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Microparticles impact coagulation after traumatic brain injury

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Abstract

Background—The pathophysiology that drives the subacute hypercoagulable state commonly seen after traumatic brain injury (TBI) is not well understood. Alterations caused by TBI in platelet and microparticle (MP) numbers and function have been suggested as possible causes; however, the contributions of platelets and MPs are currently unknown.

Materials and methods—A weight-drop technique of TBI using a murine model of moderate head injury was used. Blood was collected at intervals after injury. MP enumeration and characterization were performed using Nanoparticle Tracking Analysis, and platelet counts and coagulation parameters were determined using thromboelastometry. A MP procoagulant assay was used to compare activity between injured and sham mice.

Results—At 24 h after injury, there were no changes in circulating platelet numbers. However, there was a decrease in platelet contribution to clot formation. In contrast, there was a decline in circulating total MP numbers. When MPs from sham mice were added to the blood from head-injured animals, there was a normalization of platelet contribution to clot formation. Conversely, when MPs from TBI mice were added to sham blood, there was a significant decrease in platelet contribution to clot formation. Notably, there was an increase in MP procoagulant activity in head-injured mice.

Conclusions—MPs generated after TBI likely contribute to altered coagulation after head injury and may play a key role in the development of a posttraumatic hypercoagulable state in TBI patients.

Keywords

Platelet function; Microparticles; TBI; Hypercoagulability; Thromboelastometry

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Disclosure

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1. Introduction

Traumatic brain injury (TBI) results in significant disability and mortality among trauma populations. Mortality rates have remained relatively stable over the past 10 years suggesting a need for better understanding and treatment of this disease process [1]. One of the major pathophysiologic disturbances shown to be predictive of increased morbidity and mortality after TBI is acute traumatic coagulopathy, with reported incidences ranges from 10%–97.5% [2–5]. After this immediate hypocoagulable state, a subacute hypercoagulable state develops with the incidence of deep vein thrombosis ranging from 1.8%–44% in major trauma patients [6–8]. More specifically, the presence of head injury is predictive of an increased risk for thrombotic events [9]. Although the mechanisms behind these changes are not fully understood [10–12], the commonly proposed causes include consumptive coagulopathy [13], tissue factor release [14], and platelet dysfunction [4,15–17].

More recent investigations have begun to explore the role of microparticles (MPs) in this process, as they have been identified as markers of and possible contributors to the hypercoagulable states seen in venous thromboembolism [18], cardiovascular disease [19], cancers [20], prothrombotic pregnancy complications [21], and posttraumatic states [22,23]. MPs are defined as 0.1–1 μm vesicles that bleb off cell membranes of activated or apoptotic cells and function in cell–cell communication [24]. Procoagulant MPs are often identified as those expressing tissue factor or phosphatidylserine, and previous studies have found them to be increased in the cerebrospinal fluid and blood of patients after intracranial hemorrhage [25] and head trauma [26–28]. However, the role of these particles in altering coagulation, and more specifically platelet function, has not been explored.

In the present study, we used a murine model to characterize alterations in coagulation after TBI to determine whether MPs contribute to these changes. Our hypotheses were that (1) TBI would result in a subacute hypercoagulable state, (2) increased circulating MPs would be seen after TBI, and (3) the changes in coagulation would be due to the altered MP populations.

2. Materials and methods

2.1. Materials

Male C57BL/6 mice between ages 6 and 8 wk and 20–28 g obtained from Taconic Labs (Hudson, NY) were used for all experiments. Only male mice were used as previous research has demonstrated that higher estrogen levels can impact immune status after trauma [29]. All murine experiments were performed between 8 AM and 10 AM and were performed under protocols approved by the Institutional Animal Care and Use Committee of the University of Cincinnati.

2.2. Traumatic brain injury

Mice were anesthetized with 2% inhaled isoflurane, and a traumatic closed-head injury was induced using an established weight-drop model [30]. In short, after anesthesia, mice were placed prone on a platform, and the posterior coronal suture of the mice was aligned below the weight. A 400 g weight was then dropped 1 cm to induce head injury. Sham mice were

anesthetized and laid on the platform without head injury. Righting reflex time was measured immediately after TBI to ensure an injury of moderate severity.

