Received: 19 March 2019 | Accepted: 19 August 2019
DOI: 10.1111/jth.14617

ORIGINAL ARTICLE

jth

Phagocytosis by endothelial cells inhibits procoagulant activity of platelets of essential thrombocythemia *in vitro*

Shuting Ji[1] | Weijun Dong[2] | Yushan Qi[1] | Hong Gao[3] | Danwei Zhao[4] | Minghui Xu[1] | Tingting Li[1] | Hongyin Yu[1] | Yuting Sun[1] | Ruishuang Ma[5] | Jialan Shi[6,7] | Chunyan Gao[1]

[1]Department of Medical Laboratory Science and Technology, Harbin Medical University-Daqing, Daqing, China
[2]Department of General Surgery, The Fifth Hospital, Harbin Medical University, Daqing, China
[3]Department of Hygienic Microbiology, Public Health College, Harbin Medical University, Harbin, China
[4]Department of Endocrinology, Beijing United Family Hospital, Beijing, China
[5]The Key Laboratory of Myocardial Ischemia, Ministry of Education, Harbin Medical University, Harbin, China
[6]Department of Hematology, The First Hospital, Harbin Medical University, Harbin, China
[7]Departments of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts

Correspondence
Chunyan Gao, Department of Medical Laboratory Science and Technology, Harbin Medical University-Daqing, Daqing 163000, China.
Email: gaochunyan1234@163.com

Jialan Shi, Department of Hematology, The First Hospital, Harbin Medical University, Harbin 150001, China and Departments of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA.
Email: jialan_shi@hms.harvard.edu

Funding information
National Natural Science Foundation of China , Grant/Award Number: 81400097; Natural Science Foundation of Heilongjiang Province , Grant/Award Number: H2015109; China Postdoctoral Science Foundation , Grant/Award Number: 2014M550202 and 2015T80373; Heilongjiang Postdoctoral Science Foundation , Grant/Award Number: LBH-Z13153; Seed Fund of Harbin Medical University-Daqing , Grant/Award Number: DQ2013-05; Yu Weihan Foundation of Harbin Medical University, Grant/Award Number: 31021180167 and DQYWH201801; The Scientific Research Fund of Harbin Medical University, Grant/Award Number: 2018XN-09 and 2018XN-16

Abstract

Background: Essential thrombocythemia (ET) is characterized by thrombocytosis with increased platelet number and persistent activation. The mechanisms of thrombosis and the fate of these platelets are not clear. The aim of the present study is to explore the phagocytosis of platelets of ET patients by endothelial cells (ECs) *in vitro* and its relevance to the procoagulant activity (PCA).

Methods: Phosphatidylserine (PS) exposure on platelets was detected by flow cytometry. Phagocytosis of the platelets by ECs was performed using flow cytometry, confocal microscopy, and electron microscopy. The PCA of platelets was evaluated by coagulation time and purified coagulation complex assays.

Results: The PS exposure on platelets in ET patients is higher than that in healthy controls. The PS-exposed platelets are highly procoagulant and lactadherin reduced 80% of the PCA by blockade of PS. When cocultured, the platelets of ET patients were sequestered by ECs in a time-dependent fashion. Lactadherin enhanced phagocytosis by bridging the PS on activated platelets and the integrin $\alpha v\beta 3$ on ECs, and P-selectin played at least a partial role in this process. Furthermore, factor Xa and prothrombinase activity of PS-exposed platelets were decreased after incubation with ECs.

Conclusion: Our results suggest that phagocytic clearance of platelets by ECs occurs in ET patients, thus representing a novel mechanism to remove activated platelets

Manuscript handled by: Yukio Ozaki
Final decision: Yukio Ozaki, 19 August 2019

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.
© 2019 The Authors. *Journal of Thrombosis and Haemostasis* published by Wiley Periodicals, Inc. on behalf of International Society on Thrombosis and Haemostasis

from the circulation; lactadherin and phagocytosis could cooperatively limit the thrombophilia in ET patients.

KEYWORDS
endothelial cell, essential thrombocythemia, phagocytosis, platelet, procoagulant activity

1 | INTRODUCTION

Essential thrombocythemia (ET) is a myeloproliferative neoplasm characterized by thrombocytosis with bone marrow megakaryocytic hyperplasia and persistent platelet activation and accumulation, in which arterial and venous thrombosis represent the main causes of morbidity and mortality.[1-4] Activated platelets expose anionic phospholipids, which provide a template for the organization of the coagulation cascade.[5,6] Their active removal by phagocytes might represent a protective mechanism against thrombosis. However, the mechanisms of thrombosis and the removal process of platelets in ET remain to be investigated.

It has been reported that platelets could be cleared by leukocytes in polycythemia vera and ET.[7] However, professional macrophages may become overwhelmed by the excessive amounts of platelets in ET. Endothelial cells (ECs) are amateur phagocytes that exist in large amount on the walls of blood vessels and have potential phagocytotic ability. Our previous studies have demonstrated that ECs could clear apoptotic neutrophils, acute promyelocytic leukemia cells, and platelets of sepsis by lactadherin-mediated engulfment.[8-10] So we speculate that ECs and lactadherin cooperatively might contribute to the clearance of excessive platelets and help maintain a normal and stable coagulation state in ET patients.

Phosphatidylserine (PS), an anionic phospholipid, is usually confined to the inner leaflet of the cell membrane.[11,12] It is externalized to the outer membrane when apoptosis occurs or cells are activated. Exposed PS on platelets provides a catalytic surface for the assembly of factor tenase and prothrombinase complexes and promotes thrombin formation at the same time the activated protein C system together with protein S has the ability to down-regulate the procoagulant phenotype of platelets, thereby creating a balance between coagulation and anticoagulation in a physiological state.[13] Phosphatidylserine is also a critical “eat me” signal on activated or apoptotic cells for phagocytes.[14-16] It is well known that the removal of apoptotic cells by phagocytes could be greatly enhanced by lactadherin through its opsonin activity.[17,18] Lactadherin, also known as milk fat globule–epidermal growth factor 8, is a kind of glycoprotein secreted by macrophages and ECs. It includes two epithelial growth factor–like domains and two lectin-type C domains that are homologous with the PS-binding domains of blood clotting factor VIII. In contrast to annexin V, membrane binding of lactadherin appears proportional to PS content and is independent of Ca^{2+} concentration and membrane phosphatidylethanolamine (PE) content.[19,20] It anchors apoptotic cells to macrophage alphaV integrins via its RGD (Arg-Gly-Asp) motif in the EGF domain.[21] Considering these properties, we investigated the cooperative effect of lactadherin and phagocytosis on the platelets of ET patients *in vitro*.

The present study explored whether and, if so, how EC engulfs platelets of ET patients and affects hemostatic balance *in vitro*.

Essentials

- Essential thrombocythemia (ET) is characterized by increased platelet number and thrombocytosis.
- Increased phosphatidylserine (PS) exposure on platelets contributes to the thrombocytosis of ET.
- Platelets of ET could be phagocytosed by endothelial cells (ECs) through the PS-lactadherin-$\alpha v\beta 3$.
- Lactadherin-mediated clearance of platelets by EC limits the thrombophilia in ET patients.

2 | MATERIALS AND METHODS

2.1 | ET patients

This study was approved by the Ethics Committee of Harbin Medical University and conducted in accordance with the Helsinki Declaration. Twenty healthy controls (10 males and 10 females) and 20 ET patients (11 males and 9 females) diagnosed according to the 2008 WHO criteria were consecutively enrolled between July 2013 and October 2014 after informed consent. The patient and control characteristics are shown in Table 1. None of the healthy controls had taken any drugs affecting the coagulation system in the last 10 days prior to blood drawing and none of them had a history of thrombosis. Among the ET patients, 10 were receiving treatment with hydroxyurea, 5 with aspirin, and the remaining 5 ET patients were not receiving any treatment. Five ET patients had a positive history for arterial (n = 2) or venous (n = 3) thrombotic episodes. Blood samples were obtained when patients were admitted. No diabetes, malignant tumors, iron deficiency, or active or chronic infection was presented in these patients.

