Systemic Platelet Dysfunction Is the Result of Local Dysregulated Coagulation and Platelet Activation in the Brain in a Rat Model of Isolated Traumatic Brain Injury

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Abstract

Coagulopathy after severe traumatic brain injury (TBI) has been extensively reported. Clinical studies have identified a strong relationship between diminished platelet-rich thrombus formation, responsiveness to adenosine diphosphate agonism, and severity of TBI. The mechanisms that lead to platelet dysfunction in the acute response to TBI are poorly understood. The development of a rodent model of TBI that mimics the coagulopathy observed clinically has recently been reported. Using immunohistochemical techniques and thromboelastography platelet mapping, the current study demonstrated that the expression of coagulation (tissue factor and fibrin) and platelet activation (P-selectin) markers in the injured brain paralleled the alteration in systemic platelet responsiveness to the agonists, adenosine diphosphate and arachidonic acid. Results of this study demonstrate that local procoagulant changes in the injured brain have profound effects on systemic platelet function.

Key words: brain injury; coagulopathy; platelet dysfunction

Introduction

COAGULOPATHY is an independent prognostic risk factor after traumatic brain injury (TBI) and increases the chances of poor outcome by greater than 30-fold relative to patients without coagulopathy.¹ This is especially the case when it occurs in the acute phase of TBI (within 12 h after injury).² The underlying mechanisms for the TBI-induced coagulopathy are not completely understood but may involve release of tissue factor (TF) from the brain, a rich source of this protein,³ or even from damaged vasculature,⁴ dysregulated fibrinolysis, shock, or hypoperfusion⁵ with downstream effects on platelet function. Acute coagulopathy is prevalent in cases of severe TBI (>60%), but uncommon in mild head injury (<1%).^{6,7} The coagulopathy could be a combination of both hypo- and hypercoagulative states, potentially regulated by the extent of injury, eventually resulting in life-threatening ischemic and/or hemorrhagic events.⁸

An initial early case report demonstrated that the release of TF from the brain resulted in coagulation disorders after severe TBI.⁹ TF is the primary physiological initiating factor of blood coagulation and has been shown to be expressed on the surface of cells within the central nervous system.¹⁰ Its systemic release enveloped in active procoagulant microparticles (MPs) can be the result of

compromised blood–brain barrier after brain injury. Relevant to this hypothesis, studies have identified increased levels of procoagulant MPs in cerebrospinal fluid and blood at the onset of TBI.¹¹ The excessive release and sustained generation of these procoagulant MPs paralleled poor clinical outcome for patients with TBI.¹²

Platelet dysfunction can be the cause or result from coagulopathy after TBI and is frequently not the result of diminished platelet count.¹³ We, and others, have shown a correlation between severe TBI and platelet dysfunction.^{13,14} In addition, the severity of diminished platelet thrombotic responses to the agonist, adenosine diphosphate (ADP), as determined using thromboelastography, distinguished between survivors and non-survivors.¹³ In that study, there was a strong correlation between enhanced platelet insensitivity to agonist ADP and mortality in patient with low abbreviated injury score (AIS) non-TBI (<2) and a high AIS-head (≥3) scores. Recently, a rodent model of TBI has been developed, and initial characterizations have demonstrated that blunt force trauma results in platelet dysfunction similar to that of patients with closed TBI.¹⁵ It is hypothesized that there is an initial local activation of coagulation after TBI that then affects systemic platelet function. The aim of this study was to identify TBI-induced locally altered hemostasis through immunohistochemical (IHC) techniques.

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Methods

Injury model

Male Sprague-Dawley rats (245–285 g, Charles River Laboratories, Wilmington, MA) were studied in a constrained, non-penetrating brain injury model using the TBI 0310 (Precision Systems and Instrumentation, Fairfax, VA) as described previously.¹⁵

All animal studies were performed with prior approval from the University of Notre Dame Institutional Animal Care and Use Committee.

Blood collection

At 15, 30, and 60 min post-injury, rats were anesthetized and whole blood collected from the vena cava into a syringe containing heparin (68 U/4 mL, Sagent Pharmaceuticals, Schamburg, IL) or sodium citrate (3.2%, Sigma-Aldrich, St. Louis, MO) using a 20-gauge needle. Non-injured rats were used as controls.