2.3. Thromboelastometry

Rotational thromboelastometry (ROTEM; TEM Systems Inc, Durham, NC) analyses were performed to determine alterations in coagulation per manufacturer instructions. Whole blood collected via cardiac puncture from sham and TBI mice was anticoagulated with 10% citrate. All analyses were initiated within 10 min of whole blood collection. Overall coagulation was determined using NATEM, extrinsic pathway coagulation using EXTEM, and fibrin contribution to clot using the FIBTEM analysis. For EXTEM and FIBTEM analysis, 20 μ L of thromboplastin and cytochalasin D were added to 300 μ L of citrated blood. Clotting time (CT), clot formation time (CFT), clot lysis (LI30), α -angle (AA), and maximum clot firmness (MCF) were determined for each test. Treated samples had 30 μ L of saline or MP preparations added. MPs were isolated as described in section 2.5. Percent of platelet contribution (%MCF-Platelet) was calculated by the equation: $(\text{EXTEM}_{\text{MCF}} - \text{FIBTEM}_{\text{MCF}}) / \text{EXTEM}_{\text{MCF}}$, similar to the methods used by Kornblith *et al.* [31]. For NATEM analysis, sham mice ($n = 9$) and TBI mice at 10 min ($n = 7$), 24 h ($n = 10$), and 72 h ($n = 11$) were used. For EXTEM and FIBTEM analyses, the same treatment groups were used with 8–10 mice per group in both the untreated and MP-treated samples.

2.4. Platelet count determination

Whole blood collected via cardiac puncture from injured mice was anticoagulated with 10% citrate. Coulter AcT 10 Hematology Analyzer (Beckman Coulter, Brea, CA) was used to determine platelet counts.

2.5. MP isolation, enumeration, and characterization

The MP isolation protocol used was adapted from those previously published from our laboratory [32]. In short, whole blood collected via cardiac puncture from injured mice was anticoagulated with heparin. It was then centrifuged at 450 g for 10 min; the supernatant was collected and centrifuged at 10,000 g for 10 min to remove platelets. The platelet-free supernatant containing the MPs was then diluted at 1:1000 with Roswell Park Memorial Institute media and stained with 10 μ L per sample CD41 antibody (Clone MWReg30; BD Pharmingen, San Jose, CA). Nanoparticle Tracking Analysis (NanoSight; Malvern Instruments Ltd, Worcestershire, United Kingdom) was then used to enumerate total and CD41+ MP concentrations. MP populations were characterized in sham mice ($n = 26$) and injured mice at 30 min ($n = 13$), 3 h ($n = 9$), 24 h ($n = 21$), and 72 h ($n = 25$) after TBI. For MPs used in ROTEM analyses, platelet-free plasma was collected per previously mentioned protocols and centrifuged at 25,000 g for 30 min to pellet the MPs. MPs were then resuspended in sterile saline to a concentration of 3.0×10^8 MPs/mL and added to anticoagulated blood as stated in section 2.3. MPs isolated 24 h after injury from sham mouse blood will be referred to as sham MPs and those isolated from TBI mouse blood will be referred to as TBI MPs.

2.6. MP procoagulant activity

MP procoagulant activity was determined using a Zymuphen MP-Activity functional assay (Aniara, West Chester, OH). This assay first exposed the phospholipids on the surface of the MPs and then measured the activation of prothrombin into thrombin by these exposed phospholipids. Whole blood was collected via cardiac puncture from sham ($n = 8$) and TBI mice ($n = 7$) 24 h after injury and anticoagulated with 10% citrate. MPs were isolated per protocol in section 2.5, diluted at 1:20, and the assay was performed per manufacturer protocol.

2.7. Statistical analysis

A power calculation was performed to determine samples sizes required for all experiments. With a power of 0.8, an alpha value of 0.05 and an estimated difference between treatment groups of 25%, a sample size of six was required. Student *t*-tests were used when comparisons were made between two treatment groups. One-way analysis of variance with Tukey *post hoc* test was used to compare multiple populations. Prism 6 (GraphPad Software, La Jolla, CA) was used for all statistical analysis. Experiments containing multiple data points were used to calculate means and standard errors of the mean. A *P* value of 0.05 was considered significant.