2.2 | Reagents and monoclonal antibodies

Umbilical vein endothelial cells and EC medium were from ScienCell Research Laboratories (San Diego, California). Trypsin-EDTA was from Gibco (Grand Island, US). Monoclonal antibodies against CD142 (tissue factor, clone HTF-1), CD144-PE (clone 55-7H1), CD142-PE

TABLE 1 Characteristics of patients and controls

	Controls	ET
Sex (male/female)	10/10	11/9
Age (years)	52.9 ± 11.8	53.6 ± 14.3
JAK2 mutation (positive/ wild-type)	—	11/9
RBC count (10^{12}/L)	4.4 ± 0.4	4.6 ± 0.5
Platelet count (10^9/L)	200.8 ± 53.2	690.9 ± 43.9*
MPV (fl)	8.86 ± 0.5	8.37 ± 0.2*
WBC count (10^9/L)	6.1 ± 1.3	6.8 ± 1.7
Hydroxyurea treatment, *n* (%)	—	10 (50%)
Aspirin treatment, *n* (%)	—	5 (25%)
History of thrombosis, *n* (%)	—	5 (25%)

Note: Data are presented as mean ± SD or number (%).
Abbreviations: ET, essential thrombocythemia; MPV, mean platelet volume; RBC, red blood cell; SD, standard deviation; WBC, white blood cell.
*P value < .05.

(clone HTF-1), CD62P (P-selectin, clone AK4), and CD62P-FITC were from BD Biosciences Pharmingen (Torreyana Rd. San Diego, California, USA). Anti-Integrin alpha V beta 3 antibody was from Abcam (Cambridge, MA). Annexin V was obtained from Sigma-Aldrich (St. Louis, MO). Recombinant human MFG-E8 protein was obtained from R&D Systems (Minneapolis, MN). Alexa fluor 488-labeled lactadherin antibodies were prepared in our laboratory. CellTracker Green CMFDA and CellTracker Red CMTPX were from Molecular Probes (Invitrogen). Anti-EEA1 antibody and anti-Rab9 antibody were from Boster (Boster Biological Technology), CD41 monoclonal antibody was from Abbkine (Wuhan, Hubei, China). Alexa fluor 488-labeled goat antirabbit immunoglobulin, Cy3-labeled goat antimouse immunoglobulin, and diamidino-phenylindole were from Beyotime (Beyotime, Jiangsu, China). Gold-conjugated streptavidin was from Sigma-Aldrich. Human factor X (FX), FXa, and FIXa were obtained from Enzyme Research Laboratories (South Bend, US); human prothrombin, thrombin, FVa, and FVIIa were purchased from Haematologic Technologies Inc (Essex Junction, VT). Recombinant human factor VIII was from American Diagnostica Inc (Stamford, CT). Chromogenic substrates S-2765 and S-2238 were from Instrumentation Laboratory Company (Bedford, MA). Tyrode's buffer containing 1 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid was from our laboratory and was filtered through a 0.22-µm syringe filter from Millipore (Chaoyang District. Beijing, China).

2.3 | Platelet preparation

Blood samples were drawn from healthy subjects and ET patients with a 21-gauge needle and were collected into a 5-mL evacuated blood collection tube containing 3.2% citrate (Becton Dickinson, San Jose, CA). Platelet-rich plasmawas prepared within 30 min of blood collection by centrifugation for 13 min at 200 ×*g* at room temperature and was analyzed immediately after isolation. For the coincubation assays, the platelets were pelleted by centrifugation at 900 *g* for 17 min and washed with Tyrode's buffer at room temperature. The concentration of the platelets was adjusted to 1 × 10^8/mL in the same buffer.

2.4 | Coincubation experiments

For the phagocytosis assay, ECs were cultured in endothelial cell growth medium -2 under standard cell culture conditions (humidified atmosphere, 5% CO_2, 37 °C) in six-well culture plates. On reaching 60% to 70% confluence, the ECs were overlaid and cocultured with platelets (at an EC : platelet ratio of 1:40). In some cases, platelets were pretreated with lactadherin (2 nmol/L), anti-P selectin (20 µg/mL), or annexin V (2 nmol/L) and ECs were pretreated with anti-αvβ3 (5 µg/mL) at room temperature for 15 min. After washing and removing of free proteins containing supernatant solution, the ECs and platelets were coincubated. At various time points (0, 30, 60, 90, and 120 min), the mixture was collected from each well and the ECs were detached by 800 µL of 0.25% trypsin-EDTA solution for 2 min at 37 °C. After vigorous washing of the EC/platelet cocultures to remove non-adherent/non-phagocytosed platelets, the ECs were harvested for further analysis by flow cytometry and confocal microscopy.

2.5 | Flow cytometry

The PS exposure, P-selectin, and TF expression on platelets of normal subjects and ET patients were measured by flow cytometry (BD Aria, Becton Dickinson, San Jose, CA) based on the binding of lactadherin-Alexa fluor 488, CD62P-FITC, and CD142-PE separately. Platelets were adjusted to 1 × 10^6/mL to a final volume of 200 µL in Tyrode's buffer, and then 5 µL Alexa fluor 488-conjugated lactadherin, CD62P-FITC, and CD142-PE were added to the cell suspension separately and incubated for 10 min at room temperature in the dark. For the phagocytosis assay, the amount of phagocytosis was evaluated by measuring the percentage of platelets containing ECs. As described in "Coincubation experiments" above, platelets of healthy subjects or ET patients were pretreated with lactadherin, anti-P selectin, or annexin V and then were labeled with 1 µmol/L CMFDA at room temperature for 30 min. Then each group of platelets was incubated with ECs (pretreated with or without anti-αvβ3) for 120 min. At determined time points, the mixed cells were washed, detached, and any surface-bound platelets were removed. Then the harvested ECs were collected and labeled with CD31-PE for further analysis by flow cytometry. Ten thousand events per sample were acquired and analyzed with Flowjo Software.

2.6 | Electron microscopy

In scanning electron microscope assays, cocultured ECs and platelets were collected and fixed by immersion in 2.5% glutaraldehyde-phosphate fixative and stored at 4 °C until processed. After several rinses in 0.1 mol/L Na-cacodylate HCl buffer, cocultivations were postfixed

in 1% OsO4 and dehydrated in a graded series of ethanol (30%, 50%, 70%, 90%, and 100%; twice in 5 min). After critical drying, a layer of platinum, ˜10 nm thick, was sprayed on the samples. All images were viewed with an S-3400N Scanning Electron Microscope (Hitachi Ltd, Tokyo, Japan) with an ultra-high-resolution mode.

In transmission electron microscope experiments, the mixed cells were collected and double fixed in 2.5% glutaraldehyde and 1% OsO4. After dehydration and embedding, ultrathin sections were prepared with Reichert Jung Ultracut Ultramicrotome (Leica Microsystems Inc. Buffalo Grove, IL). Images were observed under a H7650 transmission electron microscope (Hitachi Ltd.).

In immunoelectron microscope experiments, cocultured ECs and platelets were collected and fixed using 4% paraformaldehyde (pH 7.2) at 4 °C. After dehydration and embedding, ultrathin sections were prepared with Reichert Jung Ultracut Ultramicrotome (Leica). After dehydration and embedding, specimens were blocked in 3% bovine serum albumin-phosphate-buffered saline for 30 min, and then incubated in primary antibody (CD41) for 1 h. The sections were incubated with 10 nm gold-conjugated streptavidin. Images were observed under a Hitachi H7650 transmission electron microscope.

2.7 | Confocal microscopy

Engulfment was detected as previously described.[8] Briefly, the ECs labeled with CMTPX were overlaid and cocultured with CMFDA-labeled platelets in the presence of 2 nmol/L lactadherin for 90 min. At different time points, the samples were observed and imaged by LSM 510 confocal laser scanning microscope (Zeiss). For immunofluorescent analysis of the intracellular trafficking of the platelets, coincubated platelets and ECs at determined time points were stained with markers that are commonly used to study phagocytosis [endosome antigen-1 (EEA-1) for early-stage endocytosis, Rab-9 for late-stage endocytosis].[22,23] Cells grown on coverslips were washed and fixed with prepared 4% paraformaldehyde in phosphate-buffered saline for 15 min, and then were permeabilized with 0.1% Triton X-100 in PBS for 30 min. After three washes, they were blocked in the 1% bovine serum albumin-phosphate-buffered saline for 1 h, incubated in primary antibodies (CD41, anti-EEA1, and anti-Rab9, respectively) for 12 h at 4 °C, and then secondary IgG 1 h at room temperature. Antibody dilutions were as follows: EEA1, 1:50; Rab9, 1:50; CD41, 1:50; goat antimouse or antirabbit Alexa 488 or Cy3, 1:500. 4′6-Diamidino-2-phenylindole was used at 1:3000. Confocal imaging was carried out as earlier.

