Coagulopathy often develops soon after acute traumatic brain injury and its cause remains poorly understood. We have shown that injured brains release cellular microvesicles that disrupt the endothelial barrier and induce consumptive coagulopathy. Morphologically intact extracellular mitochondria accounted for 55.2% of these microvesicles, leading to the hypothesis that these extracellular mitochondria are metabolically active and serve as a source of oxidative stress that activates platelets and renders them procoagulant. In testing this hypothesis experimentally, we found that the extracellular mitochondria purified from brain trauma mice and those released from brains subjected to freeze-thaw injury remained metabolically active and produced reactive oxygen species. These extracellular mitochondria bound platelets through the phospholipid-CD36 interaction and induced α-granule secretion, microvesiculation, and procoagulant activity in an oxidant-dependent manner, but failed to induce aggregation. These results define an extracellular mitochondria-induced and redox-dependent intermediate phenotype of platelets that contribute to the pathogenesis of traumatic brain injury-induced coagulopathy and inflammation.

Introduction

Traumatic brain injury (TBI) induces coagulopathy that promotes secondary bleeding and propagates cerebral injury,1 resulting in poor clinical outcomes of the patients.2-6 Laboratory findings suggest that coagulopathy results from a hypercoagulable state that rapidly develops into consumptive coagulopathy.7 This consumptive coagulopathy has been recapitulated in animal models of TBI with thrombotic and hemorrhagic manifestations in the pulmonary and cerebral microvasculature.8,9 Despite strong clinical and laboratory evidence of its presence and association with poor clinical outcomes, the pathogenesis of TBI-associated coagulopathy remains poorly understood.

We have recently shown in mouse models that cellular microvesicles released from traumatically injured brains disrupt endothelial cell junctions through a synergistic action with platelets.2 These brain-derived microvesicles are highly procoagulant due to the abundant expression of surface-exposed anionic phospholipids and tissue factor. The sudden and substantial release of brain-derived microvesicles into the circulation results in a consumptive coagulopathy that is characterized by progressive fibrinogen depletion from plasma and fibrin deposition in the vasculature.8
We also found that 55.2±12.6% of annexin-V-binding microvesicles in peripheral blood of TBI mice were morphologically intact extracellular mitochondria (exMT). These exMT promoted coagulation through the surface-exposed anionic phospholipid cardiolipin. The findings raise an important question: are these exMT metabolically active and, if so, do they affect platelet function through redox-dependent mechanisms? The question is raised because exMT can be viable, transferred between cells, and influence the function of target cells. The influence of mitochondria on target cells has mostly been reported as protective, likely due to increased energy supply. However, reactive oxygen species (ROS) are generated during ATP production in mitochondria and are known to activate platelets. Several studies have shown that platelets in patients with TBI are activated but aggregate poorly. This platelet phenotype has also been reproduced in rat and swine models of TBI, in which platelets are activated, accumulate on the pia mater, and contribute to thrombosis in the lesion boundary zone. The underlying mechanism for this unique TBI-associated platelet phenotype remains poorly understood. Here we discuss results from a study that was designed to investigate the effect of exMT on platelet activation and procoagulant activity through in vitro experiments and in mouse models.

Methods

Mouse models

C57BL/6 male mice (12-20 weeks and 22-25 g), obtained from the Jackson Laboratory (Bar Harbor, ME, USA) were subjected to fluid percussion injury and blood samples were collected to quantify plasma exMT (Online Supplementary Methods). This mouse protocol was approved by the Institutional Animal Care and Use Committee of the BloodWorks Research Institute.

Flow cytometry

Platelet activation

We used an LSR II flow cytometer (Beckon Dickinson, San Jose, CA, USA) to detect platelet activation through several measurements: CD62p expression, the binding of PAC-1 antibody (BD Biosciences), which recognizes the active conformation of the fibrinogen receptor αIIbβ3, annexin V binding, platelet-bound fibrinogen and coagulation factor V, and the formation of platelet-leukocyte complexes (Online Supplementary Methods).

