortality. The mechanism underlying tMVI is not fully understood. The present study aims to determine plasma levels of von Willebrand factor (VWF), a disintegrin and metalloprotease with thrombospondin type 1 repeats (ADAMTS) 13 activity, and human neutrophil peptides (HNP) 1–3 and to correlate these biomarkers with functional outcomes after moderate-severe TBI. Thirty-one consecutive TBI patients (Glasgow Coma Scale [GCS] range, 3–12) were enrolled into the study between February 2010 and November 2014. Blood samples were collected on 0, 1, 2, 3, and 5 days after admission and analyzed for plasma levels of VWF antigen (VWF_{Ag}), collagen-binding activity (VWF_{Ac}), ADAMTS13 activity, and HNP1–3 proteins. Mean values of plasma VWF_{Ag}, VWF_{Ac}, and HNP1–3 were significantly increased in TBI patients compared to those in healthy controls ($n=30$). Conversely, mean plasma values of ADAMTS13 activity in TBI patients were significantly decreased during the first 2 days after admission. This resulted in a dramatic reduction in the ratio of ADAMTS13 activity to VWF_{Ag} or ADAMTS13 to VWF_{Ac} in all 5 post-TBI days. Cluster analysis demonstrated that high median plasma levels of VWF_{Ag} and HNP1–3 were observed in the cluster with a high mortality rate. These results demonstrate that a relative deficiency of plasma ADAMTS13 activity, resulting from activation of neutrophils and endothelium, may contribute to the formation of microvascular thrombosis and mortality after moderate-severe TBI.

Keywords: ADAMTS13; coagulopathy; inflammation; traumatic brain injury; von Willebrand factor

Introduction

TRAUMATIC BRAIN INJURY (TBI) is a devastating disease that is newly diagnosed in 2.8 million people each year.^{1,2} Whereas many recover, 5.3 million Americans live with chronic TBI-related disabilities.³ Despite the widespread impact of the disease, treatment options remain woefully inadequate. Consensus statements recommend use of biochemical and imaging biomarkers to target injury-specific therapies in TBI.⁴

One such injury-specific phenotype, or endophenotype, is traumatic microvascular injury (tMVI), which is known to worsen outcome after TBI.⁵ tMVI is often attributed to disseminated intravascular

coagulation (DIC)⁶; however, much about this pathophysiology remains unknown. Recent evidence suggests that cellular elements, specifically platelets,⁷ the endothelium,⁸ inflammatory mediators,⁹ the fibrinolytic¹⁰ system, as well as damage-associated molecular pattern molecules,¹¹ are crucial to tMVI pathology. However, the mechanisms underlying tMVI remain unknown. Further investigation of this process may guide early treatment decisions to mitigate the thromboembolic complications.

This pilot study focuses on serial determinations of plasma levels of von Willebrand factor (VWF), derived from vascular endothelium,¹² a disintegrin and metalloprotease with thrombospondin type 1 repeats (ADAMTS13), a metalloprotease that cleaves VWF,¹³ and

¹Departments of Neurology, ²Neurosurgery, and ³Anesthesiology and Critical Care, the University of Pennsylvania Perelman School of Medicine, Philadelphia, Pennsylvania.

⁴Division of Laboratory Medicine, Department of Pathology, The University of Alabama at Birmingham, Birmingham, Alabama.

⁵Department of Pathology, Keck School of Medicine of USC, Los Angeles, California.

⁶Department of Acute, Chronic, and Continuing Care, The University of Alabama at Birmingham, Birmingham, Alabama.

*These authors contributed equally to this work.

human neutrophil peptides (HNP) 1–3,¹⁴ released from activated and degranulated polymorphic neutrophils in patients post-TBI. We hypothesize that dysregulation of VWF activity may contribute to the mechanism of tMVI and/or mortality in patients after moderate-severe TBI. Specifically, we aimed to determine whether VWF activity is increased days after initial TBI, and whether the increased activity may be related to immune-mediated suppression of ADAMTS13 activity, as observed in other forms of brain injury.¹⁵

Methods

Patients

Patients were prospectively enrolled in a cohort study at an academic level 1 trauma center, during the period between February 2010 and November 2014, after approval from the local institutional review board. Informed consent was obtained from each patient's legal representative. Eligibility for enrollment included patients 18 years of age or older with TBI, Glasgow Coma Scale (GCS) <12 (or higher GCS on admission, but >2-point fall in the first 8 h with an abnormal head computed tomography scan), and admission to the Neurological Intensive Care Unit. Patients on anticoagulation or -thrombotic therapy at time of TBI were excluded from the study. Patients presenting with severe coagulopathy were excluded from the study (all patients had an international normalized ratio [INR] <1.4 upon enrolment). No patient received plasma, platelets, or other hemostatic agents preceding enrollment. Controls were healthy subjects not taking antithrombotic medications; all controls consented for the study.

Patient demographic information, including age, sex, and race, was obtained, as was admission GCS. Comorbidity data, including diabetes mellitus, hypertension, coronary artery disease, previous venous thromboembolism, and history of liver and renal failure, were collected. Tobacco use, ethanol use, and positive blood alcohol test on admission were also recorded. Mechanism of injury was noted, as were patterns of bleeding: epidural, subdural, subarachnoid, and contusion. Other admission other variables obtained include vital signs (systolic blood pressure, diastolic blood pressure, and triage temperature), laboratory results (PT [prothrombin time], INR, aPTT [activated partial thromboplastin time], pH, and base deficit), and volume of resuscitation fluids.