2.8 | Factor Xa and prothrombinase assays

The formations of FXa and prothrombinase in each group (4×10^4 platelets, 10^3 ECs or coincubated cells) at various time points were performed with a two-step amidolytic substrate assay using our previous protocols.[24] For the intrinsic FXa formation assay, each group was incubated with 5 nmol/L FVIII, 130 nmol/L FX, 1 nmol/L FIXa, 0.2 nmol/L thrombin, and 5 mmol/L Ca^{2+} in FXa buffer (1 mL 10 × Tris-buffered saline, 200 μL 10% bovine serum albumin, 8.8 mL double distilled H_2O) for 5 min at 25 °C. The activation of extrinsic FXa was performed with the addition of 130 nmol/L FX, 1 nmol/L FVIIa, and 5 mmol/L Ca^{2+} to the samples for 5 min at 25 °C. The reaction was then stopped by the addition of EDTA to the 7-mmol/L final concentration. After the addition of 10 μL S-2765 (0.8 mmol/L) to each reaction, the quantity of FXa formed was determined immediately at 405 nm in kinetic mode on a Universal Microplate Spectrophotometer (PowerWave XS; Bio-Tek, Winooski, VT). Results were evaluated against the rate of substrate cleavage of a standard dilution of FXa. To measure the production of prothrombinase, the samples were incubated with 1 nmol/L FVa, 0.05 nmol/L FXa, 1 μmol/L prothrombin, and 5 mmol/L Ca^{2+} in prothrombinase buffer (1 mL 10 × Tris-buffered solution, 50 μL 10% bovine sserum albumin, 8.95 mL digital droplet H_2O) for 5 min at 25 °C. The production of thrombin was measured using chromogenic substrate S-2238 after the addition of EDTA. Inhibition of coagulation complexes by lactadherin or anti-TF was measured as follows. Cells were incubated with varying concentrations of lactadherin (0-128 nmol/L) or anti-tissue factor (TF) (0-51.2 μg/mL) for 10 min at 25 °C in Tyrode's buffer. The mixture was then incubated with the specified clotting factors according to the preceding protocols. The quantity of thrombin or FXa formation was assessed as previously described.[24]

2.9 | Coagulation time

Procoagulant activity (PCA) was determined by a one-stage recalcification time assay as described previously.[24] All the platelet-poor plasma that was used in the coagulation time study was from healthy volunteers; 100 μL of platelet (1×10^7) from healthy subjects and ET patients was incubated with ECs in platelet-poor plasma for 5 min at 37 °C separately. After 180 s, 100 μL of warmed 25 mmol/L $CaCl_2$ was added to start the reaction and the clotting time was immediately measured by a KC4A-coagulometer (Amelung, Labcon, Heppenheim, Germany). All clotting assays were performed in triplicate. For the inhibition assay of coagulation time, 50 μL lactadherin (final concentration 128 nmol/L) or anti-TF (final concentration 25.6 μg/mL) was incubated with 100 μL platelet suspension for 10 min at 37 °C. Clotting time was then recorded as previously after addition of 100 μL platelet-poor plasma and 50 μL of warmed 50 mmol/L $CaCl_2$.

To determine the procoagulant activity of cocultured platelets of ET patients and ECs, platelets (1×10^7) were cocultured with ECs (2.5×10^5) in 12-well culture plates for 90 min. The mixed cells were then harvested and resuspended in 100 μL Tyrode's buffer. Platelets of healthy subjects that were cocultured with ECs were used as control. Clotting time was determined as described earlier.

2.10 | Statistical analysis

Numerical variables were tested for normal distribution with the Kolmogorov-Smirnov test. Data are expressed as mean ± standard deviation (SD), and statistical analysis was made by unpaired

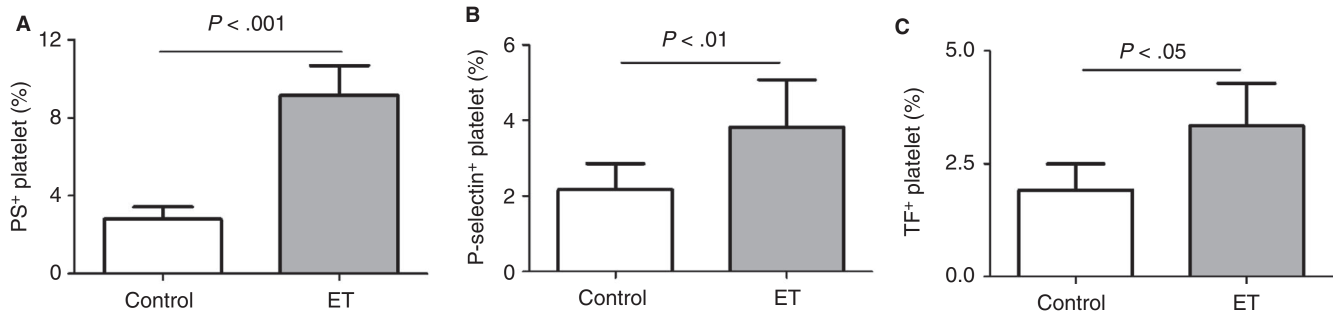


FIGURE 1 Activation of platelets in ET. Flow cytometry analysis of A, PS exposure; B, p-selectin; and C, TF expression on platelets from healthy subjects (n = 10) and ET patients (n = 10). Platelets were incubated with Alexa fluor 488-labeled lactadherin, CD62P-FITC, and CD142-PE in the dark for 10 min at room temperature separately. Results are expressed as mean ± SD. P values < .05 were considered significant. ET, essential thrombocythemia; PS, phosphatidylserine; SD, standard deviation

Student t test or analysis of variance as appropriate. P < .05 was considered statistically significant. Linear regression analysis was used to detect any relation between mean platelet volume (MPV) levels and thromboembolic events. P < .05 was considered statistically significant.

3 | RESULTS

3.1 | Subject characteristics

Characteristics of ET patients and healthy subjects are summarized in Table 1. Compared with those of healthy controls, the numbers of red blood cells and white blood cells have no significant difference in the ET group. Platelet counts in the ET group were significantly higher than those in the healthy group (P < .05). The MPV in ET patients was significantly lower than that in healthy subjects (P < .05). There was no relation between MPV and thromboembolic event rate in the ET group (data not shown).

3.2 | Platelets in ET patients are activated

To evaluate platelet activation in ET patients, the PS exposure, P-selectin, and TF expression were detected by flow cytometry separately. The average level of PS exposure on the platelet membrane in patients with ET was 9.14 ± 0.49%, which was significantly higher than in healthy subjects (2.79 ± 0.2%, P < .001) (Figure 1A). P-selectin expression of the platelet in the ET group (2.16 ± 0.7%) was elevated significantly in comparison to that in healthy controls (3.82 ± 1.2%) (P < .01) (Figure 1B). Compared with healthy controls (1.52 ± 0.5%), the ET group had a significantly higher percentage of TF-positive platelets (3.34 ± 0.9%) (P < .05) (Figure 1C). All these results reveal that platelets were activated in ET patients.