Calcium influx

We used flow cytometry to measure the exMT-induced calcium influx in platelets labeled with 5 μM eFlour 514 Calcium Sensor Dye (Biosciences, San Diego, CA, USA) for 10 min at 37°C.

Reactive oxygen species production

ExMT were suspended in Ca2+-free HEPES buffered Tyrode solution (138 mM NaCl, 5.5 mM glucose, 10 mM HEPES, 12 mM NaHCO3, 2.9 mM KCl, 0.4 mM NaH2PO4, 0.4 mM MgCl2, and 0.1% BSA; pH 7.2) and incubated for 30 min at 37°C with 10 μM of DCFH-DA dye (ThermoFisher). As controls, labeled exMT were treated with either 50 μM N-acetylcysteine (NAC, Life Technologies, Grand Island, NY, USA), which is a precursor of the antioxidant glutathione, or 200 μM tert-butyl hydroperoxide (TBHP, Life Technologies), which increases intracellular ROS production. ExMT fixed with 5% paraformaldehyde were also examined as a control.

Image flow cytometry for the extracellular mitochondria-platelet interaction

As we previously described, platelet-rich plasma was incubated for 30 min at 37°C with exMT labeled with MitoTracker Green and an APC-conjugated CD41α antibody (BD Bioscience). The platelets were washed in phosphate-buffered saline and fixed in 1% paraformaldehyde. Images were acquired at 60X magnification to collect ≥20,000 cells from each sample using the Amnis ImageStream® X Mk II system (Amnis, Seattle, WA, USA). To distinguish exMT bound to the platelet surface from those internalized, platelets were digested with 1% trypsin for 10 min at 37°C after incubation with exMT. Mouse mitochondrial-specific DNA was then amplified from these exMT-treated and trypsinated platelets using a protocol modified from our previous study.

CD36-extracellular mitochondria interaction

For in vitro experiments, platelet-rich plasma was incubated for 30 min at 37°C with a monoclonal CD36 antibody (Abcam, ab17044, Cambridge, MA, USA) or isotype IgG, followed by incubation with MitoTracker Green-labeled exMT and a PE-CD41α antibody for 30 min. For in vivo experiments, blood samples from non-injured mice infused with MitoTracker Green-labeled exMT were analyzed for exMT-bound platelets (MitoTracker Green/CD41α), platelet counts (CD41α/platelet counts in 60 s), platelet CD62p expression, and platelet-leukocyte complexes (CD41a/CD45). The CD36 antibody used in this study recognizes both mouse and human CD36.

Statistical analysis

Categorical (frequency) variables were expressed as percentages and continuous variables were expressed as the mean ± standard error of mean. The quantitative data were analyzed using SigmaPlot V. 11.2 (SYSTAT Software Inc., San Jose, CA, USA) for paired t tests, one way or repeated measures analysis of variance (ANOVA) as specified in each analysis. A P value of ≤0.05 was considered statistically significant.

Results

Extracellular mitochondria bound platelets in vivo and in vitro

Structurally intact (MitoTracker Green+) (Figure 1A) and cardioliopin-exposed (Figure 1B) exMT were detected in the peripheral blood of mice with acute TBI. The exMT formed complexes with approximately 12.3 ± 5.8% of circulating platelets that remained detectable for 6 h after TBI (Figure 1C). Low levels of exMT-platelet complexes were also detected in sham-treated mice and likely resulted from surgery-induced injuries. When co-incubated for 30 min at 37°C in vitro, purified exMT from brains subjected to freeze-thaw injury also formed complexes with platelets (Figure 1D) in a dose-dependent manner (Figure 1E). Transmission electron microscopy showed that exMT interacted with the body (Figure 1F) and filopodia (Figure 1G) of platelets.