3. Results

3.1. TBI does not alter overall clot strength

We analyzed whole blood from sham and injured mice at 10 min, 24 h, and 72 h after head trauma using the NATEM test on the ROTEM. We did not observe any differences in MCF at any time point after TBI compared with sham (Fig. 1). In the subacute phase of injury at 24 h after TBI, there were also no differences in CT, CFT, AA, or LI30. From these data, we conclude that in our TBI model, overall clot firmness is unchanged and overall coagulation is unchanged at 24 h after head injury.

3.2. Decreased platelet contribution to clot formation is seen after TBI

Evaluation of the extrinsic pathway after TBI demonstrated increased EXTEM_{MCF} at 24 h after injury compared with sham with no differences seen at 10 min and 72 h after TBI (Fig. 2A). CT, CFT, LI30, and AA were not different at any time point on the EXTEM analysis (data not shown). Evaluation of fibrin function using FIBTEM analysis with platelet inhibition demonstrated increased FIBTEM_{MCF} at 24 h after TBI compared with sham with no differences seen at the other injury time points compared with sham (Fig. 2B). CT, CFT, LI30, and AA were not different at any time point on the FIBTEM analysis (data not shown). The calculated %MCF-Platelet was found to be significantly lower 24 h after TBI compared with that of sham but unchanged 10 min and 72 h after injury (Fig. 2C). There were no significant changes in whole blood platelet counts after injury (Fig. 2D). Altogether, these data suggest that although overall clot formation remains unchanged after injury, 24 h after TBI there is a reduced platelet contribution to clot formation that is not the result of the altered platelet counts. Thus, there is another mediator that is replacing the role of platelets in clot formation after TBI.

3.3. Circulating MP populations are altered after TBI

Overall MP populations were unchanged 30 min after injury, significantly decreased 3 h and 24 h after injury, and rebounded to sham levels by 72 h after injury (Fig. 3A). Platelet-derived MP populations were not significantly changed after injury (data not shown); however, the proportion of total MPs that are PMPs were altered over time with a trend toward increased PMP contribution at 24 h after injury compared with 30 min and 72 h after injury (Fig. 3B). These findings demonstrate that MP populations are altered after injury, with decreased overall circulating MPs 24 h after injury and a significantly increased proportion of PMPs at this time point compared with immediately after injury and 3 d after injury.

3.4. MPs isolated from sham and TBI mice contribute to alterations seen in coagulation after head injury

MPs were isolated from sham and TBI mice at 24 h after injury, concentrations normalized, and MP suspensions added back to sham and TBI whole blood 24 h after injury. Saline was used as a control to ensure any changes in coagulation parameters were not due to dilution of whole blood samples. ROTEM analysis using EXTEM and FIBTEM tests were performed. When sham MPs or TBI MPs were added to sham whole blood, there were no changes in the extrinsic pathway compared with saline (EXTEM test, data not shown). However, when platelet function was blocked on the FIBTEM test, there was a significant increase in FIBTEM_{MCF} when TBI MPs were added to sham whole blood compared with saline and no change seen with addition of sham MPs to sham blood (Fig. 4A). This resulted in a significantly lower %MCF-Platelet (Fig. 4B). There were no changes in CT, CFT, LI30, or AA on EXTEM or FIBTEM tests (data not shown). Sham MPs and TBI MPs were then added to whole blood from head-injured mice 24 h after TBI. No changes were seen in any EXTEM test parameters (data not shown). When sham MPs were added to whole blood from mice 24 h after TBI, there was a significant decrease in FIB-TEM_{MCF} compared with saline and no change seen when TBI MPs were added (Fig. 4C). This resulted in a significant increase in %MCF-Platelet in the sham MP-treated group compared with saline (Fig. 4D). There were no changes in CT, CFT, LI30, or AA with the addition of either population of MPs compared with saline (data not shown). These data suggest that addition of sham MPs or TBI MPs to blood from TBI or sham mouse blood, respectively, results in alterations to the platelet contribution to clot formation consistent with that seen in the MP donor. In summary, TBI MPs caused a significant decrease in platelet contribution to clot formation in sham blood, similar to that seen in TBI mice, and sham MPs caused a significant increase in platelet contribution to clot formation in TBI blood, consistent with coagulation profile seen in sham mice.