3.3 | Procoagulant activity

To explore the contribution of PS externalization and TF expression to the hypercoagulable state of ET patients, we investigated the PCA of platelets using intrinsic, extrinsic FXa, and prothrombinase assays. The production of the three procoagulant enzyme complexes was increased in the ET groups (P < .001) compared with the controls (Figure 2A). To determine the necessity of exposed PS on platelets of ET patients to support procoagulant reactions, inhibition studies were performed with lactadherin and anti-TF. The dose-dependent experiments indicate that the amount of thrombin and FXa complex production gradually decreased with increasing concentrations of lactadherin (varying concentration from 0 to 256 nmol/L). Anti-TF antibody (varying concentration from 0 to 51.2 μg/mL) had no significant effect (Figure S1); 128 nmol/L lactadherin blocked production of the three procoagulant enzyme complexes significantly (P < .001) (Figure 2A). The PCA of platelets from healthy individuals and ET patients was assessed further by recalcification-time assays. The results showed that with identical numbers of platelets, the coagulation time was significantly reduced in the ET patients compared with the controls (P < .05) (Figure 2B). In order to explore the contribution of PS and TF to the PCA of platelets in ET patients, we also performed coagulation inhibition assays. The PS and TF were blocked with lactadherin and anti-TF antibody, respectively. The PCA of platelets was almost entirely inhibited by 128 nmol/L lactadherin, whereas 25.6-μg/mL anti-TF did not affect the coagulation time of platelets significantly (Figure 2B).

3.4 | Phagocytosis of platelets by ECs *in vitro*

It is unknown whether ECs are able to engulf platelets of ET patients mediated by lactadherin. To answer this question, we performed microscopic assays on cocultured ECs with platelets of ET patients to observe the interactions between the two kinds of cells. After incubation for 30 min, images of representative confocal microscopy (Figure 3A-C) showed that CMFDA-stained platelets (green) adhered with CMTPX-labeled ECs (red) and were taken up by ECs (60 min). After incubation for 90 min, platelets were undergoing degradation in ECs (yellow). For trafficking of the platelets of ET through early phagosomes to a late-stage phagosome in ECs, we costained platelets and markers that are commonly used to study phagocytosis [early endosome antigen-1 (EEA-1) for early-stage endocytosis, Rab-9 for late endosome]. After coincubation for 30 min,

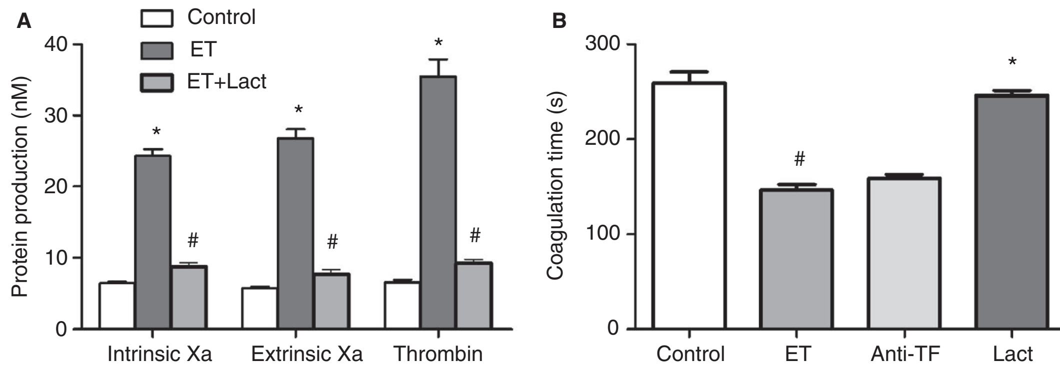


FIGURE 2 Procoagulant activity and inhibition assay. A, Formation and inhibition assays of procoagulant enzyme complexes. FXa and thrombin production of 4×10^4 platelets in each group is shown. Intrinsic FXa formation was measured in the presence of FIXa, FVIII, and thrombin. Extrinsic FXa production was assessed in the presence of FVIIa. Thrombin generation was investigated in the presence of FXa and FVa. The capacity of 128 nmol/L lactadherin to block procoagulant enzyme complexes on platelets from 10 ET patients was evaluated. Results displayed are mean ± SD for triplicate samples of independent experiments (n = 10). *P < .001 vs control, $^{\#}P$ < .001 versus ET patients. B, Coagulation times and inhibition assay. Coagulation times of 100 μL platelets (1×10^7) of ET patients and healthy subjects were detected in the absence or presence of 128 nmol/L lactadherin or 25.6 μg/mL anti-TF antibody separately. Results displayed are mean ± SD for triplicate samples of independent experiments (n = 10). $^{\#}P$ < .001 versus healthy control, *P < .001 versus ET patients. ET, essential thrombocythemia; FXa, factor Xa; SD, standard deviation

the platelets are initially internalized into a phagosome through successive fusion with early endosomes that express early endosome antigen-1 EEA-1(Figure 3E). After coincubation for 60 min, the early phagosome matures to a late-stage phagosome by acquiring Rab protein-9, which has long been implicated in the regulation of transport between the trans-Golgi network and endosome. Figure 3F shows the Rab-9-positive endosomes (green) together with the CD41- labeled platelets (red) distributed throughout the cytoplasm in ECs. These results showed the dynamic phagocytosis process.

Scanning electron microscopy sterically depicted the dynamic phagocytosis process during which the platelets were taken up and then engulfed by ECs (Figure 3G,H). Further, transmission electron microscopy clearly displayed the ECs containing internalized platelets after coincubation for 90 min (Figure 3I). Then, immunoelectron microscope were used to confirm the internalized platelet further. After 120 min of coincubation, the engulfedgol platelets were incorporated and accumulated in the nucleus in ECs (Figure 3J). At the same time, cells were stained with CD41 and gold-conjugated streptavidin to visualize platelets. Arrows in Figure 3K show immunogold particles on the surface of the fused platelets, which indicate platelets were indeed devoured by ECs.

To explore the amount of phagocytosis of platelets by ECs and the role of lactadherin, the percentage of phagocytosis was further measured by flow cytometry. Representative scattergrams from flow cytometry were shown to illustrate the phagocytosis of platelets by ECs. The CMFDA-positive population in ECs was defined as internalized platelets. After incubation for 90 min, a few platelets were adhered or engulfed by ECs in the healthy subject with or without lactadherin (Figure 4A,B). Of the ECs 4.86% engulfed platelets of ET patients in the absence of lactadherin (Figure 4C) and 14.3% of ECs contained platelets of ET patients in the presence of lactadherin (Figure 4D). We found that, compared with PS-exposed ET platelets without lactadherin opsonization, target platelets pretreated with lactadherin showed a greater enhanced phagocytosis by ECs in a time-dependent manner. After 60-min incubation, the percentage of ECs that contained the intracellular platelets of ET patients in the presence of lactadherin was 5.30 ± 0.98%, which was significantly higher than that without lactadherin 3.18 ± 0.21% (P < .05). After 90-min incubation, lactadherin opsonization enhanced the phagocytic percentage, peaking to 15.6 ± 1.8% (Figure 4E). For subsequent phagocytosis assays, mixed ECs and ET platelets as earlier were subjected to different treatments for 90 min (Figure 4F). When we blocked the integrin on ECs by anti-$\alpha v\beta 3$, the percentage of ECs that engulfed ET platelets was dramatically suppressed as compared with that with only lactadherin treatment (P < .001). Another PS binding protein, annexin V, markedly inhibited the uptake compared to the buffer control (P < .05). Furthermore, the phagocytic effect was also inhibited by the P-selectin (Figure 4F). Our present results showed that the phagocytosis process was time-dependent and mostly mediated by the PS-lactadherin - $\alpha v\beta 3$ signal pathway.