Platelet mapping by thromboelastography

Platelet responses to the agonists, ADP and arachidonic acid (AA), were determined using the TEG[®] Thromboelastograph[®] Hemostasis Analyzer System PlateletMapping[®] Assay (Haemonetics Corporation, Braintree, MA) as described previously.¹⁴ Essentially, MA_{Thrombin} (maximum amplitude of the curve) was determined by adding a vial of kaolin (Haemonetics Corporation) to 1 mL citrated blood, and the MA_{Fibrin} was determined by adding 10 μ L of Activator-F (Haemonetics Corporation) to 360 μ L of heparinized blood. For the MA_{Analyte} (effectiveness of analyte toward stimulation of platelets), AA (1 mM, final concentration, Haemonetics Corporation) or ADP (50 mM, final concentration, Haemonetics Corporation) as well as 10 μ L of Activator-F were added to 360 μ L heparinized blood. The analyte-specific response (%) was calculated using the following equation: $(MA_{Analyte} - MA_{Fibrin}) / (MA_{Thrombin} - MA_{Fibrin}) \times 100$.

Immunohistochemistry for P-selectin, TF, and fibrin(ogen) expression

At 0, 15, and 30 min post-injury and after blood collection, the rats were then perfused with saline followed by fixation with 4% paraformaldehyde. The brains were then removed and 2 mm coronal sections (A–J) were sliced using a bread-loaf matrix.

Sections were embedded in paraffin, and sections B, C, and D were further sectioned into 4 μ m sections. Antigen-retrieval for all the IHC stains was accomplished by heat-induced epitope retrieval in citrate buffer, pH 6.0 (Life Technologies, Inc., Carlsbad, CA), and endogenous peroxidase activity was blocked using Peroxoblock (Life Technologies). For P-selectin immunostaining, the primary antibody was goat-anti-mouse CD 62P polyclonal antibody (R&D Systems, Minneapolis, MN), and the secondary antibody was horseradish peroxidase (HRP)-conjugated donkey-anti-goat IgG polyclonal antibody (Jackson ImmunoResearch Laboratories, West Grove, CA). For TF immunostains, the primary antibody was rabbit-anti-human TF monoclonal antibody (ABCAM, Cambridge, MA), and the secondary antibody was HRP-conjugated donkey-anti-rabbit IgG polyclonal antibody (Jackson ImmunoResearch Laboratories).

For fibrin(ogen) immunostains, the primary antibody was goat-anti-mouse fibrin(ogen) polyclonal antibody (Nordic MUBio, Susteren, The Netherlands), and the secondary antibody was HRP-conjugated donkey-anti-goat IgG polyclonal antibody (Jackson ImmunoResearch Laboratories). Blocking against host secondary antibody for P-selectin and fibrin(ogen) immunostains was by Serum Free Protein Block (Dako, Carpinteria, CA) and for the TF immunostain, normal donkey serum (Dako). The chromogenic substrate for all the immunostains was Nova Red (Vector Laboratories, Burlingame, CA).

Quantitation of IHC stains

To quantitate the extent of expression of P-selectin, TF, or fibrin(ogen) in various regions of the brain, sections from the 2 mm sections B, C, and D were microscopically evaluated for positive IHC staining (P-selectin, TF, and fibrin[ogen]). The time points consisted of 15 min, 30 min, and 1 h post-injury, and control samples (noninjured).

The positive (+) and negative (–) stained slides were identified and categorized according to six different regions of the brain. They included the superior aspect of the impact site (re: superior sagittal sinus, pia, layer 1, and layer 2/3 of the secondary motor cortex); the inferior aspect (ventral edge of hypothalamus); the subarachnoid spaces; the third, fourth ventricles, and subfornical organ; the lateral ventricles (re: ependyma and choroid plexus); and the deep cortical layers of supplemental somatosensory cortex and caudate putamen.

A score of 1 for each region was given for (+) staining (maximum = 6). The individual staining results were quantified according

FIG. 1. Immunohistochemistry of P-selectin (activated platelets), tissue factor, and fibrin(ogen). (A) Hematoxylin and eosin (H&E) stain of control brain. (B) H&E staining of injured brain at 15 min post-injury, and (C) fibrin(ogen) immunostaining showing a fibrin clot in the superior sagittal sinus near the anterior cingulate cortex and secondary motor cortex (15 min post-injury). (D) P-selectin and (E) fibrin(ogen) were detected in the subarachnoid space at the level of the piriform area of the cortex (30 min post-injury). (F) Tissue factor was observed in the paraventricular nucleus of the thalamus (30 min post-injury). A, B, C = 4X; D, E, F = 20X.

FIG. 2. Relative distribution in brain tissue and comparative platelet agonist responses in blood platelets. (A) Immunohistochemical quantitation of P-selectin (activated platelets), tissue factor, and fibrin(ogen) in brain tissue from non-injured (control [CTL]), and 15 min, 30 min, 1 h post-injury samples. $n = 10$ (CTL); 9 (15 min); 10 (30 min); and 12 (1 h). (B) Adenosine diphosphate (ADP)- and arachidonic acid (AA)-induced platelet responses for control, 15 min, 30 min, and 1 h post-injury. For ADP non-injured (CTL) $n = 10$; 15 min = 9; 30 min = 10; 1 h = 12. For AA non-injured (CTL) $n = 9$; 15 min = 9; 30 min = 10; 1 h = 12. All p values are relative to control sample.

to the average of the sum of all (+) regions per rat for the given time point. Three sections/rat were analyzed and the number of rats/time point was 10 for controls, 9 for 15 min injury, 10 for 30 min injury, and 12 for 1 h injury.

