Elevated procoagulant microparticles expressing endothelial and platelet markers in essential thrombocythemia

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ABSTRACT

Background

Most cell types, including blood - and vascular cells, produce microparticles upon activation. Since cellular microparticles are known to be elevated in thromboembolic diseases, we hypothesized a role for microparticles in the pathogenesis of thrombosis in essential thrombocythemia.

Design and Methods

In plasma samples from 21 patients with essential thrombocythemia and ten healthy subjects, the levels and the cellular origin of microparticles were determined by flowcytometric analysis, while the microparticle-associated procoagulant activity was measured using a thrombin generation assay.

Results

Patients with essential thrombocythemia had significantly higher numbers of circulating annexin V-positive microparticles than controls (median 4500 *vs.* 2500×10^6 events/L; p=0.039), including significantly higher numbers of microparticles positive for the platelet marker CD61 (p=0.043), the endothelial markers CD62E (p=0.009) and CD144 (p=0.021), and for tissue factor (p=0.036). CD62E was co-expressed with the platelet marker CD41 on microparticles, suggesting a bilineage origin of such microparticles, which were observed only in patients with risk factors for thrombosis. Patients with essential thrombocythemia had higher plasma levels of mature von Willebrand factor (p=0.045) but similar propeptide levels compared to controls. In thrombin generation analyses, microparticle-rich plasma from patients with essential thrombocythemia had a shorter lag time (p=0.001) and higher peak height (p=0.038) than plasma from controls. Peak height correlated significantly with the total number of microparticles (R=0.634, p<0.001).

Conclusions

Patients with essential thrombocythemia had higher number of circulating microparticles with platelet and endothelial markers, suggesting ongoing platelet and endothelial activation. This was confirmed by an increased level of mature von Willebrand factor, an abnormal mature von Willebrand factor/propeptide ratio, and a hypercoagulable state reflected in thrombin generation. These findings suggest a role for microparticles in thrombosis in essential thrombocythemia.

Key words: microparticles, essential thrombocythemia, E-selectin, thrombin generation, von Willebrand factor.

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Introduction

Cellular microparticles are plasma membrane vesicles of <1.5 μm in diameter, mainly composed of lipids and proteins, which are released into the circulation by blood-cells and vascular cells during cellular activation or apoptosis.[1] Microparticles are heterogeneous, differing in size, as well as in phospholipid and protein composition. In addition, microparticles display some specific cell surface proteins that indicate their cellular origin. Depending on the cellular process and the cellular origin triggering their formation, the outer surfaces of cellular microparticles may contain phosphatidylserine, which provides a suitable anionic phospholipid surface for assembly of the tenase and prothrombinase complexes, and they may express tissue factor (TF), the primary initiator of coagulation.[2] Such phosphatidylserine- and/or TF-bearing microparticles may contribute to the pathogenesis of thrombosis in different diseases, including cancer–associated thrombosis and sepsis.[3] Indeed, the numbers and characteristics of circulating microparticles have been found to be altered in many vascular diseases associated with an increased risk of both arterial and venous thrombosis. In particular, elevated numbers of platelet-derived microparticles have been described in diabetes mellitus,[4] acute coronary syndrome,[5] myocardial infarction[6] and disseminated intravascular coagulation.[7] Elevated levels of endothelial-derived microparticles have been found in venous thromboembolism[8] and in the antiphosholipid syndrome.[9] The majority (more than 90%) of microparticles in healthy controls are of platelet origin, whereas less than 10% originate from granulocytes and less than 5% from endothelial cells, red blood cells and monocytes.[10]

Essential thrombocythemia (ET) is a chronic myeloproliferative disease characterized by an increased risk of both arterial and venous thrombosis. At the time of diagnosis approximately 20% of ET patients have had a major thrombotic event and approximately another 20% will subsequently have an event.[11] This makes venous and (more often) arterial thrombosis the leading causes of morbidity and mortality in ET. Established risk factors for thrombosis in ET are older age (over 60 years) and previous thrombotic events.[12] Recently, leukocytosis has been identified as an additional risk factor.[11,13,14] Numerous mechanisms, including blood hyperviscosity and quantitative/qualitative abnormalities of blood cells, have been advocated to be at the origin of the hypercoagulable state in these patients.[15] An increased number of platelets,[16] abnormal function of platelets,[12] activation of platelets and leukocytes,[17,18] their interaction to form platelet-leukocyte aggregates,[17] and endothelial activation may all contribute to the increased thrombotic state in ET, which is still not completely understood.