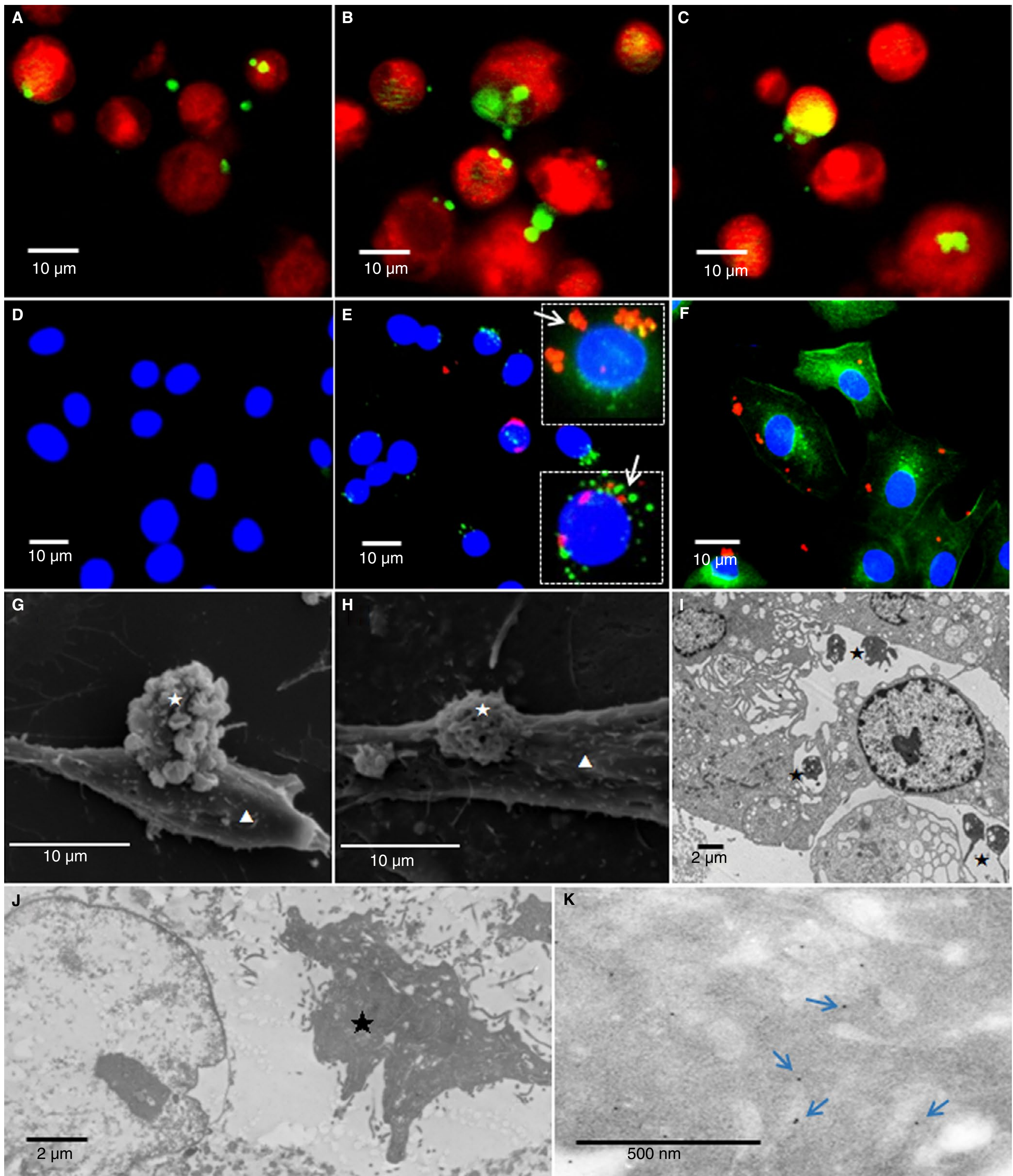
3.5 | Engulfment decreases the PCA of platelets

The implication that ECs were able to phagocytose PS exposed platelets motivated us to explore the contribution of phagocytosis to coagulation. The clearance of platelets by ECs could decrease the PCA induced by ET patients' platelets, which was demonstrated by further coagulation experiments on cocultured ECs and platelets in the presence of lactadherin. With the coculture time extended, the coagulation time of cocultured cells was gradually prolonged and reached statistical significance at 60 min (Figure 5A). We next tested the capacity of the mixed cells to support individual enzyme complexes that contribute to PCA. Accordingly, the ability of cocultured

cells (platelets + ECs) to promote conversions of FX and prothrombin to FXa and thrombin was also decreased with coculture time, respectively. A 1-h increase in coculture time made a significant decrease in productions of active enzymes ($P < .05$) (Figure 5B). These results suggest that the elimination of platelets by ECs led to alleviation of coagulant stress.

4 | DISCUSSION

In the present study, we identify that increased PS exposure on platelets contributes to the hypercoagulable state of ET patients. Dependent on the exposed PS, platelets of ET patient are tethered and endocytosed by ECs in the presence of lactadherin *in vitro*.



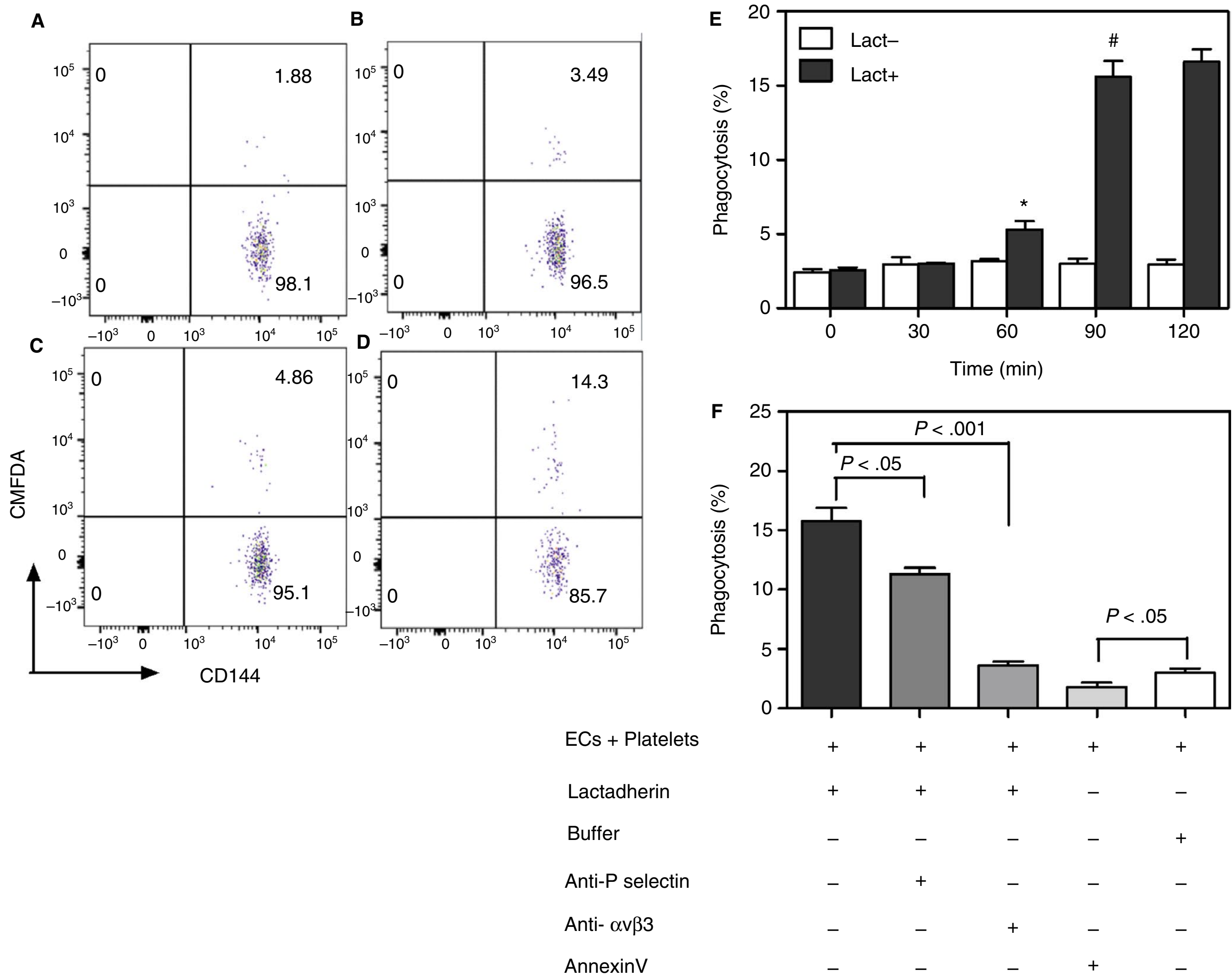


FIGURE 4 Number of platelets phagocytosed by ECs. Platelets (4×10^{7}) of healthy subjects and ET patients were stained separately with 1 μmol/L CMFDA (green) for 10 min at room temperature, and then overlaid with an EC (~1×10^{6}) monolayer in the presence or absence of 2 nmol/L lactadherin and cocultured for 120 min. After the non-adherent platelets were washed, the ECs were detached and labeled with CD144-PE. A representative set of scattergrams from flow cytometric analysis is shown to illustrate the phagocytosis of platelets by ECs. The CMFDA and CD31 double-positive population were defined as the platelets engulfed by ECs. The percentage of ECs contained the intracellular platelets of healthy subjects A, in the absence of lactadherin and B, in the presence of lactadherin. The phagocytic percentage of platelets from ET patients D, with and C, without lactadherin after they were cocultured for 90 min. E, The percentage of phagocytosis was counted at 0, 30, 60, 90, and 120 min by flow cytometry. *P < .05 versus 30-min coculture. #P < .001 versus 60-min coculture. F, Mixed cells as earlier were subjected to different treatments and cocultured for 90 min for subsequent phagocytosis assays by flow cytometry. Mixed cells (ET platelets + ECs) treated with Tyrode's buffer were used as control. All data were representative of at least three independent experiments and values were expressed as mean ± SD; P value < .05 was considered significant. EC, endothelial cell; ET, essential thrombocythemia