Plasma levels of von Willebrand factor antigen

Plasma VWF antigen (VWFAg) was determined by an enzyme-linked immunosorbent assay (ELISA), as described previously.¹⁵ Briefly, a Maxisorp plate (Nalge Nunc International, Rochester, NY) was coated with a polyclonal rabbit antihuman VWF antibody at 1:2000 dilution (Dako, Glostrup, Denmark) at 4°C overnight. Plasma samples at various dilutions and standards were incubated at room temperature for 2 h after blocking with 1% casein in phosphate-buffered saline (PBS). After being washed 3 times with PBS containing 0.05% Tween-20, a horseradish peroxidase (HRP)-conjugated polyclonal rabbit antihuman VWF (Dako) immunoglobulin G (IgG) at 1:3000 was applied as the detection antibody. After the additional wash steps, a tetramethylbenzidine (TMB) solution (Invitrogen, Camarillo, CA) was added for color reaction. Absorbance at 450 nm was determined with a ThermoMax190 microtiter plate reader (Molecular Devices, Sunnyvale, CA). Normal pooled human plasma (NHP; George King Bio-Medical, Overland Park, KS) was used as the standard, defined as having 100% of VWFAg.

Plasma levels of von Willebrand factor activity

Plasma VWF activity (VWFAc) was determined by its ability to bind type III collagen.¹⁵ A microtiter plate was coated with 100 μ L

of type III collagen (6 μ g/mL; Southern Research Institute, Birmingham, AL) in 20 mM of acetic acid. After blocking with 1% casein in PBS, diluted patient plasma or a standard was added to the wells and incubated at room temperature for 2 h. Bound VWF was detected by addition of a HRP-conjugated antihuman VWF IgG (1:3,000), followed by TMB. NHP was defined to have 100% of VWFAc.

Plasma a disintegrin and metalloprotease with thrombospondin type 1 repeats 13 activity

Plasma ADAMTS13 activity was determined by its ability to cleave a FRETs-VWF73 peptide. A recombinant human VWF fragment (rVWF73) from the central A2 domain of VWF was expressed and fluorescein-labeled, and purified to homogeneity using the protocol described previously.¹⁶ The final concentration of FRET-rVWF73 was determined by a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA) at the absorbance of 280 nm with a correction at 495 nm. Purified FRET-rVWF73 (1 μ mol/L) was incubated with either 2.5 μ L of NHP or patient plasma in 5 mmol/L Bis-Tris, pH 6.0, 25 mmol/L CaCl₂, and 0.005% Tween-20 at 25°C. Rate of fluorescence generation was monitored every 2 min for 60 min on a GeminiXPS microtiter plate reader (Molecular Devices). Relative activity (%) was determined based on the standard curve generated with NHP. ADAMTS13 activity in the pooled NHP was defined as having 100% of proteolytic activity.

Plasma levels of human neutrophil peptides 1–3

Plasma concentrations of HNP1-3 were determined by a commercially available ELISA method (Hycult Biothec, Plymouth, PA), following the manufacturer's recommendation.¹⁷ Detection ranges from 156 to 10,000 pg/mL.

Statistical analysis

The Kruskal–Wallis test was used to determine differences in median values of plasma levels of VWFAg, VWFAc, ADAMTS13 activity, and HNP1-3 among various time points in TBI patients compared to those in healthy controls. Because of low sample size, for outcome association purposes, the statistical analysis is not inference based, but rather exploratory in nature. Data were explored using cluster analysis for repeated measures of on days 2, 3, and 5 for all biomarkers. Generated clusters were used to summarize both quantitative and categorical variables. The first step in cluster analysis involved finding the optimal number of clusters. The “elbow method” using a plot of within-cluster error measure indicated two clusters. Consequently, a k-means clustering algorithm was used to perform the cluster analysis. The two-cluster solution for all the variables was performed. Cluster analysis was performed using R statistical software (R Foundation for Statistical Computing, Vienna, Austria). The Everitt and Hothorn (2009) R function was used to plot the cluster elbows.¹⁸ The cluster analysis requires complete data (no missing data); therefore, only 11 observations were available for use in this analysis.

Results

Demographics

During the study period, a total of 32 patients were enrolled with a median age of 42 years (range, 18–85). Twenty-seven were male (84%) and 13 were of African-American descent (40.6%). The most common mechanism of injury was falls (17; 53%). Seventeen (53%) patients presented in coma; 42% with a GCS of 3. Only 4

patients died (mortality rate of 12.5%). Other key demographics variables are summarized in Table 1.

Elevated plasma levels of von Willebrand factor antigen and von Willebrand factor collagen-binding activity in patients with traumatic brain injury

Plasma VWF antigen and activity were determined by an ELISA-based assay as described in the Methods section. The ability to bind type III collagen is proportional to the size of circulating VWF multi-mers.¹⁹ Therefore, collagen-binding activity represents the distribution of plasma VWF multi-mers. Median plasma levels of VWFAg (95% confidential interval, *n*) in TBI patients on admission and day 1, 2, 3, and 5 after admission were 249% (172–363; *n* = 10; *p* = 0.114), 281% (216–363; *n* = 17; *p* = 0.0021), 383% (306–480; *n* = 20; *p* < 0.00001), 446% (335–593; *n* = 20; *p* < 0.0001), and 458% (361–580; *n* = 18; *p* < 0.0001), respectively, which was significantly higher than 135% (113–160; *n* = 30) in healthy controls (Fig. 1A). Also, median plasma levels of VWFAc (95% confidential interval, *n*) in TBI patients on admission and day 1, 2, 3, and 5 after admission were 203% (132–312; *n* = 10; *p* = 0.044), 244% (185–322; *n* = 17; *p* < 0.0001), 298% (248–359; *n* = 20; *p* < 0.0001), 284% (235–342; *n* = 20; *p* < 0.0001), and 307% (248–383; *n* = 18; *p* < 0.0001), respectively, which was significantly higher than 79% (53–116; *n* = 30) in healthy controls (Fig. 1B). However, ratios of VWFAc to VWFAg (0.65–0.87) in all days except for day 1 (*p* = 0.0218) in patients with TBI were similar to that in healthy controls (0.63; *p* > 0.05; Fig. 1C). These results suggest that both VWFAg and VWFAc in TBI patients were proportionally increased.