In the present study, we investigated the numbers and the procoagulant potential of circulating microparticles in a group of patients with ET, using flow cytometry and a thrombin generation assay, respectively. In addition, levels of mature von Willebrand factor (vWF) and propeptide, and soluble E-selectin were determined in the same plasma samples as a measure of ongoing endothelial activation.

Design and Methods

Study subjects

Twenty-one consecutive ET patients (8 males and 13 females, median age: 58, range: 34-83 years) were enrolled at the Hematology Department of Bergamo Hospital (Italy), after giving informed consent. Patients were diagnosed as having ET according to the Polycythemia Vera Study Group (PVSG) criteria.[19] All investigations were approved by the local ethical committee (Comitato di Bioetica, Ospedali Riuniti, Bergamo, Italy). The patients' characteristics are shown in Table 1. Nine patients were heterozygous carriers for the *JAK2*^{V617F} mutation. At the time of the sample collection 18 patients were receiving treatment with aspirin, eight with hydroxyurea and three patients were not receiving any treatment. The sample size was chosen based on a power calculation using data from a preceding unpublished pilot study in which the mean number of microparticles in control subjects was 3400×10^6/L and that in ET patients was 7500×10^6/L. With an α of 0.05 this calculation showed that nine controls and 18 ET patients would provide the analysis with sufficient power. In addition, we investigated ten healthy controls (6 males and 4 females, median age 46, range 19-59). None of the healthy controls was on antiplatelet medication at the time of blood collection. Controls were significantly younger than ET patients (p=0.015).

Blood collection and isolation of microparticles

Blood samples were drawn early in the morning, before any therapy, with a 21-gauge needle after applying a light tourniquet. After discarding the first 3 mL, blood was collected into a 5 mL tube containing 3.2% citrate (BD, Plymouth, UK). Plasma was prepared within 30 min after blood collection by centrifugation for 20 min at 1,550 x g at room temperature, without brake. Aliquots of plasma were snap-frozen in liquid nitrogen, and then stored at -75°C until use. In order to isolate the microparticles, 250 μL of plasma were thawed on ice for 60 min and then centrifuged for 30 min at 17,570 x g at 20°C. Subsequently, 225 μL of supernatant (i.e. microparticle-free plasma) were removed. The remaining 25 μL containing the microparticle pellet were resuspended in 225 μL of phosphate-buffered saline (PBS; 154 mM NaCl, 1.4 mM phosphate, pH 7.4), containing 10.9 mM trisodium citrate to prevent coagulation activation. Samples were centrifuged for 30 min at 17,570 x g at 20 °C; thereafter, 225 μL of supernatant were removed and the microparticle pellet was resuspended in 125 μL of PBS.

Phenotypic analysis of plasma-derived microparticles

Flowcytometric analysis was used to quantify and characterize plasma-derived microparticles, as previously described.[20] Briefly, 5 μL of microparticle sample were diluted in 35 μL PBS containing 2.5 mM $CaCl_2$ (pH 7.4). The samples were then incubated for 30 min at room temperature in the dark with 5 μL annexin V-allophycocyanin (Caltag Laboratories, Burlingame, CA, USA) and/or 5 μL fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, peridinin-chlorophyll-protein complex

Table 1. Characteristics of study subjects at enrollment into the study. Data are presented as median (range) or number (%).

	Controls	ET patients
Male/female	6/4	13/8
Age (years)	46 (19-59)	58 (34-83)
Platelets ($\times 10^9$/L)	ND*	510 (315-1340)
White blood cells ($\times 10^9$/L)	ND	7.2 (3.13-14.7)
Polymorphonuclear cells ($\times 10^9$/L)	ND	4.46 (1.71-10.2)
Hematocrit (%)	ND	40.5 (31.5-47.6)
Patients' therapy	-	18 (85%)
Aspirin	-	18 (85%)
Hydroxyurea	-	7 (38%)
History of thrombosis	-	6 (28%)

Not determined.

(PerCP)- labeled anti-human monoclonal antibodies, or 5 μL isotype-matched control monoclonal antibodies.