FIGURE 3 Phagocytosis of platelets by ECs. Platelets from ET patients were incubated with cultured ECs in the presence of lactadherin at indicated time point. Confocal microscopy images showing that A, after incubation for 30 min, the CMFDA-stained platelets (green) adhere to CMTPX-labeled ECs (red); B, after incubation for 60 min, platelets (green) were endocytosed by ECs (red); C, platelets were undergoing degradation in ECs after incubation for 90 min. The second panel shows the intracellular trafficking patterns for the ET platelets in ECs. D, No EEA-1 or Rab-9 staining was found in the cytoplasm of ECs without platelets. E, Platelets (red) colocalized with EEA-positive early phagosomes (green) in ECs after 15 min of incubation. Arrow indicates the platelets and EEA-1 phagosomes accumulate in tight clusters. F, After 30 min of incubation, CD41- positive platelets (red) together with Rab-9- positive late endosome (green) distributed throughout the cytoplasm in ECs. G, Scanning electron microscopic examination of adhered (stars) platelets to the surface of ECs (triangles) after 30 min of incubation. H, Grasped (star) and internalized platelets by ECs after 60 min incubation. I, Transmission micrograph showing engulfed platelets (star) in ECs after 90 min of incubation. J, Immunoelectron microscopic analysis showing large piece of platelets aggregates (stars) close to ECs after 120 min of incubation. K, High-magnification image of the platelets in the EC. Black particles (arrow) indicate immunogold-labeled CD41 on the surface of the platelets. EC, endothelial cell; EEA-1, the early endosome marker; ET, essential thrombocythemia

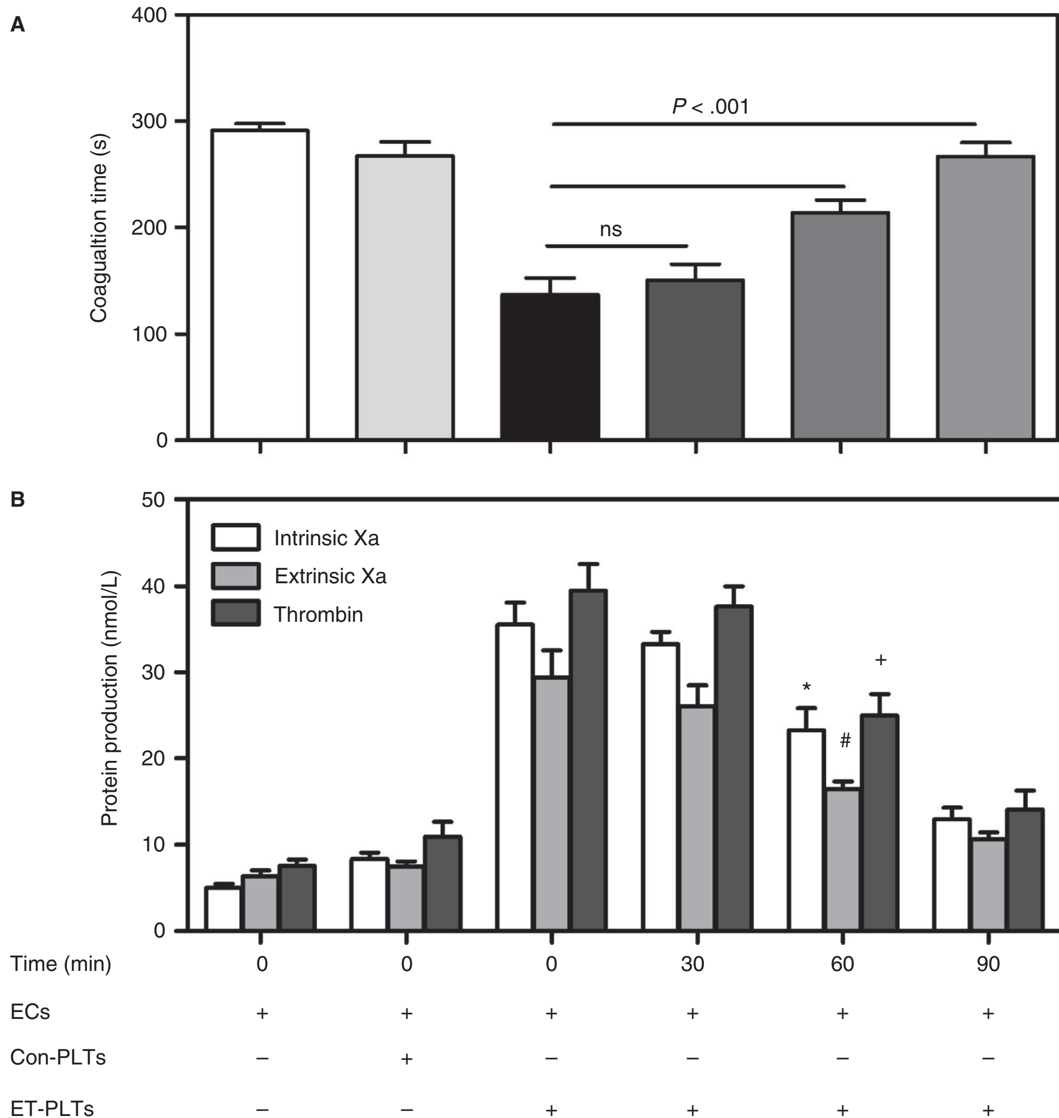


FIGURE 5 Coagulation assays for cocultured ECs and platelets. Platelets from healthy subjects and ET patients were cocultured with or without ECs (40:1 in cell number) for coagulation time determination at various time points, respectively. A, There was significant prolonged coagulation time by 60 min of coculture compared with 0 min and 30 min in platelets + ECs group. B, In a separate experiment, mixed cells were incubated with various cofactors to determine the production of FXa and thrombin at different coculture time points. Coculture of platelets and ECs decreased the production of FXa and thrombin by 60 min compared with 0 min and 30 min ($^{*\,\#\,+}P < .05$). All data were representative of at least three independent experiments and values were expressed as mean ± SD; P value < .05 was considered significant. Con-PLT, platelets of healthy subjects; EC, endothelial cell; ET, essential thrombocythemia

Lactadherin and phagocytosis could cooperatively inhibit both the number and the PCA of platelets in ET patients (Figure 6).