3.5. Circulating MPs in TBI mice have increased procoagulant activity 24 h after injury

To further elucidate the mechanism for MP-induced changes to clot formation, we next performed a MP procoagulant assay. At 24 h after injury, MP procoagulant activity was significantly increased compared with that of sham MPs (Fig. 5). From these data, we conclude that MPs isolated from TBI mice have an intrinsically higher procoagulant activity than those isolated from sham mice.

4. Discussion

This study used a murine model to characterize the subacute hypercoagulable state after TBI and to determine whether MPs contribute to these changes. We demonstrated that in a murine model of TBI, there is no change in overall coagulation 24 h after injury (Fig. 1). Despite the lack of change in overall coagulation, we detected a significant decrease in platelet contribution to clot formation at 24 h after injury (Fig. 2). In addition to the altered platelet contribution 24 h after injury, there was an overall decline in circulating MP populations with a trend toward an increase in the proportion of PMPs (Fig. 3). When TBI MPs were added to sham blood, a similar decline in platelet contribution was seen, with a contrasting normalization of platelet contribution when sham MPs were added back to TBI blood 24 h after injury (Fig. 4). Furthermore, procoagulant MP activity was also increased in injured mice at this time (Fig. 5). All together, these data suggest that MPs are affecting the coagulation changes seen in these animals.

Traumatic injury, and more specifically TBI, is known to result in alterations in coagulation with an early coagulopathy and later development of a subacute hypercoagulable state. Viscoelastic coagulation testing, including thromboelastometry, is now more commonly used to characterize these postinjury states as its sensitivity for detecting the balance between thrombosis and LI30 [33]. Using this technology, human studies have shown that these tests are able to detect hypercoagulable states missed by common coagulation parameters such as plasma prothrombin time or activated partial thromboplastin time [34], and furthermore, that increased clot firmness on admission thromboelastography predicts increased rates of pulmonary embolism [35], highlighting the clinical importance of these tests. In addition, similar to our murine study, other human studies have shown that platelet contribution to clot strength steadily decreases over the 72 h after trauma, with a resulting increase in fibrinogen contribution [31]. Animal models have investigated altered platelet function after head injury [36]; however, our study is the first to demonstrate similar temporal changes in coagulation, specifically the platelet contribution to clot strength, after TBI using thromboelastometry in a murine model. With these new data, we are closer to both understanding the mechanisms behind coagulation changes after TBI and also to identifying appropriate therapeutic targets to prevent thromboembolic complications. As anticoagulation is often contraindicated in TBI patients immediately after injury, identifying specific mediators that can be targeted to prevent unwanted clot formation maybe key to minimizing life-threatening thromboembolic events.

Previous studies have investigated the time course of circulating MPs in blood [27,28] and cerebrospinal fluid [27] in human subjects after TBI, but our study is the first to describe these changes in a murine model. Interestingly in our model, circulating MP levels were significantly decreased at 24 h after injury compared with those of controls, whereas human studies suggest increased populations are present [25–28]. Similar to Nekludov *et al.* [28], we demonstrated a relative increase in PMPs and a similar decline in MPs soon after injury followed by an increase in MP levels at 72 h. In comparison with human studies where functional prothrombinase assays [25,27] and flow cytometry [26,28] were used for MP enumeration, MP populations in our study were determined using Nanoparticle Tracking Analysis. The different techniques used to identify MP populations may explain the

differences seen as prothrombinase assays do not determine overall MP populations, and flow cytometry can only detect MPs down to a size of 200 nm, whereas Nanoparticle Tracking Analysis can reliably detect particles down to 50 nm [37].

As MP release increases with endothelial injury, platelet activation, and increased inflammation [38], and they have been shown to be procoagulant [24], it is not unexpected that they play a role in coagulation changes after injury. Platelet-derived MPs have been shown to be more prothrombotic than platelets themselves [39], and lower circulating PMPs are seen in patients that suffer from hemorrhagic complications after trauma [40]. In parallel with these studies, we noted a relative increase in PMPs 24 h after TBI and an associated increase in clot firmness at this time. By adding sham MPs and TBI MPs to whole blood *ex vivo*, we were also able to demonstrate that MPs may be responsible for these changes on thromboelastometry. The MPs induced measureable changes in the FIBTEM_{MCF} that were not affected by platelet inhibition with cytochalasin D. Although we did not isolate PMPs specifically in these experiments, we also demonstrated that circulating MPs from mice 24 h after injury have significantly increased procoagulant activity. Combined with the associated increase in PMPs at this time point, we believe that the PMPs are likely the cause of these changes.