Reduced plasma levels of a disintegrin and metalloprotease with thrombospondin type 1 repeats 13 activity and the ratios of a disintegrin and metalloprotease with thrombospondin type 1 repeats 13 activity to von Willebrand factor antigen or von Willebrand factor collagen-binding activity in patients with traumatic brain injury

To assess the hemostatic balance between ADAMTS13 and VWF, we determined plasma ADAMTS13 activity and the ratio of ADAMTS13 to VWFAg or to VWFAc. As shown, plasma levels of ADAMTS13 activity, median (95% confidential interval, *n*), were 55 IU/dL (42–73; *n* = 10) on admission, 63 IU/dL (48–83; *n* = 17) on day 1, and 68 IU/dL (54–85; *n* = 20) on day 2. These values were statistically different from controls. However, the difference was not statistically significant on day 3 (*p* = 0.125) and day 5 (*p* = 0.102) when compared to controls (Fig. 2A). Interestingly, median ratios of ADAMTS13 activity to VWFAg (0.16–0.23; Fig. 2B) or ratios of ADAMTS13 to VWFAc (0.22–0.27; Fig. 2C) in TBI patients during all days after admission appeared to be significantly lower in TBI patients than that in healthy controls (0.68 or 0.99; *p* < 0.0001), suggesting a relative deficiency of plasma ADAMTS13 activity in these patients.

Increased plasma levels of human neutrophil peptides 1–3 in patients with traumatic brain injury

TBI results in acute systemic inflammation, which activates neutrophils and releases granular contents (e.g., HNPs). As shown, median levels of plasma HNP1–3 (95% confidential interval, *n*) in patients with TBI on admission and day 1, 2, 3, and 5 after admission were 7.7 ng/mL (3.0–20.1; *n* = 9; *p* = 0.0596), 11.4 ng/mL (6.1–21.3; *n* = 18; *p* < 0.0001), 14.8 ng/mL (9.1–24.2; *n* = 20;

FIG. 1. Plasma VWF antigen, VWF collagen binding activity, and the ratios of VWF activity to antigen in healthy controls and TBI patients. (A and B) Plasma VWF antigen (VWFAg) and collagen-binding activity (VWFAc), respectively, in healthy control and in patients on admission (D0), day 1 (D1), 2 (D2), 3 (D3), and 5 (D5) post-TBI are shown as the medians $\pm$ 95% confidential intervals. (C) Ratios of plasma VWFAc to VWFAg in patients and healthy controls are also shown as the medians $\pm$ 95% confidential intervals. Kruskal–Wallis analysis determined the significance of the differences between the control and patient values at various days after TBI. Symbols (*, **, ***, and ****) indicate the *p* values < 0.05, < 0.01, < 0.005, and < 0.0001, respectively. n.s., not significant; TBI, traumatic brain injury; VWF, von Willebrand factor.

FIG. 2. Plasma ADAMTS13 activity and the ratios of ADAMTS13 to VWF in healthy controls and TBI patients. (A) Plasma ADAMTS13 activities in healthy controls and patients on admission (D0), day 1 (D1), 2 (D2), 3 (D3), and 5 (D5) post-TBI are shown as the medians $\pm$ 95% confidential intervals. (B and C) Ratios of plasma ADAMTS13 activity to (VWF) or (VWFAc), respectively, in healthy controls and TBI patients are shown as the medians $\pm$ 95% confidential intervals. Symbols (n.s., *, **, ***, and ****) indicate the p values >0.05 , <0.05 , <0.01 , <0.005 , and <0.0001 , respectively. ADAMTS13, a disintegrin and metalloprotease with thrombospondin type 1 repeats 13; n.s., not significant; TBI, traumatic brain injury; VWF, von Willebrand factor.

$p < 0.0001$), 16.3 ng/mL (10.3–25.8; $n = 19$; $p < 0.0001$), and 19.2 ng/mL (10.4–35.5; $n = 17$; $p < 0.0001$) were significantly higher than that in healthy controls 2.6 ng/mL (2.3–3.0; $n = 27$; Fig. 3), consistent with the acute inflammatory state post-TBI.