There are likely to be multiple risk markers of thrombosis in ET patients including elevated platetet count, platelet-neutrophil aggregates, JAK2 V617F mutation, and expression of P-selectin and TF on the surface of platelets.[2,25,26] But the pathogenesis of the thrombosis seen in ET patients is not fully understood. In this study and our previous study,[27] we observed that the levels of PS exposure and P-selectin and TF expression on the platelets were markedly increased in ET patients, respectively. The increased PS exposure of platelets in ET patients supports the assembly of intrinsic/extrinsic FXa and prothrombinase, promotes the coagulation cascade reaction, and contributes to hypercoagulability in ET patients. Although we found significant increase of TF-positive platelet, the anti-TF antibody could not inhibit the PCA of the ET patients' platelets significantly. The reason may be that most exposed TF is encrypted and has little PCA until activated by exposed PS,[28] while the TF-expressed platelets of ET patients did not expose PS through the flow cytometry analysis in our present study (data not shown), which indicates the TF on the platelet of ET is inactivated. By lactdherin inhibiton assay, we demonstrated that the major PCA of platelets in ET is PS dependent. This led us to

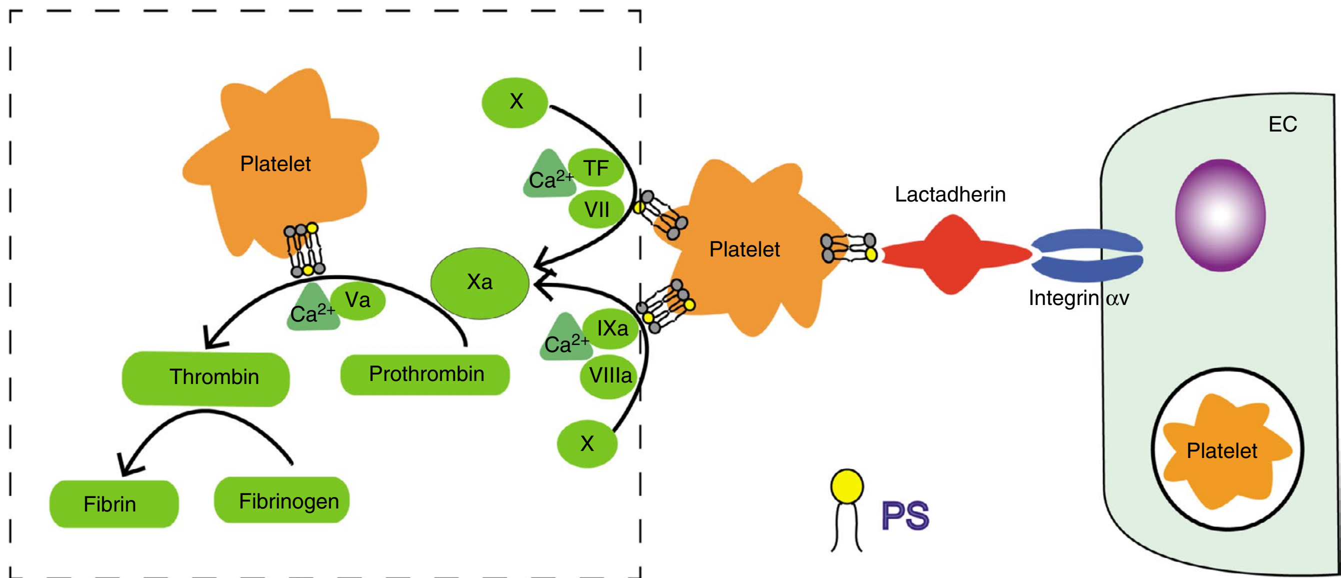


FIGURE 6 Mechanism diagram of lactadherin-mediated phagocytosis that reduces PCA of platelets in ET. Externalized PS on the outer membrane of platelets in ET provides binding sites for FXa and prothrombinase complexes and increases thrombin and fibrin production. EC phagocytosis of activated platelets via the axis of PS-lactadherin-integrin $\alpha v\beta 3$, which blocks the coagulation cascade and further reduces thrombus formation. Lactadherin-mediated phagocytosis of platelet by ECs may provide a new strategy for preventing thrombosis in ETs. EC, endothelial cell; ET, essential thrombocythemia; FXa, factor Xa; PCA, procoagulant activity; PS, phosphatidylserine

investigate how to remove these activated platelets and modulate the coagulation in ET patients.

Known platelet clearance mechanisms include antibody-mediated clearance by spleen macrophages, as in immune thrombocytopenia.[29,30] Platelets of chilling or sepsis are cleared in the liver by macrophages, that is, Kupffer cells and hepatocytes, through lectin-mediated recognition of platelet glycans.[31-33] The tyrosine kinase receptors Tyro3, Axl, and Mer and their ligands protein S and Gas6 are involved in the uptake of platelet microparticles in ECs.[34] However, relatively little is known about the removal process of the activated platelets in ETs. A large number of platelets activate or enter apoptosis, which may be overwhelmed for phagocytes by professional macrophages in ET patients. It is therefore not surprising that platelets were cleared by amateur phagocytes, such as ubiquitously distributed ECs. It was reported that platelets could be internalized by liver sinusoidal endothelial cells.[35,36] However, the effect of the phagocytosed platelets on hemostasis balance has been unclear until recently. In the current study, we explored the phagocytosis of platelets of ET patients by ECs *in vitro* as well as the effects of lactadherin in enhancing this process. Results from flow cytometry and confocal and electron microscopy assays of cocultured ECs and platelets *in vitro* showed that the platelets could be trapped and endocytosed by ECs in the presence of lactadherin. Moreover, the intracellular trafficking patterns for the platelets through EEA1-positive early phagosomes within 30 min, followed by subsequent localization to Rab 9-positive late phagosomes within 60 min, were observed in ECs.

In this study, no significant difference was found in the level of phagocytosis with and without lactadherin in healthy subjects. Furthermore, inhibition of phagocytosis of ET platelets in the presence of annexin V, a protein that has a PS-binding domain but no integrin-binding domain, indicated a major role for PS in this process. Although αv-integrins are present on ECs, neither $\alpha v\beta 3$ nor $\alpha v\beta 5$ can bind PS without lactadherin. Lactadherin can bridge the PS on activated platelets and the αv integrin on ECs and may therefore facilitate engulfment of platelets. When blocking the $\alpha v\beta 3$ or $\alpha v\beta 5$ integrin on ECs, the percentage of phagocytosis of platelets was decreased significantly. These results suggest that PS- lactadherin-integrin plays a critical role in platelet engulfment and removal. P-selectin also plays a key role in mediating platelet adhesion to ECs from our present study. Whether the PS-protein S/Gas6-Tyro3-Axl-Mer pathway and/or the Del-1-dependent manner was involved in the process of phagocytosis of platelets by ECs needs to be further investigated. Although the precise mechanism of platelet phagocytosis by ECs remains unclear, the PS, integrin αv, and lactadherin enhancement as well as P-selectin expression may be collectively involved in this complex process. Further *in vivo* studies will be necessary to indicate the mechanism.

From our previous results, the major PCA of platelets in ET is PS dependent, and ECs were able to recognize, engulf, and ultimately dispose of PS-exposed platelets mediated by lactadherin. However, the contribution of phagocytosis by scavengers to the PCA of platelets is still unclear. Experimentally, the PCA of platelets of ET was suppressed when cocultured with EC in the presence of lactadherin. Coagulation time by cocultured ECs and platelets was markedly prolonged, compared with that of platelets alone. Similarly, the presence of ECs also led to decreased production of FXa and thrombin,

indicating that ECs were able to restore coagulation function to normal by phagocytosis of the procoagulant platelets. Moreover, this important role of ECs requires support from lactadherin, which plays a crucial part in the recognition, adhesion, and endocytosis of platelets. Meanwhile lactadherin functions, *in vitro*, as a potent anticoagulant by competing with blood coagulation proteins FVIII and FV for phospholipid binding sites, inhibits the FXa and prothrombinase complex, and then decreases the PCA of platelets.[19] These results suggest that the clearance of platelets by ECs may actually contribute, at least in part, to the maintenance of a stable coagulation state of ET.

In summary, we demonstrate the phagocytosis of activated platelets of ET patients by ECs via the pathway of PS-lactadherin-integrin $\alpha v\beta 3$ *in vitro*. Lactadherin-enhanced engulfment of platelets by ECs may be used to decrease the amount of platelets and inhibit PCA in ET patients.

Addendum

Chunyan Gao and Jialan Shi conducted experiments, wrote the manuscript, interpreted data, and gave final approval of the version to be published; Shuting Ji, Weijun Dong, and Hong Gao collected patient samples, designed experiments, and analyzed data; Yushan Qi, Danwei Zhao, Minghui Xu, Tingting Li, Hongyin Yu, and Yuting Sun conducted experiments, analyzed data, and edited the manuscript.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Natural Science Foundation of China (81400097), the Natural Science Foundation of Heilongjiang Province (H2015109), Project Funded by Heilongjiang Postdoctoral Science Foundation (LBH-Z13153), Project Funded by China Postdoctoral Science Foundation (2014M550202, 2015T80373), Yu weihan Foundation of Harbin Medical University (31021180167), Yu Weihan Foundation of Harbin Medical University-Daqing (DQYWH201801), Seed Fund of Harbin Medical University- Daqing (DQ2013-05), and the Scientific Research Fund of Harbin Medical University-Daqing (2018XN-09, 2018XN-16).