The Amnis® imaging flow cytometer detected exMT on the surface of platelets (Figure 2A, top panel), being endocytosed by platelets (Figure 2A, middle panel) and fused with platelet membranes (Figure 2A, bottom panel). The internalization of exMT by platelets was further validated by co-incubation of human platelets with mouse exMT for 30 min at 37°C followed by the amplification of mouse mitochondria-derived DNA from exMT-treated platelets that were trypsinated to remove surface-bound exMT.
Quantitatively, 13.7 ± 9.1% of platelets were exMT-bound after 30 min co-incubation (Figure 2C), which was consistent with the level of exMT-bound platelets found in TBI mice (Figure 1C). This exMT-platelet interaction was blocked by the phosphatidylserine-binding proteins annexin V and lactadherin (Figure 2C) and partially by a CD36 antibody (Figure 2D), which blocks the CD36-mediated binding of endothelial microvesicles to platelets, suggesting that exMT bind platelets through cardiolipin exposed on the mitochondria and CD36 expressed on platelets. Furthermore, ATP production was dose-dependently increased in the exMT-treated platelets at levels that were significantly higher than in platelets activated with ADP (Figure 2E). We were unable to determine whether the ATP increase was caused by platelet-bound exMT or by exMT-induced ATP production of platelets. Finally, when added to platelets that had been treated with exMT, FITC-conjugated annexin V bound 16.2 ± 6.3% of platelets (Figure 2F).

Extracellular mitochondria were metabolically viable and activated platelets

We found that exMT from TBI mice stimulated platelets to express CD62p and this activity was partially blocked by the antioxidant glutathione (GSH) (Figure 3A). One pitfall of this experiment was that exMT purified from plasma of TBI mice were derived from multiple cells. It was technically challenging to separate brain-derived exMT from those of other cells, without damaging the viability of exMT. To address this concern, we tested exMT from brains subjected to freeze-thaw injury in vitro. This metabolic viability was further validated by the lack of ROS production from paraformaldehyde-fixed exMT (Figure 3C). After 30 min of incubation with exMT, both mouse (Online Supplementary Figure S2) and human platelets (Figure 3D) expressed CD62p on their surfaces. The exMT-induced CD62p expression was reduced by the antioxidants GSH (20 μM), L-cysteine (0.5 mM), and NEM (2 mM) (Figure 3E). Testing the three thiol-modifying agents was necessary because GSH and L-cysteine reversibly interact with extracellular and intracellular oxidants, respectively, whereas NEM forms irreversible sulfur bonds with thiols. The exMT-treated platelets also increased calcium influx (Online Supplementary Figure S3), formed complexes with leukocytes (Figure 3F), and
released von Willebrand factor (Figure 3G). The exMT-induced CD62 expression and platelet-leukocyte aggregation were detected in 10-23% of platelets, which was consistent with the percentages of exMT-bound platelets found in TBI mice (Figure 1). We used platelets stimulated with collagen and thrombin as the control because this exMT-induced platelet phenotype resembled that of “coated platelets”. Together, these data suggested that exMT from TBI mice and those released from brains subjected to freeze-thaw injury in vitro were metabolically viable and activated platelets in an oxidant-dependent manner.

Using hopping probe ion conductance microscopy, we continuously monitored morphological changes of platelets adherent to fibrinogen in real-time (Figure 4A, top panel). After stimulation with exMT, adherent platelets underwent drastic membrane disintegration as exemplified in the middle panel of Figure 4A. This exMT-induced membrane disintegration was prevented by 20 μM GSH (Figure 4A, bottom row). Consistent with the exMT-induced platelet disruption, CD41a+ platelet-derived membrane microvesicles were detected in the supernatant of exMT-treated platelets (Figure 4B) and platelet counts were reduced after treatment with exMT (Figure 4C). The production of platelet microvesicles and the reduction of platelet counts were partially blocked by the anti-oxidant GSH.