Elevated von Willebrand factor antigen and human neutrophil peptides 1–3 are clustered into a high-mortality group

Because cluster analysis requires complete data, only 11 observations were available and the analysis revealed two descriptive clusters. Whereas there was no patient who died of 7 in Cluster 1, there was 1 who died of 4 in Cluster 2. Not surprisingly, patients in Cluster 1 were also older and spent more time in the triage, which naturally made them at higher risk for mortality when compared to the ones in Cluster 2 (Table 2). Further, plasma levels of VWFag (mean $\pm$ standard error of the mean [SEM]) on day 2, 3, and 5 in Cluster 1 were lower than corresponding levels in Cluster 2 ($397 \pm 40.8\%$ vs. $591 \pm 314\%$ on day 2; $402 \pm 46.9\%$ vs. $674 \pm 343\%$ on day 3; and $446 \pm 60\%$ vs. $643 \pm 293\%$ on day 5). Similarly, plasma levels of HNP1-3 on day 2 (10.0 ± 2.7 ng/mL), 3 (8.2 ± 1.7 ng/mL), and 5 (12.5 ± 4.0 ng/mL) in Cluster 1 were lower than those on day 2 (36.8 ± 2.2 ng/mL), 3 (30.9 ± 3.3 ng/mL), and 5 (33.3 ± 4.0 ng/mL) in Cluster 2 (Table 2). Taken together, these results suggest that high plasma levels of VWF antigen and HNP1-3, resulting in relative deficiency of plasma ADAMTS13 activity, may be associated with increased mortality.

Discussion

Our present study demonstrates a significant increase in plasma levels of VWF (antigen and collagen-binding activity) and HNP1-3 with a concurrent decrease of plasma ADAMTS13 activity in patients after acute TBI. This results in markedly reduced ratios of plasma ADAMTS13 to VWF antigen or activity in these patients when compared to that in healthy controls. This moderate deficiency of plasma ADAMTS13 activity may be important for

FIG. 3. Plasma levels of HNP1-3 in healthy controls and TBI patients. Plasma concentrations of HNP1-3 in healthy controls and patients on admission (D0), day 1 (D1), 2 (D2), 3 (D3), and 5 (D5) post-TBI are shown as the medians $\pm$ 95% confidential intervals. Symbols (*, **, ***, and ****) indicate the p values <0.05 , <0.01 , <0.005 , and <0.0001 , respectively. HNP1-3, human neutrophil peptides 1–3; n.s., not significant; TBI, traumatic brain injury; VWF, von Willebrand factor.

TABLE 1. DEMOGRAPHIC INFORMATION IN PATIENTS WITH TBI

Variable	Median (range or %)
Age (years)	42 (18–85)
Sex (% male)	27 (64.3)
Race (% African American)	13 (40.6)
Mechanism of injury	
- Fall	17 (53)
- Motor vehicle accident	4 (12.5)
- Car versus pedestrian	4 (12.5)
- Assault	2 (6.25)
- Others	5 (16.1)
Presence of	
- Subdural hematoma	18 (56.2)
- Epidural hematoma	5 (15.6)
- Subarachnoid hematoma	22 (68.8)
Admission Glasgow Coma Score (3–8)	17 (53)
Hospital length of stay (days)	12 (2–59)
Intensive care unit length of stay (days)	10 (2–27)
Disposition	-
- Dead	- 4 (12.5)
- Skilled nursing facility	- 5 (15.6)
- Acute rehabilitation facility	- 13 (40.6)
- Home	- 10 (31.2)

TBI, traumatic brain injury.

development of microvascular thrombosis in the parenchyma of the brain after moderate-severe TBI, and possibly contributes to mortality (Fig. 4). These data are consistent with evidence of the development of a late developing platelet-mediated hypercoagulable state after TBI.²⁰

Microthrombi are frequently observed in histological samples of TBI patients and in experimental models.^{21–23} Microthrombi are found not only in the brain, but also in remote organs, such as the kidney, liver, and spleen, supporting the theory of DIC.²⁴ Autopsy studies confirm a higher concentration of microthrombi in specimens from patients with TBI than in those from patients with non-neurological demise.²⁵ The timing of microthrombus formation coincides with an increased concentration of fibrin degradation products, suggesting a local hyperfibrinolytic state.²⁵ The number of intravascular microthrombi per square inch correlates linearly with the burden of coagulopathy defined by the number of abnormal serum coagulation tests, including the PT, aPTT, and platelet count.⁶ Further, in a randomized, nested controlled trial of trauma patients, treatment with an antifibrinolytic improved outcomes in TBI patients.²⁶ These findings serve to correlate coagulation abnormalities to microthrombi deposition, thus serving as a plausible causal link between intravascular thrombosis and traumatic coagulopathy.

Additionally, systemic inflammation promotes thrombosis in TBI. Production and release of inflammatory cytokines, including tumor necrosis factor- α , interleukins (i.e., IL-1 β , IL-6, and IL-18), or interferons, are increased.²⁷ These inflammatory cytokines are known to not only suppress ADAMTS13 production from hepatic stellate cells and endothelial cells,²⁸ but also stimulate release of procoagulant and -inflammatory VWF from Weibel–Palade bodies of endothelium and α -granules of megakaryocytes and platelets.¹² Such an imbalance between ADAMTS13 and VWF may lead to an enhanced formation of ultra-large VWF polymers on the activated endothelial surface or at the site of vascular injury where platelets and neutrophils adhere and aggregate, resulting in microvascular