Some sections were stained with hematoxylin and eosin for morphological assessment of the tissue.

Statistical analysis

Injured rats were compared with noninjured control rats using unpaired t testing (GraphPad Prism, GraphPad software 5.0a, San Diego, CA). Data are expressed as the mean $\pm$ standard error of the mean.

Results

As reported earlier, hemorrhaging was observed in the subarachnoid spaces after constrained blunt force injury to the brain in a rat model of TBI.¹⁵ To further characterize downstream events after injury, we assessed activation of the blood coagulation pathway as well as platelet activation and function. In this study, a fibrin-enriched clot was observed in the superior sagittal sinus close to the site of impact (Fig. 1A–C) as early as 15 min post-injury.

In addition, evidence for activation of platelets (positive for P-selectin) was observed in the subarachnoid space at the level of the piriform area of the cortex (Fig. 1D). An extensive accumulation of fibrin(ogen) was seen in the same region of the brain (Fig. 1E) indicating local activation of the blood coagulation cascade. In support of this, the initiating factor of the extrinsic pathway of coagulation, TF, was observed in the form of MPs in the paraventricular nucleus of the thalamus (Fig. 1F).

To determine if the pattern of appearance of these activated platelet and coagulation markers parallel the ADP and AA responses in circulating platelets, ADP and AA platelet responses were assessed at 0–1 h post-injury and compared with the expression of P-selectin, TF, and fibrin(ogen) in brain tissue over the same time frame. Interestingly, when ADP or AA responses in circulating platelets were normal, expression of these markers was suppressed (Fig. 2A, B). In contrast, when responses to platelet agonists were suppressed, expression of the hypercoagulant markers was enhanced, implicating an early burst of local thrombin expression, the result of TF expression and potentially a break in the blood–brain barrier as causative events in systemic platelet dysfunction.

Discussion

Previous clinical studies have identified a strong correlation of diminished platelet responsiveness to ADP agonist as measured by TEG platelet mapping (TEG PM) and severity of TBI as determined by a Glasgow Coma Scale (GCS) score ≤ 8 .¹³ This altered platelet responsiveness was shown not to be the result of changes in platelet count. To further characterize platelet dysfunction in TBI, we developed a rat model of constrained blunt force TBI. Using this model, we have shown that, accompanying brain parenchymal injury, local subarachnoid hemorrhaging occurred and systemic both ADP- and AA-induced platelet responses were rapidly diminished post-injury. Interestingly, in that model, coagulation profiles (prothrombin time (PT) and activated partial thromboplastin time (aPTT)) were unchanged. Further, administration of the thrombin inhibitor, Refludan,[®] before injury, protected systemic platelet responses to ADP indicating a potential role for thrombin in the regulation of systemic platelet dysfunction after TBI.¹⁵

In the current study, we correlated temporal changes in ADP- and AA-induced platelet activation, as measured by TEG PM, with altered expression of coagulation markers TF, fibrin(ogen), and P-selectin in the rat brain after injury. In all three markers, the extent of localized expression of these proteins inversely correlated with systemic platelet responses to ADP and AA agonists, potentially supporting the hypothesis that there is an immediate but recoverable systemic exhausted platelet effect. It has been reported that there is a transient expression of TF on monocytes and platelet-monocyte complexes.¹⁶ In addition, patients with TBI with progressive hemorrhagic injury have increased plasma levels of D-dimer, a plasmin-derived fibrin degradation product.¹⁷ Breakdown of the blood–brain barrier, as evident by subarachnoid hemorrhaging observed in this model, would result in transient exposure of subendothelial TF leading to local activation of blood coagulation and release into the systemic circulation.

Procoagulant MPs, derived from the damaged endothelium and/or activated platelets, have been identified in the cerebrospinal fluid and blood of patients with TBI.¹¹ In this study, we have identified TF-enriched particles in the paraventricular nucleus of the thalamus. In addition, increases in thrombin-antithrombin complex have been identified in cerebrovenous blood in patients with isolated severe

head injury.⁴ Therefore, local activation of blood coagulation in the brain can have profound systemic effects on platelet function, and its sustained activation can lead to poor clinical outcomes.

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Author Disclosure Statement

No competing financial interests exist.

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