In contrast to their induction of α-granule secretion, exMT at comparable doses failed to induce platelet aggregation (Figure 4D) and did not enhance the formation of platelet thrombosis on the collagen matrix under arterial shear stress (Online Supplementary Figure S4A-C). Furthermore, the exMT-treated platelets aggregated normally in response to collagen (Figure 4E) and ADP (Figure 4F), and were moderately primed for activation by sub-threshold concentrations of ADP and collagen (Online Supplementary Figure S4D). Neither the binding of PAC-1 antibody (Online Supplementary Figure S5), which recognizes the active conformation of integrin αIIbβ3, nor the surface density of the integrin αIIbβ3 (Figure 4G) was changed after exMT treatment, as compared to platelets stimulated with collagen.

**Extracellular mitochondria-treated platelets were procoagulant**

ExMT-treated platelets also expressed anionic phos-
phospholipids as indicated by annexin V binding (Figure 2F) and had elevated levels of fibrinogen (Figure 5A) and coagulation factor Va on their surfaces (Figure 5B), suggesting that exMT-treated platelets could promote coagulation.33,34 Consistent with this notion, exMT-treated platelets accelerated clot formation at a level comparable to that of collagen-activated platelets but lower than that of purified phosphatidylserine micelles (Figure 5C). When added to platelet-rich plasma, exMT-treated platelets significantly accelerated and enhanced thrombin-induced clot retraction (Figure 5D), which measures the integrin αIIbβ3-dependent retraction of platelet-bound fibrin fibers.35,36 The effect of exMT was comparable to that of collagen-stimulated platelets. This procoagulant activity was independent of tissue factor, which was not detected on the surface of exMT-treated platelets (Online Supplementary Figure S6).

**Anti-CD36 antibody blocked extracellular mitochondria-induced platelet activation**

To measure exMT-induced platelet activation in vivo, non-injured mice were infused with purified exMT. Thirty minutes after infusion, platelets became exMT-bound (Figure 6A), expressed CD62p (Figure 6B), and formed complexes with leukocytes (Figure 6C). Mice infused with exMT also developed thrombocytopenia (Figure 6D). MitoTracker-Green-labeled exMT were detected in approximately 50% of platelet-leukocyte aggregates (Figure 6E). These phenotypic changes of platelets were prevented by infusing mice with exMT, together with a CD36 antibody but not isotype IgG.

**Discussion**

We investigated whether morphologically intact but cardiolipin-exposed exMT are metabolically active and, if so, whether they activate platelets through ROS. We have made several novel observations that define an exMT-induced and redox-dependent intermediate phenotype of platelets.

First, despite the cardiolipin translocation,4 exMT found in TBI mice or released from brains subjected to freeze-thaw injury produced ATP and ROS (Figure 3B, C). Because metabolic activity of mitochondria requires a functional membrane, exMT that interact with platelets...
are likely those with an intact membrane. The metabolically active exMT formed complexes with platelets (Figures 1 and 2) which remained detectable in the circulation for at least 6 h after TBI (Figure 1C). Cardiolipin on exMT and CD36 on platelets mediated the exMT-platelet interaction because: (i) cardiolipin is the dominant anionic phospholipid expressed on exMT and (ii) the exMT-platelet interaction was blocked by the phospholipid binding proteins annexin V and lactadherin (Figure 2C) and by an antibody against CD36 (Figure 2D), which is a phospholipid receptor that is expressed on platelets and promotes phospholipid-mediated endocytosis.

Second, exMT induced platelets to secrete their α-granule proteins (Figures 3 and 5) but failed to induce them to aggregate (Figure 4D) or promote platelet thrombus formation on the collagen matrix under arterial shear stress (Online Supplementary Figure S4A-C). There are several possible explanations for this apparent discrepancy. (i) Platelet-bound exMT may have interfered with the fibrinogen coupling of platelets through steric hindrance. This is unlikely because exMT-bound platelets aggregated after stimulation with collagen or ADP at levels comparable to those of platelets that had not been treated with exMT (Figure 4E, F). (ii) ExMT only activated 10–25% of platelets, insufficient to induce platelet aggregation. A similar effect was observed with platelets simultaneously treated with two antibodies against the integrins αIIb and β3, suggesting that α-granule secretion alone is insufficient to induce platelet aggregation, but may prime platelets for activation by other agonists. (iii) Some or all exMT-stimulated platelets underwent drastic membrane disintegration to produce microvesicles (Figure 4A, B), thereby becoming unavailable for aggregation. The third possibility is supported by the reduction of platelet counts after exMT treatment (Figure 4C). This exMT-induced intermediate platelet phenotype resembles "coat-
Mitochondria-induced platelet procoagulant activity