TABLE 2. CLUSTER ANALYSIS OF DESCRIPTIVE RESULTS IN TBI PATIENTS

Variable (SEM) ^a	Cluster 1 (n = 7, 0 died)	Cluster 2 (n = 4, 1 died)
Age in years	42 (9.8)	63 (11.5)
No. of female (%)	1 (13)	0 (0)
Fall as mechanism of injury (%)	3 (43)	2 (50)
Subdural hematoma present (%)	0 (0)	1 (25)
GCS on admission	2.3 (0.84)	3 (1.22)
Blood pressure on admission (mm Hg)	151/89 (16.8/7.14)	119/71 (11.9/7.76)
INR on admission	1.04 (0.037)	1.2 (0.082)
aPTT on admission in sec	34 (1.53)	30.2 (1.24)
VWFAg%		
Day 2	397 (40.8)	591 (314)
Day 3	402 (46.9)	674 (343)
Day 5	446 (60.1)	643 (293)
VWFAc% on		
Day 2	319 (44.8)	320 (111)
Day 3	292 (21.8)	312 (105)
Day 5	301 (17.8)	419 (157)
ADAMTS13%		
Day 2	52 (6.23)	63 (12.6)
Day 3	58 (8.65)	69 (16.7)
Day 5	49 (17.8)	69 (0.16)
HNP1-3 ng/mL		
Day 2	10 (2.69)	36.8 (2.19)
Day 3	8.3 (1.67)	30.9 (3.31)
Day 5	12.5 (4)	33.3 (4.04)

A k-means clustering algorithm was fit using the following quantitative variables: VWFAg, VWFAc, ADAMTS13, and HNP1-3 on days 2, 3, and 5 after admission results were all used. Therefore, only 11 observations were available for analysis. Cluster 1 had no mortality whereas Cluster 2 had 25% mortality. Although the sample size is small, the results highlighted major differences between two clusters in terms of age, blood pressure at admission, VWFAg days 2, 3, and 5, and HNP1-3 days 2, 3, and 5 after admission.

^aAll data presented in the parenthesis are standard errors of the means.

TBI, traumatic brain injury; SEM, standard error of the mean; GCS, Glasgow Coma Scale; INR, international normalized ratio; aPTT, activated partial thromboplastin time; VWFAg, von Willebrand factor antigen; VWFAc, von Willebrand factor activity; ADAMTS13, a disintegrin and metalloprotease with thrombospondin type 1 repeats 13; HNP1-3, human neutrophil peptides 1–3.

inflammation and thrombosis (Fig. 4), similar to the pathology observed in patients with thrombotic thrombocytopenic purpura (TTP).²⁹ Microthrombi in parenchymal microvessels in patients after TBI appear to be rich in platelets, VWF, and fibrin, which is also the characteristic findings in tissues obtained from patients who died of TTP.³⁰ Whereas TTP is caused by severe deficiency of plasma ADAMTS13 activity (<10 IU/dL),³¹ relative deficiency of plasma ADAMTS13 activity has been shown to be a risk factor for development of many other thrombotic disorders, including myocardial infarction,^{32,33} acute ischemic stroke,^{33,34} preeclampsia,³⁵ and cerebral malaria.³⁶

ADAMTS13 is known to have both anti-thrombotic and anti-inflammatory activity^{37,38} and therefore may have a potential for therapy in patients with acute brain injury, resulting from various insults. Mice lacking ADAMTS13 activity showed a larger infarct size compared to their wild-type controls that resulted from ischemia-perfusion injury.^{39,40} Infusion of recombinant ADAMTS13 into both wild-type and *Adamts13*^{-/-} mice reduces acute inflammation and

FIG. 4. The diagram depicts how TBI may perturb the ADAMTS13/VWF function, resulting in thrombus formation. When a patient suffers from TBI, acute inflammation results in activation of neutrophils and vascular endothelial cells and release of HNP1-3 and VWF, respectively. Locally released HNP1-3 may bind to VWF, which blocks its proteolysis by ADAMTS13; additionally, increased VWF results in consumption of VWF. Inflammatory cytokines released during the acute inflammation may suppress the synthesis of ADAMTS13 from hepatic stellate cells and endothelial cells while triggering the release of VWF from endothelial cells. The net results are the reduction of ADAMTS13 or decrease in the ratio of ADAMTS13 to VWF. The relative deficiency may cause platelet adhesion and aggregation, and thrombus formation in the brain or elsewhere. ADAMTS13 (or A13), a disintegrin and metalloprotease with thrombospondin type 1 repeats 13; HNP1-3, human neutrophil peptides 1–3; TBI, traumatic brain injury; VWF, von Willebrand factor.

ischemic injury after experimental TBI in mice, which depends on VWF.⁴⁰ In fact, recombinant ADAMTS13, in conjunction with tissue plasminogen activator, enhances its thrombotic efficacy and reduces hemorrhagic complications in animal stroke models.^{41–43}

HNP1-3, the most abundant protein constituent of neutrophil azurophilic granules,⁴⁴ consists of 29–30 amino acid residues that form a tertiary structure by three disulfide bonds.⁴⁵ Molecular weight of HNPs1-3 is approximately 3.5 kDa. HNP1, 2, and 3 are nearly identical except for the first residue. During acute infection or inflammation, neutrophils are activated and degranulated. The released cationic HNP1-3 bind to microbial membranes, resulting in

killing of invaded microorganisms.¹⁴ In the meantime, tissue plasminogen activation⁴⁶ enhances fibrin formation⁴⁷ and inhibits proteolytic cleavage of VWF by ADAMTS13, at least *in vitro*.⁴⁸ Therefore, HNP1-3 may not only provide an important innate immunity by killing the microorganisms, but also cause a collateral damage to host cells by inducing microvascular thrombosis. As shown, plasma levels of HNP1-3 are dramatically elevated 24 h post-TBI, which persists throughout 5 days of hospital stay. Similar kinetics in plasma levels of platelet factor 4, released from platelet activation, was observed after experimental injury.⁴⁹ Whether HNP1-3 is merely an inflammatory marker or has its specific biological activity triggering microvascular thrombosis remains to be determined.