For the phenotypic characterization of microparticles the following cell-specific monoclonal antibodies were used: anti-CD8-PE (SK1, IgG_1), anti-CD14-PE (MΦP9, IgG2b), anti-CD15-PE (HI98, IgM), anti-CD20-PE (L27, IgG_1), anti-CD45-PerCP (H130, IgGλ, κ), anti-CD61-FITC (VI-PL2, IgG_1), anti-CD63-FITC (H5C6, IgG_1), anti-CD146-PE (P1H12, IgG1), labeled isotype controls IgG_1 (X40) and IgG_{2a} (X39), all from Becton Dickinson (San Jose, USA); IgG_{2b}-PE, anti-glycophorin A-FITC (JC159, IgG_1) and anti-CD41-FITC (5B12, IgG_1) from DAKO (Glostrup, Denmark); anti-CD144-FITC (BMS158FI, IgG1) from Bender MedSystems (Vienna, Austria); anti-CD106-FITC (1.G11B1, IgG_1) from Calbiochem (Darmstadt, Germany); anti-CD54-PE (K562, IgG_1), anti-CD66b-FITC (80H3, $IgG_1\kappa$) and anti-CD62P-PE (CLB-Thromb/6, IgG_1) from Immunotech (Marseille, France); anti-CD62E-PE (HAE-1f, IgG_1) from Kordia (Leiden, The Netherlands); anti-CD4-PE (CLB-T4/2, IgG_1) and anti-CD66acde-PE (CLB-gran/10, IH4Fc, IgG_1) from Sanquin (Amsterdam, The Netherlands).

For TF measurement on microparticles, anti-TF-FITC from American Diagnostics (VD8, IgG_1, Stamford, CT, USA) was used in the same experimental conditions.

After incubation, 760 μL PBS/calcium buffer were added and the samples were analyzed on a FACS Calibur using Worklist Manager (BD) for 1 min during which the flowcytometer analyzed approximately 55 μL of the suspension. Forward scatter (FSC) and sideward scatter (SSC) were set at logarithmic gain. To distinguish microparticles from events due to noise, microparticles were identified on the basis of their specific FSC and SSC characteristics, with gates set using *in vitro* platelet activation and microparticle generation data (*results not shown*), and by annexin V positivity (Figure 1A and 1B).[21] To identify annexin V-positive events, a threshold was set in a microparticle sample prepared without calcium. The number of microparticles per liter of plasma was calculated as previously described # $MPs_{per\ minute}$ *(Volume(V) $_{tube}$/V_{minute}) *(V_{end}/ V_{start}) * (1000/ $V_{labeled}$)= # events/mL.[7] Data were analyzed with CellQuest-pro software (Becton Dickinson).

Thrombin generation measurements

Thrombin generation in platelet-poor but microparticle-rich plasma, as prepared for microparticle isolation