Although our study establishes an important connection between altered coagulation and the role of MPs in this process, there are limitations to the study. First, as our study was only performed in head-injured mice, it may not be applicable to all injury models. We also focused on the subacute hypercoagulable changes seen after injury and did not focus this investigation on the early coagulopathic changes immediately after injury. In addition, although we were able to demonstrate that MPs isolated from injured and sham mice altered coagulation profiles, we have yet to identify the definitive cell derivation of the MPs responsible for this change, which we speculate are PMPs. We also did not perform analyses to determine whether coagulation factors intrinsic to MPs were present in the MP preparation, including tissue factor, phosphatidylserine, and other mediators such as fibrinogen, and this will be an area of future research. Furthermore, we have yet to perform *in vivo* studies to verify altered coagulation when MPs are injected into injured and sham mice. Finally, we were unable to elucidate on a molecular level how MPs may alter platelet function during coagulation. When these limitations are addressed, we speculate that the knowledge gained will robustly advance our knowledge of the coagulation process after TBI.

5. Conclusions

Platelet contribution to clot formation is decreased 1 d after TBI despite unchanged platelet counts. Circulating MP populations are also altered at this time point with a significant increase in procoagulant MP activity. This suggests that MPs likely contribute to the altered platelet role in coagulation and to the development of a post-TBI hypercoagulable state.

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and M.D.G. did the approval of the final version. P.L.J., J.W.K., and C.C.C. did the critical revisions approval of the final version.

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Fig. 1.

Clot strength is unchanged after TBI. Whole blood NATEM_{MCF} analysis for sham ($n = 9$) and TBI blood 10 min ($n = 7$), 24 h ($n = 10$), and 72 h ($n = 11$) after injury. Data are expressed as the mean $\pm$ standard error of the mean. No statistically significant changes on one-way analysis of variance with Tukey *post hoc* test compared with sham.

Fig. 2.

Thromboelastometry measurements after TBI demonstrate decreased platelet contribution to clot formation. Whole blood from sham and TBI mice was collected 10 min, 24 h, and 72 h after injury and was analyzed using thromboelastometry. (A) EXTEM_{MCF}, (B) FIBTEM_{MCF}, (C) platelet contribution to clot formation, and (D) whole blood platelet counts. Samples sizes are 8–10 for each group. Data are expressed as the mean $\pm$ standard error of the mean. * $P < 0.05$ based on one-way analysis of variance with Tukey *post hoc* test compared with sham.

Fig. 3.

Circulating MP populations are altered after TBI. MPs were isolated from sham ($n = 26$) and TBI blood 30 min ($n = 13$), 3 h ($n = 9$), 24 h ($n = 21$), and 72 h ($n = 24$) after injury. (A) Total MP concentrations and (B) proportion of total MPs that are PMPs. Data are expressed as the mean $\pm$ standard error of the mean. * $P < 0.01$ based on one-way analysis of variance with Tukey *post hoc* test compared with sham.

Fig. 4.

Serum MPs isolated from sham and TBI mice alter coagulation. MPs were isolated from whole blood from sham and TBI mice 24 h after injury. MP concentrations were normalized to 3.0×10^8 MPs/mL in 30 μ L sterile saline were then added to whole blood from sham and TBI mice and thromboelastometry performed. (A) FIBTEM_{MCF} and (B) platelet contribution to clot formation after saline, sham MPs, and TBI MPs are added to sham whole blood. (C) FIBTEM_{MCF} and (D) platelet contribution to clot formation after saline, sham MPs, and TBI MPs are added to TBI whole blood. Data are expressed as the mean $\pm$ standard error of the mean. Samples sizes are 8–10 for each group. * $P < 0.01$ based on one-way analysis of variance with Tukey *post hoc* test compared with sham.

Fig. 5.

MP procoagulant activity is increased in TBI mice. Procoagulant activity of MPs isolated from sham ($n = 8$) and TBI ($n = 7$) blood was determined. Data are expressed as the mean $\pm$ standard error of the mean. * $P = 0.04$ based on Student t -test compared with sham.