There are limitations to this exploratory pilot study. Observed differences may be attributed to small sample size or confounding variables, such as admission blood pressure, a known determinant of outcome in patients with TBI. Although there is an apparent difference in admission blood pressure between the two groups, blood pressure was maintained above the recommended treatment targets per the Brain Trauma Foundation.⁴⁸ Additionally, in the cluster analysis, the sample size was relatively small because of the requirement of all data points in each patient for analysis.

We conclude that the elevated plasma levels of VWF, reduced ADAMTS13 activity, and elevated HNP1-3 in patients post-TBI may explain the underlying mechanism of microvascular thrombosis found in vessels of brain parenchyma and other organ tissues despite a seemingly hypocoagulability revealed by other routine laboratory tests, including low platelet count, prolongation in prothrombin time, and activated thromboplastin time. Our findings may provide a rationale for supporting future clinical trials with recombinant ADAMTS13 as a novel therapy in patients with TBI.

Acknowledgment

The study was, in part, supported by grants from National Heart, Lung, and Blood Institute (NHLBI) R01-HL126724 and R01-HL115187 (to X.L.Z.).

Author Disclosure Statement

X.L.Z. is a speaker for Alexion and serves as a consultant for Ablynx and BioMedica.

References

1. Taylor, C.A., Greenspan, A.I., Xu, L., and Kresnow, M.J. (2015). Comparability of national estimates for traumatic brain injury-related medical encounters. *J. Head Trauma Rehabil* 30, 150–159.
2. Cuthbert, J.P., Harrison-Felix, C., Corrigan, J.D., Bell, J.M., Haarbauer-Krupa, J.K., and Miller, A.C. (2015). Unemployment in the United States after traumatic brain injury for working-age individuals: prevalence and associated factors 2 years postinjury. *J. Head Trauma Rehabil* 30, 160–174.
3. Faul, M., and Coronado, V. (2015). Epidemiology of traumatic brain injury. *Handb. Clin. Neurol.* 127, 3–13.
4. Narayan, R.K., Michel, M.E., Ansell, B., Baethmann, A., Biegon, A., Bracken, M.B., Bullock, M.R., Choi, S.C., Clifton, G.L., Contant, C.F., Coplin, W.M., Dietrich, W.D., Ghajar, J., Grady, S.M., Grossman, R.G., Hall, E.D., Heetderks, W., Hovda, D.A., Jallo, J., Katz, R.L., Knoller, N., Kochanek, P.M., Maas, A.I., Majde, J., Marion, D.W., Marmarou, A., Marshall, L.F., McIntosh, T.K., Miller, E., Mohberg, N., Muizelaar, J.P., Pitts, L.H., Quinn, P., Riesenfeld, G., Robertson, C.S., Strauss, K.I., Teasdale, G., Temkin, N., Tuma, R., Wade, C., Walker, M.D., Weinrich, M., Whyte, J., Wilberger, J., Young, A.B., and Yurkewicz, L. (2002). Clinical trials in head injury. *J. Neurotrauma* 19, 503–557.