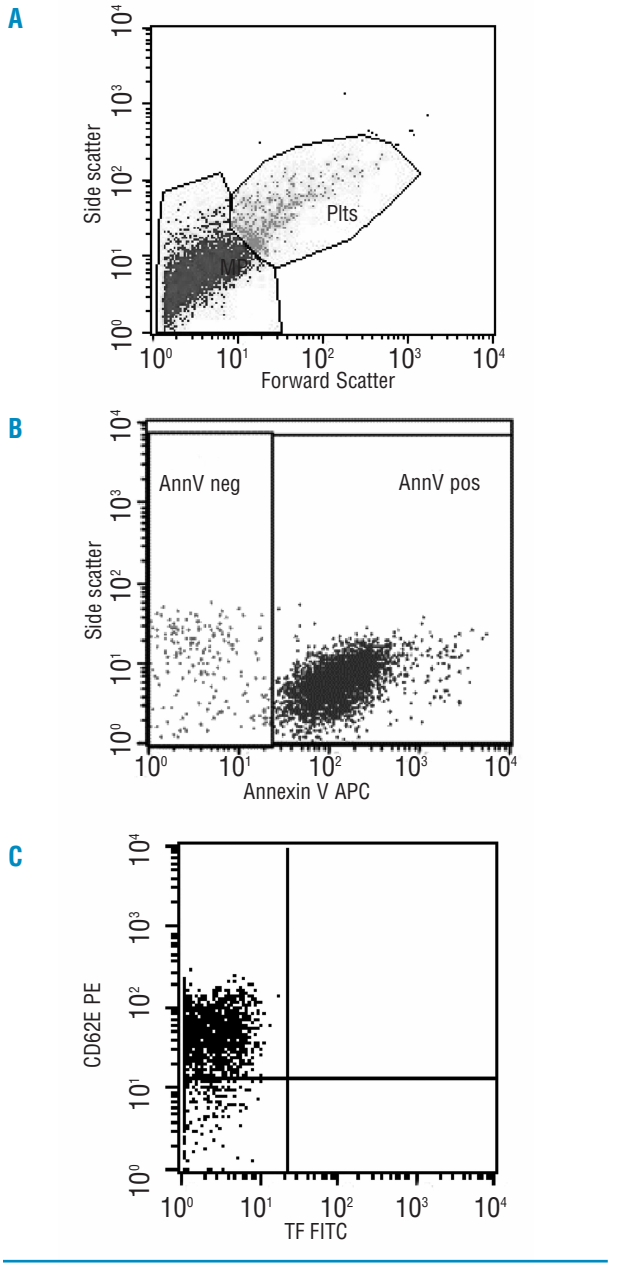

Figure 1. Flow cytometric analysis of microparticles. A representative set of scattergrams from flowcytometric microparticle analysis in a sample from an ET patient is shown to illustrate microparticle and subpopulation definition. Panel A: Forward and side scatter were used to define the microparticle (MP) and platelet (Plts) gates as shown. Panel B: Events defined as microparticles were then selected for their annexin V binding, determined by positivity for annexin V-allophycocyanin fluorescence (on the x-axis). Panel C: Annexin V-positive microparticles were further examined for expression of other antigens by co-labeling with PE - and FITC- labeled antibodies as is shown here for CD62E-PE and TF-FITC binding.

(described above), was measured with the calibrated automated thrombogram method (Thrombinoscope BV, Maastricht, The Netherlands).[22,23] Thrombin generation was triggered in 80 μL of plasma by different conditions; 1 pM of TF and 4 μM of phospholipids, 4 μM of phospholipids alone, and buffer (no exogenous TF or phospholipids added), using reagents obtained from Thrombinoscope BV. Thrombin generation was meas-

ured as fluorescence, read in a Fluoroskan Ascent reader (Thermo Labsystems OY, Helsinki, Finland) equipped with a 390/460 filter set and thrombin generation curves were calculated with the Thrombinoscope software (Thrombinoscope BV). Three parameters were derived from the thrombin generation curves: lag time (initiation phase of coagulation), endogenous thrombin potential, and peak height. Lag time was defined as the time to reach 1/6 of the peak height. Validation of the calibrated automated thrombogram method showed normalization of non-time-dependent parameters to be mandatory to obtain acceptable inter-assay variations.[23] Intra-assay variations for normalized parameters are typically below 6%, and inter-assay variations below 8%.[23] Therefore, each thrombin generation measurement includes normal pooled plasma and both the endogenous thrombin potential and peak height values are expressed as the ratio of patient's value to the value in normal pooled plasma, expressed in percentages.

Plasma markers of endothelial activation

Plasma concentrations of soluble E-selectin were determined by a commercially available enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions (Diaclone, Cedex, France). Mature vWF and propeptide plasma levels were measured by ELISA as described previously.[24] The half-life of mature vWF is four times that of the propeptide half-life, and due to this difference in half-life their relative concentration is a distinctive indicator of ongoing chronic as opposed to acute endothelial activation.[25]

Statistical analysis

We established the statistical significance of differences in microparticle numbers between groups with the non-parametric Mann-Whitney U and Kruskal-Wallis tests, where appropriate. Bivariate correlations were estimated by Spearman's rank correlation (R). All tests for statistical significance were two-tailed and p values less than 0.05 were considered statistically significant. Analyses were performed using SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

Number and phenotypic characterization of microparticles

The total number of microparticles is significantly higher in ET patients (median: 4500×10^6/L) than in controls (2500×10^6/L, p=0.039). For most study subjects, more than 90% of circulating microparticles bound to annexin-V, indicating the presence of phosphatidylserine on their membrane (Figure 1A and 1B).

The phenotypic characterization of microparticles depicted in Figure 2 showed that the subset composition of the microparticle population (i.e. of platelet -, leukocyte -, endothelial cell or erythrocyte origin) is similar in ET and controls. The large majority of microparticles is of platelet origin in both groups of subjects, as determined by positivity for the platelet markers CD41 and/or CD61 (CD41 ET *vs.* controls: median±SD 95±0.34% *vs.* 95.7±0.7%; p=0.15). Accordingly, the number of platelet-derived microparticles was greater in ET patients than in controls (CD61 median 4000 *vs.* 2400×10^6/L; p=0.043). The levels of the two platelet activation markers CD62P and CD63 were lower on microparticles from ET patients than on those from controls, and this difference was statistically significant for CD63 (median 5.5 *vs.* 40×10^6/L, p<0.001).