Figure 5. Extracellular mitochondria induced procoagulant activity of platelets and enhanced clot retraction. (A-C) Platelets incubated with extracellular mitochondria (exMT) at a ratio of 1:1 or 1:5 for 30 min at 37°C expressed increased amounts of fibrinogen (A) (n=24, one-way analysis of variance, ANOVA) and coagulation factor Va (B) (n=24, one-way ANOVA), and had shortened clotting time (C) (n=24, one-way ANOVA). Platelets stimulated with 5 μg/mL of collagen or 50 nM of thrombin were again used as controls to mimic “coated platelets.” (D) Clot retraction in platelet-rich plasma was induced by 1 U/mL of thrombin in the presence of phosphate-buffered saline (PBS, left panel), exMT (1:1 ratio to platelets, middle panel), or 5 μg/mL of collagen (right panel). The graph in the right panel summarizes the results from multiple experiments (n=27, one-way ANOVA, *P<0.05 vs. PBS-treated). FVa: activated factor V; PS: phosphatidylserine.

Figure 6. CD36 antibody reduced extracellular mitochondria-induced platelet activation. Non-injured mice were infused with 2 x 10^8/mouse of MitoTracker-Green-labeled extracellular mitochondria (exMT), together with CD36 antibody or isotype IgG. (A-F) Blood samples were collected 30 min after infusion to measure levels of exMT-bound platelets (A), CD62p+ platelets (B), platelet-leukocyte complexes (C), platelet counts (D), and platelet-leukocyte-exMT tri-complexes (E) by flow cytometry (n=16, one-way analysis of variance). (F) Schematic summary of the study. An injured brain releases metabolically active exMT that interact with platelets. These exMT induce α-granule secretion by platelets, but do not induce platelet aggregation. As a result, the exMT-bound platelets and platelet-derived microvesicles promote the systemic consumptive coagulopathy and inflammation that are consistently found during the acute phase of traumatic brain injury. WBC: white blood cell; ROS: reactive oxygen species; VWF: von Willebrand factor; FVa: activated factor V; MV: microvesicles.
ed platelets", which are defined as a subpopulation (12-30%) of platelets that express procoagulant activity upon stimulation by collagen and thrombin. This procoagulant activity was also detected in exMT-treated platelets (Figure 5).

Third, the metabolic viability of exMT also suggests a potential role of ROS in the development of this intermediate phenotype because ROS are known to activate platelets. We have shown that ROS from exMT activate platelets (Figure 3) and induced platelet microvesiculation (Figure 4) in an oxidant-dependent manner. A physical contact between exMT and platelets appears to be required for the ROS-induced platelet secretion because ROS primarily affect exMT-bound platelets. This direct exMT-platelet interaction may concentrate ROS activity on exMT-bound cells or protect the oxidants from plasma antioxidants.

In summary, we have shown that exMT-bound platelets develop an intermediate phenotype using ROS as mediators. This intermediate phenotype is characterized by granule secretion, procoagulant activity and poor aggregation (Figure 6F). As a result, these procoagulant platelets remain in the circulation to promote non-focal coagulation, similar to the consumptive coagulopathy found in TBI patients and mouse models. While previous studies have found protective effects of exMT on target cells, our study demonstrates that these exMT could also have detrimental effects by making platelets procoagulant and prothrombotic. The findings from this study could have much broader implications regarding exMT in other pathologies in which hypercoagulable and inflammatory states develop, such as severe infections, autoimmune diseases, and cancer.

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