5. Golding, E.M., Robertson, C.S., and Bryan, R.M., Jr. (1999). The consequences of traumatic brain injury on cerebral blood flow and autoregulation: a review. *Clin. Exp. Hypertens.* 21, 299–332.
6. Stein, S.C., Chen, X.H., Sinson, G.P., and Smith, D.H. (2002). Intravascular coagulation: a major secondary insult in nonfatal traumatic brain injury. *J. Neurosurg.* 97, 1373–1377.
7. Morel, O., Morel, N., Freyssinet, J.M., and Toti, F. (2008). Platelet microparticles and vascular cells interactions: a checkpoint between the haemostatic and thrombotic responses. *Platelets* 19, 9–23.
8. Sabatier, F., Roux, V., Anfosso, F., Camoin, L., Sampol, J., and Dignat-George, F. (2002). Interaction of endothelial microparticles with monocytic cells in vitro induces tissue factor-dependent pro-coagulant activity. *Blood* 99, 3962–3970.
9. Sillesen, M., Rasmussen, L.S., Jin, G., Jepsen, C.H., Imam, A., Hwabejire, J.O., Halaweish, I., DeMoya, M., Velmahos, G., Johansson, P.I., and Alam, H.B. (2014). Assessment of coagulopathy, endothelial injury, and inflammation after traumatic brain injury and hemorrhage in a porcine model. *J. Trauma Acute Care Surg.* 76, 12–19; discussion, 19–20.
10. Boudreau, R.M., Johnson, M., Veile, R., Friend, L.A., Goetzman, H., Pritts, T.A., Caldwell, C.C., Makley, A.T., and Goodman, M.D. (2017). Impact of tranexamic acid on coagulation and inflammation in murine models of traumatic brain injury and hemorrhage. *J. Surg. Res.* 215, 47–54.
11. Braun, M., Vaibhav, K., Saad, N.M., Fatima, S., Vender, J.R., Baban, B., Hoda, M.N., and Dhandapani, K.M. (2017). White matter damage after traumatic brain injury: a role for damage associated molecular patterns. *Biochim. Biophys. Acta* 1863, 2614–2626.
12. Wagner, D.D., and Bonfanti, R. (1991). von Willebrand factor and the endothelium. *Mayo Clin. Proc.* 66, 621–627.
13. Zheng, X.L. (2013). Structure-function and regulation of ADAMTS-13 protease. *J. Thromb. Haemost.* 11, Suppl. 1, 11–23.
14. Ganz, T., Selsted, M.E., Szklarek, D., Harwig, S.S., Daher, K., Bainton, D.F., and Lehrer, R.I. (1985). Defensins. Natural peptide antibiotics of human neutrophils. *J. Clin. Invest.* 76, 1427–1435.
15. Kumar, M., Cao, W., McDaniel, J.K., Pham, H.P., Raju, D., Nawalinski, K., Frangos, S., Kung, D., Zager, E., Kasner, S.E., Levine, J.M., and Zheng, X.L. (2017). Plasma ADAMTS13 activity and von Willebrand factor antigen and activity in patients with subarachnoid haemorrhage. *Thromb. Haemost.* 117, 691–699.
16. Lu, R.N., Yang, S., Wu, H.M., and Zheng, X.L. (2015). Unconjugated bilirubin inhibits proteolytic cleavage of von Willebrand factor by ADAMTS13 protease. *J. Thromb. Haemost.* 13, 1064–1072.
17. Cao, W., Pham, H.P., Williams, L.A., McDaniel, J., Siniard, R.C., Lorenz, R.G., Marques, M.B., and Zheng, X.L. (2016). Human neutrophil peptides and complement factor Bb in pathogenesis of acquired thrombotic thrombocytopenic purpura. *Haematologica* 101, 1319–1326.
18. Everitt, B. (2004). Cluster analysis is a generic term for a wide range of numerical methods for examining data. *Stat. Methods Med. Res.* 13, 343–345.
19. Gerritsen, H.E., Turecek, P.L., Schwarz, H.P., Lammle, B., and Furlan, M. (1999). Assay of von Willebrand factor (vWF)-cleaving protease based on decreased collagen binding affinity of degraded vWF: a tool for the diagnosis of thrombotic thrombocytopenic purpura (TTP). *Thromb. Haemost.* 82, 1386–1389.
20. Massaro, A.M., Doerfler, S., Nawalinski, K., Michel, B., Driscoll, N., Ju, C., Patel, H., Quattrone, F., Frangos, S., Maloney-Wilensky, E., Sean Grady, M., Stein, S.C., Kasner, S.E., and Kumar, M.A. (2015). Thromboelastography defines late hypercoagulability after TBI: a pilot study. *Neurocrit. Care* 22, 45–51.
21. Stein, S.C., and Smith, D.H. (2004). Coagulopathy in traumatic brain injury. *Neurocrit. Care* 1, 479–488.
22. Hekmatpanah, J., and Hekmatpanah, C.R. (1985). Microvascular alterations following cerebral contusion in rats. Light, scanning, and electron microscope study. *J. Neurosurg.* 62, 888–897.
23. Stein, S.C., Young, G.S., Talucci, R.C., Greenbaum, B.H., and Ross, S.E. (1992). Delayed brain injury after head trauma: significance of coagulopathy. *Neurosurgery* 30, 160–165.
24. Kaufman, H.H., Hui, K.S., Mattson, J.C., Borit, A., Childs, T.L., Hoots, W.K., Bernstein, D.P., Makela, M.E., Wagner, K.A., Kahan, B.D., and Gildenberg, P.L. (1984). Clinicopathological correlations of disseminated intravascular coagulation in patients with head injury. *Neurosurgery* 15, 34–42.
25. Lafuente, J.V., and Cervos-Navarro, J. (1999). Craniocerebral trauma induces hemorheological disturbances. *J. Neurotrauma* 16, 425–430.
26. CRASH-2 Collaborators (Intracranial Bleeding Study). (2011). Effect of tranexamic acid in traumatic brain injury: a nested randomised, placebo controlled trial (CRASH-2 Intracranial Bleeding Study). *BMJ* 343, d3795.
27. Balu, R. (2014). Inflammation and immune system activation after traumatic brain injury. *Curr. Neurol. Neurosci. Rep.* 14, 484.
28. Cao, W.J., Niiya, M., Zheng, X.W., Shang, D.Z., and Zheng, X.L. (2008). Inflammatory cytokines inhibit ADAMTS13 synthesis in hepatic stellate cells and endothelial cells. *J. Thromb. Haemost.* 6, 1233–1235.
29. Zheng, X.L., and Sadler, J.E. (2008). Pathogenesis of thrombotic microangiopathies. *Annu. Rev. Pathol.* 3, 249–277.
30. Hosler, G.A., Cusumano, A.M., and Hutchins, G.M. (2003). Thrombotic thrombocytopenic purpura and hemolytic uremic syndrome are distinct pathologic entities. A review of 56 autopsy cases. *Arch. Pathol. Lab. Med.* 127, 834–839.
31. Zheng, X.L. (2015). ADAMTS13 and von Willebrand factor in thrombotic thrombocytopenic purpura. *Annu. Rev. Med.* 66, 211–225.
32. Horii, M., Uemura, S., Uemura, M., Matsumoto, M., Ishizashi, H., Imagawa, K., Iwama, H., Takeda, Y., Kawata, H., Nakajima, T., Fujimura, Y., and Saito, Y. (2008). Acute myocardial infarction as a systemic prothrombotic condition evidenced by increased von Willebrand factor protein over ADAMTS13 activity in coronary and systemic circulation. *Heart Vessels* 23, 301–307.
33. Andersson, H.M., Siegerink, B., Luken, B.M., Crawley, J.T., Algra, A., Lane, D.A., and Rosendaal, F.R. (2012). High VWF, low ADAMTS13, and oral contraceptives increase the risk of ischemic stroke and myocardial infarction in young women. *Blood* 119, 1555–1560.
34. Sonneveld, M.A., de Maat, M.P., Portegies, M.L., Kavousi, M., Hofman, A., Turecek, P.L., Rottensteiner, H., Scheifflinger, F., Koudstaal, P.J., Ikram, M.A., and Leebeek, F.W. (2015). Low ADAMTS13 activity is associated with an increased risk of ischemic stroke. *Blood* 126, 2739–2746.
35. Aref, S., and Goda, H. (2013). Increased VWF antigen levels and decreased ADAMTS13 activity in preeclampsia. *Hematology* 18, 237–241.
36. Lowenberg, E.C., Charunwatthana, P., Cohen, S., van den Born, B.J., Meijers, J.C., Yunus, E.B., Hassan, M.U., Hoque, G., Maude, R.J., Nuchongsin, F., Levi, M., and Dondorp, A.M. (2010). Severe malaria is associated with a deficiency of von Willebrand factor cleaving protease, ADAMTS13. *Thromb. Haemost.* 103, 181–187.
37. Chauhan, A.K., Kisucka, J., Brill, A., Walsh, M.T., Scheifflinger, F., and Wagner, D.D. (2008). ADAMTS13: a new link between thrombosis and inflammation. *J. Exp. Med.* 205, 2065–2074.
38. Chauhan, A.K., Motto, D.G., Lamb, C.B., Bergmeier, W., Dockal, M., Plaimauer, B., Scheifflinger, F., Ginsburg, D., and Wagner, D.D. (2006). Systemic antithrombotic effects of ADAMTS13. *J. Exp. Med.* 203, 767–776.
39. Zhao, B.Q., Chauhan, A.K., Canault, M., Patten, I.S., Yang, J.J., Dockal, M., Scheifflinger, F., and Wagner, D.D. (2009). von Willebrand factor-cleaving protease ADAMTS13 reduces ischemic brain injury in experimental stroke. *Blood* 114, 3329–3334.
40. Khan, M.M., Motto, D.G., Lentz, S.R., and Chauhan, A.K. (2012). ADAMTS13 reduces VWF-mediated acute inflammation following focal cerebral ischemia in mice. *J. Thromb. Haemost.* 10, 1665–1671.
41. Denorme, F., Langhauser, F., Desender, L., Vandenbulcke, A., Rottensteiner, H., Plaimauer, B., Francois, O., Andersson, T., Deckmyn, H., Scheifflinger, F., Kleinschnitz, C., Vanhoorelbeke, K., and De Meyer, S.F. (2016). ADAMTS13-mediated thrombolysis of t-PA-resistant occlusions in ischemic stroke in mice. *Blood* 127, 2337–2345.
42. Nakano, T., Irie, K., Hayakawa, K., Sano, K., Nakamura, Y., Tanaka, M., Yamashita, Y., Satho, T., Fujioka, M., Muroi, C., Matsuo, K., Ishikura, H., Futagami, K., and Mishima, K. (2015). Delayed treatment with ADAMTS13 ameliorates cerebral ischemic injury without hemorrhagic complication. *Brain Res.* 1624, 330–335.
43. Wang, L., Fan, W., Cai, P., Fan, M., Zhu, X., Dai, Y., Sun, C., Cheng, Y., Zheng, P., and Zhao, B.Q. (2013). Recombinant ADAMTS13 re-

