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Full Length Article

Platelet hyperactivation, apoptosis and hypercoagulability in patients with acute pulmonary embolism☆

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ABSTRACT

Changes in systemic redox balance can alter platelet activation and aggregation. Acute pulmonary embolism (PE) is a systematic inflammatory disease associated with mechanical shear stress, increased thrombin, catecholamines, serotonin and hemolysis, which cumulatively can hyperactivate platelets and accelerate their turnover. We tested the hypothesis that platelets from patients with moderately severe PE will show hyperstimulation and a pre-apoptotic phenotype associated with microparticles (MPs) in plasma. Blood for platelet respiration and thromboelastography (TEG) was obtained at diagnosis and 24 h later from patients ($n = 76$) with image-proven PE, SBP > 90 mm Hg and right ventricular dysfunction demonstrated by echocardiogram or elevated biomarkers. Controls ($n = 12$) were healthy volunteers. At diagnosis, platelets from PE patients had significantly elevated baseline oxygen consumption compared with controls, explained primarily by accelerated electron transport and oxygen wasting with no measurable extramitochondrial oxygen consumption. On thromboelastography, unstimulated thrombin-independent maximum amplitude was increased with PE, 19 ± 14.1 vs.10.5 ± 7.8 mm in controls ($p = 0.002$). Compared with controls, platelets from PE patients showed elevated mitochondrial reactive oxygen species with decreased mitochondrial Bcl-2 protein content and increased cytosolic cytochrome C, coincident with strong annexin V binding, P selectin release from lysed platelets and in plasma MPs compared to controls ($p < 0.05$).
These results show evidence of platelet hyperactivation and apoptosis in patients with acute PE, and provide preliminary theoretical basis for further exploration of platelet inhibition in patients with more severe PE.

1. Introduction

Acute pulmonary embolism (PE) causes death and disability secondary to right ventricular damage and failure [1]. More severe pulmonary emboli are also associated with a systemic inflammatory and hypercoagulable state that may worsen outcomes [2–5]. While much of the inflammatory and coagulation axis amplification occurs secondary to preexisting thrombophilic conditions, significant evidence implicates PE itself as a cause and perpetuator of increased inflammation, elevated thrombin generating potential and platelet activation in acute and chronic PE [6–9], representing a vicious cycle where inflammation precipitates pulmonary emboli which in turn worsen inflammation and coagulation. Platelet hyperactivity has also been observed in patients with the metabolic syndrome and ischemic stroke [10,11]. Consequences of hyperinflammation and hypercoagulability range from an increased risk of venous thromboembolism (VTE) recurrence to acute disseminated intravascular coagulation associated with right ventricular failure and risk of cardiovascular collapse [12–14].

PE causes numerous stimuli that predispose to platelet activation, including increased thrombin [6,15], platelet activating factor [16], shear forces, and hemolysis with resultant ADP release [17–21]. In addition, free hemoglobin mediated platelet activation [22], increase in circulating catecholamines, hypoxemia, and reduced L-arginine needed for nitric oxide synthesis [5,21] all perpetuate and enhance platelet reactivity. In clinical populations, it has been observed that patients with

Abbreviations: PE, pulmonary embolism; TEG, thromboelastography; PS, phosphatidyl serine; VTE, venous thromboembolism; ROS, reactive oxygen; MP, microparticle; MA, maximum amplitude; AA, arachidonic acid; ADP, adenosine diphosphate; ETS, electron transport system.

☆ Subject terms: Platelets.TOC category: Translational.TOC sub category: Thrombosis.

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PE demonstrated increased mean platelet volume in proportion to PE severity, which probably represents an increase in young (reticulated) platelets, suggesting increased turnover [22–24].

Hyperactivated platelets manifest several apoptotic-like morphological changes, including phosphatidyl serine (PS) exposure, cell shrinkage, and microparticles (MPs) formation. Apoptotic platelets shed phosphatidyl serine-bearing MPs, which further increase plasma thrombin generating potential [25–28]. Mitochondrial membrane depolarization from multiple agonist stimulation appears to be integral to the outward transport of PS and MP shedding in human platelets [29–32]. Increased platelet mitochondrial oxygen consumption provides metabolic evidence of global cellular hyperstimulation and serves as an early biomarker of apoptosis, reactive oxygen (ROS) production and MP shedding [33–41]. Altered platelet mitochondrial respiration has been observed in a numerous acute and chronic disease states [42–46], though its presence and relevance to venous thromboembolism has not been described.

This work tests the hypothesis that platelets from patients with acute intermediate risk PE receiving heparin treatment exhibit hyperstimulated platelets that generate excessive reactive oxygen species and lead to a pre-apoptotic state in association with hypercoagulability.

2. Materials and methods

2.1. Study population

This study was approved by the Institutional Review Boards at Indiana University School of Medicine and University of Mississippi Medical Center. All patients gave written informed consent to have their platelets used in this study. Data were collected as part of a preplanned ancillary study to a registered randomized clinical trial NCT01939301.