CONFLICT OF INTEREST

No conflict of interest exists.

REFERENCES

1. Jensen MK, de Nully Brown P, Lund BV, Nielsen OJ, Hasselbalch HC. Increased circulating platelet leukocyte aggregates in myeloproliferative disorders is correlated to previous thrombosis, platelet activation and platelet count. *Eur J Haematol*. 2001;66:143–151.
2. Falanga A, Marchetti M, Vignoli A, Balducci D, Barbui T. Leukocyte-platelet interaction in patients with essential thrombocythemia and polycythemia vera. *Exp Hematol*. 2005;33:523–530.
3. Arellano-Rodrigo E, Alvarez-Larran A, Reverter JC, Villamor N, Colomer D, Cervantes F. Increased platelet and leukocyte activation as contributing mechanisms for thrombosis in essential thrombocythemia and correlation with the JAK2 mutational status. *Haematologica*. 2006;91:169–175.
4. Tefferi A, Barbui T. Polycythemia vera and essential thrombocythemia: 2017 update on diagnosis, risk-stratification, and management. *Am J Hematol*. 2017;92:94–108.
5. Carobbio A, Thiele J, Passamonti F, et al. Risk factors for arterial and venous thrombosis in WHO-defined essential thrombocythemia: international study of 891 patients. *Blood*. 2011;117:5857–5859.
6. Wood JP, Silveira JR, Maille NM, Haynes LM, Tracy PB. Prothrombin activation on the activated platelet surface optimizes expression of procoagulant activity. *Blood*. 2011;117:1710–1718.
7. Maugeri N, Malato S, Femia EA, et al. Clearance of circulating activated platelets in polycythemia vera and essential thrombocythemia. *Blood*. 2011;118:3359–3366.
8. Gao C, Li W, Xie R, et al. Endothelial cell phagocytosis of senescent neutrophils decreases procoagulant activity. *Thromb Haemost*. 2013;109:1079–1090.
9. Xie R, Gao C, Li W, et al. Phagocytosis by macrophages and endothelial cells inhibits procoagulant and fibrinolytic activity of acute promyelocytic leukemia cells. *Blood*. 2012;119:2325–2334.
10. Ma R, Xie R, Yu C, et al. Phosphatidylserine-mediated platelet clearance by endothelium decreases platelet aggregates and procoagulant activity in sepsis. *Sci Rep*. 2017;7:4978.
11. Vance JE, Steenbergen R. Metabolism and functions of phosphatidylserine. *Prog Lipid Res*. 2005;44:207–234.
12. Wesseling MC, Wagner-Britz L, Nguyen DB, et al. Novel insights in the regulation of phosphatidylserine exposure in human red blood cells. *Cell Physiol Biochem*. 2016;39:1941–1954.
13. Somajo S, Koshiar RL, Norström E, Dahlbäck B. Protein S and factor V in regulation of coagulation on platelet microparticles by activated protein C. *Thromb Res*. 2014;134:144–152.
14. Wesseling MC, Wagner-Britz L, Nguyen DB, et al. Pathophysiologic implications of membrane phospholipid asymmetry in blood cells. *Blood*. 1997;89:1121–1132.
15. Ravichandran KS, Lorenz U. Engulfment of apoptotic cells: signals for a good meal. *Nat Rev Immunol*. 2007;7:964–974.
16. Li W. Eat-me signals: keys to molecular phagocyte biology and "appetite" control. *J Cell Physiol*. 2012;227:1291–1297.
17. Toda S, Hanayama R, Nagata S. Two-step engulfment of apoptotic cells. *Mol Cell Biol*. 2012;32:118–125.
18. Abdel-Monem H, Dasgupta SK, Le A, Prakasam A, Thiagarajan P. Phagocytosis of platelet microvesicles and β2– glycoprotein I. *Thromb Haemost*. 2010;104:335–341.
19. Shi J, Gilbert GE. Lactadherin inhibits enzyme complexes of blood coagulation by competing for phospholipid binding sites. *Blood*. 2003;101:2628–2636.
20. Shi J, Pipe SW, Rasmussen JT, Heegaard CW, Gilbert GE. Lactadherin blocks thrombosis and hemostasis in vivo: correlation with platelet phosphatidylserine exposure. *J Thromb Haemost*. 2008;6:1167–1174.
21. Andersen MH, Berglund L, Rasmussen JT, Petersen TE. Bovine PAS-6/7 binds alpha v beta 5 integrins and anionic phospholipids through two domains. *Biochemistry*. 1997;36:5441–5446.
22. Beas AO, Taupin V, Teodorof C, Nguyen LT, Garcia-Marcos M, Farquhar MG. Gαs promotes EEA1 endosome maturation and shuts down proliferative signaling through interaction with GIV (Girdin). *Mol Biol Cell*. 2012;23:4623–4634.
23. Chu J, Smelkinson MG, Dorward DW, Zarember KA, Gallin JI. Early intracellular trafficking of granulibacter bethesdensis in human macrophages. *Infect Immun*. 2017;85(6):pii: e00847-16.
24. Gao C, Xie R, Yu C, et al. Thrombotic role of blood and endothelial cells in uremia through phosphatidylserine exposure and microparticle release. *PLoS ONE*. 2015;10(11):e0142835.

25. Panova-Noeva M, Marchetti M, Spronk HM, et al. Platelet induced thrombin generation by the calibrated automated thrombogram assay is increased in patients with essential thrombocythemia and polycythemia vera. *Am J Hematol*. 2011;86:337–342.
26. Falchi L, Bose P, Newberry KJ, Br VS, Haematol J. Approach to patients with essential thrombocythaemia and very high platelet counts: what is the evidence for treatment? *Br J Haematol*. 2017;176:352–364.
27. Tong D, Yu M, Guo L, et al. Phosphatidylserine-exposing blood and endothelial cells contribute to the hypercoagulable state in essential thrombocythemia patients. *Ann Hematol*. 2018;97:605–616.
28. Bach RR. Tissue factor encryption. *Arterioscler Thromb Vasc Biol*. 2006;26:456–461.
29. Jensen MK, de Nully Brown P, Lund BV, Nielsen OJ, Hasselbalch HC. Increased platelet activation and abnormal membrane glycoprotein content and redistribution in myeloproliferative disorders. *Br J Haematol*. 2000;110:116–124.
30. Li R, Hoffmeister KM, Falet H. Glycans and the platelet life cycle. *Platelets*. 2016;27:505–511.
31. Arthur CM, Patel SR, Sullivan HC, et al. CD8 + T cells mediate antibody-independent platelet clearance in mice. *Blood*. 2016;127:1823–1827.
32. Hoffmeister KM, Felbinger TW, Falet H, et al. The clearance mechanism of chilled blood platelets. *Cell*. 2003;112:87–97.
33. Hoffmeister KM, Josefsson EC, Isaac NA, Clausen H, Hartwig JH, Stossel TP. Glycosylation restores survival of chilled blood platelets. *Science*. 2003;301:1531–1534.
34. Happonen KE, Tran S, Mörgelin M, et al. Gas6-Axl protein interaction mediates endothelial uptake of platelet microparticles. *J Biol Chem*. 2016;291:10586–10601.
35. Peng Q, Yeh H, Wei L, et al. Mechanisms of xenogeneic baboon platelet aggregation and phagocytosis by porcine liver sinusoidal endothelial cells. *PLoS ONE*. 2012;7(10):e47273.
36. Paris LL, Chihara RK, Sidner RA, Tector AJ, Burlak C. Differences in human and porcine platelet oligosaccharides may influence phagocytosis by liver sinusoidal cells in vitro. *Xenotransplantation*. 2012;19:31–39.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Ji S, Dong W, Qi Y, et al. Phagocytosis by endothelial cells inhibits procoagulant activity of platelets of essential thrombocythemia *in vitro*. *J Thromb Haemost*. 2020;18:222–233. https://doi.org/10.1111/jth.14617