- duces tissue plasminogen activator-induced hemorrhage after stroke in mice. *Ann. Neurol.* 73, 189–198.
44. Rice, W.G., Ganz, T., Kinkade, J.M., Jr., Selsted, M.E., Lehrer, R.I., and Parmley, R.T. (1987). Defensin-rich dense granules of human neutrophils. *Blood* 70, 757–765.
 45. Selsted, M.E., Harwig, S.S., Ganz, T., Schilling, J.W., and Lehrer, R.I. (1985). Primary structures of three human neutrophil defensins. *J. Clin. Invest.* 76, 1436–1439.
 46. Higazi, A.A., Ganz, T., Kariko, K., and Cines, D.B. (1996). Defensin modulates tissue-type plasminogen activator and plasminogen binding to fibrin and endothelial cells. *J. Biol. Chem.* 271, 17650–17655.
 47. Horn, M., Bertling, A., Brodde, M.F., Muller, A., Roth, J., Van Aken, H., Jurk, K., Heilmann, C., Peters, G., and Kehrel, B.E. (2012). Human neutrophil alpha-defensins induce formation of fibrinogen and thrombospondin-1 amyloid-like structures and activate platelets via glycoprotein IIb/IIIa. *J. Thromb. Haemost.* 10, 647–661.
 48. Pillai, V.G., Bao, J., Zander, C.B., McDaniel, J.K., Chetty, P.S., Seeholzer, S.H., Bdeir, K., Cines, D.B., and Zheng, X.L. (2016). Human neutrophil peptides inhibit cleavage of von Willebrand factor by ADAMTS13: a potential link of inflammation to TTP. *Blood* 128, 110–119.
 49. Lu, D., Mahmood, A., Goussev, A., Qu, C., Zhang, Z.G., and Chopp, M. (2004). Delayed thrombosis after traumatic brain injury in rats. *J. Neurotrauma* 21, 1756–1766.

Address correspondence to:

X. Long Zheng, MD, PhD

Division of Laboratory Medicine

Department of Pathology

The University of Alabama at Birmingham

WP230K

619 19th Street South

Birmingham, AL 35249

E-mail: xzheng@uabmc.edu;

longzheng01@gmail.com