Patients were recruited from one of three hospitals and emergency departments at Indiana University Medical Center in Indianapolis, IN, and the emergency department and hospital at the University of Mississippi, Medical Center in Jackson, MS. Patients had image-proven PE, SBP > 90 mm Hg and right ventricular dysfunction as demonstrated by echocardiogram (hypokinesis, dilation [>42 mm] or tricuspid annular plane systolic excursion <16 mm) or elevated biomarkers (troponin elevated by local standards, or brain natriuretic peptide >100 pg/mL) or RV/LV ratio > 1.0 on CT pulmonary angiography. Patients treated with fibrinolysis or those who took aspirin or thienopyridines within the past 24 h were excluded. All patients were being treated with either unfractionated or low molecular weight heparin at the time of enrollment.

2.2. Blood collection and platelet preparation

Blood was drawn by qualified phlebotomists using 18 gauge needles. Samples were obtained within 24 h of PE diagnosis at diagnosis and 24 h later (±2 h) into two (blue-top) tubes containing sodium citrate and three K_2EDTA. Blood samples were transported at room temperature (~23 °C) and analyzed within 1 h. Twenty healthy blood donors served as controls after providing written informed consent. The protocol for their selection and blood collection is shown in the data supplement.

2.3. Platelet mapping assay for the thromboelastography (TEG)

All chemicals and kits for the TEG experiments were purchased from Haemonetics Corporation (Braintree, MA). Blood protein and platelet contribution to coagulation was simultaneously assessed by measuring the time to coagulation (R time, protein only) angle (α, protein + platelet) and maximum amplitude (MA, platelet) on TEG (4-channel Haemoscope 5000) with heparin to inhibit native thrombin generation and coagulation initiation with reptilase, with heparinase to assess platelet function in the presence of native thrombin generation. In separate vials, TEG was performed on blood containing heparin + ADP (2 μM) and heparin + arachidonic acid (AA; 1 mM) to assess agonist responsiveness of platelets using two different stimulation pathways. These agonists were purchased from Haemonetics and are used in the standard "platelet mapping" assays in clinical practice.

2.3.1. High-resolution respirometry

All chemicals for platelet mitochondrial experiments were purchased from Sigma-Aldrich (St Louis, MO). The EDTA tubes were centrifuged 15 min at 300 ×*g* in room temperature, to yield a platelet-rich plasma (PRP). This PRP was pipetted off and centrifuged for 5 min at 4600 ×*g*, at room temperature, producing platelet poor plasma and a platelet pellet. The pellet was dissolved in 1 mL of the subject's own plasma by gentle pipetting to obtain a highly enriched PRP. Absence of nucleated cells was confirmed by fluorescent microscopic analysis of smears from the PRP stained with Hoechst dye 33,342 (2′-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5′-bi-1H-benzimidazole trihydrochloride trihydrate) excited with ultraviolet light and with detection at 480 nm. Platelets (200×10^6/mL) were suspended in mitochondrial respiration medium-MiR05 [47] containing glucose (5.5 mM) + octanoate (0.05 mM) and respiration was measured at a constant temperature of 37 °C in a high-resolution oxygraph (Oxygraph-2k Oroboros Instruments, Innsbruck, Austria) [47,48]. Data were recorded with DatLab software 4.3. (Oroboros Instruments, Innsbruck, Austria) with sampling rate set to 2 s. Intact platelet oxygen consumption was measured at baseline state, then in the presence of 2 μL ATP synthase inhibitor oligomycin (4 μg/mL) to evaluate the contribution of respiration independent of ADP phosphorylation ("Leak" state). Next, oxygen consumption was measured in the presence of the

Table 1
Clinical characteristics of 76 patients with PE.

Clinical feature	Mean or N	SD or %
Objective measurements and demographics		
Mean age (years)	57.3	14.5
Respiratory rate (breaths/min)	23.3	19.1
SpO2% (room air)	96.6	5.3
Systolic BP (mm Hg)	126.0	21.4
Diastolic BP (mm Hg)	76.6	13.9
Heart rate (beats/min)	87.5	13.1
MtHb%	0.9	0.6
CoHb%	3.3	2.4
Height (cm)	169.2	16.9
Weight (kg)	100.8	32.6
Platelets (10^3/μL)	212.2	70.5
MPV (fl)	8.8	1.2
Hemoglobin (g/dL)	12.3	2.5
Caucasian race	47	62%
Female gender	40	53%
Associated medical conditions		
Aspirin at home	20	26%
Bronchodilator use in the past 4 h	10	13%
Prior lung disease	26	34%
Surgery in the past 4 weeks	11	15%
Trauma in the past 4 weeks	7	10%
Coronary artery disease	9	12%
Coronary artery disease	3	4%
Prior myocardial infarction	7	10%
Heart failure	3	4%
Malignancy under treatment	11	15%
Solid tumor	4	5%
Hematologic	4	5%
Metastatic	3	4%
Connective tissue disease	14	18%
Lupus	3	4%
Scleroderma	0	0%
Rheumatoid arthritis	5	7%
Other	5	7%
Smoking	35	46%