With regard to endothelium-derived microparticles, the number of microparticles expressing the endothelial marker CD62E was significantly higher in ET patients than in controls (median 875 *vs.* 14×10^6/L; p=0.007) (Figure 1C) as was the number expressing CD144 (p=0.021). Microparticles of granulocyte (CD66b and CD66acde) and of monocyte (CD14) origin were present in low but significantly higher numbers in ET patients. Microparticles derived from T cells (CD4 or CD8), B cells (CD20), intracellular adhesion molecule (ICAM)-positive

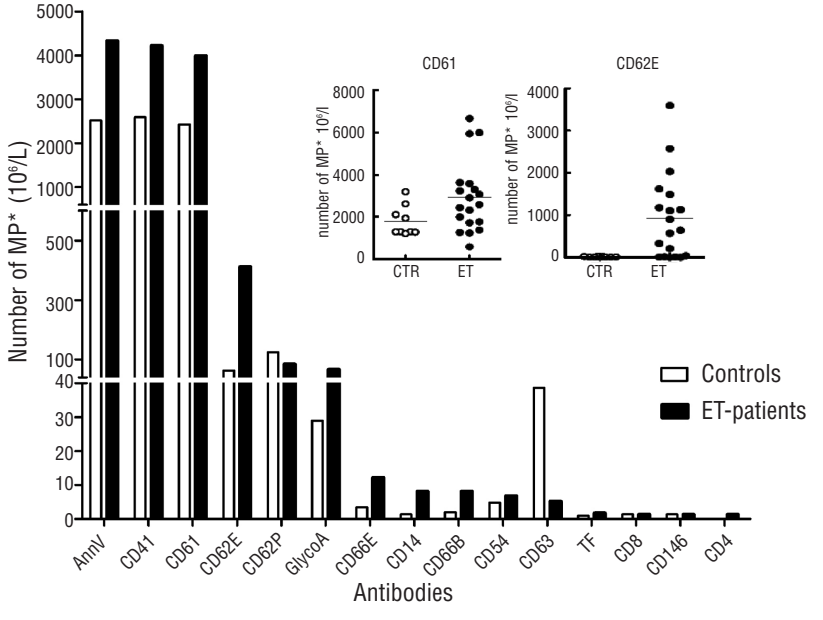

Figure 2. Size of circulating microparticle (MP) subpopulations in ET patients and controls. Bar graph: Number of microparticles with specific cellular origins as defined by marker positivity in plasma from ET patients (ET) and controls (CTR). Data are presented as medians. Numbers of microparticles positive for CD15, CD20, CD45, CD106 and CD144 were too low to be shown adequately in this graph. Inset: dotplot of the same data presented individually for the platelet marker CD61 and the endothelial marker CD62E.

cells (CD54) and vascular cell adhesion molecule (VCAM)-positive cells (CD106) account for less than 1% of all microparticles and their numbers were not different between ET patients and controls (*data not shown*).

Relation of microparticles with the presence of the JAK2^{V617F} mutation or pharmaceutical treatment

Neither *JAK2*^{V617F} mutation status nor the treatment given (hydroxyurea or aspirin) affected the number or the cellular origin of microparticles, including TF-positive microparticles in ET patients, (*data not shown*).

CD41- and CD62E-positive microparticles

Since more than 95% of microparticles were positive for CD41 and CD61, we suspected that the CD62E-positive microparticles, constituting 27% of all microparticles in ET patients, but 1% in controls, could also co-express a platelet marker. We, therefore, analyzed the microparticles with a combination of CD41-FITC/CD62E-PE monoclonal antibodies. Indeed, in both groups 90% of the CD62E positive microparticles also expressed CD41. The CD62E-positive microparticles constituted 24% of the CD41-positive microparticles in ET patients and 1% in controls. When patients with ET were classified according to a risk score, allocating one point for a history of thrombosis, age over 60 years, platelet count over 1000×10^9/L, and the presence of a cardiovascular risk factor (for example hypertension or diabetes),[26] microparticles with combined expression of CD41/CD62E were increased only in patients with one or more risk factors (Figure 3, p=0.045). This correlation was not observed for microparticles originating from other cells.

Plasma markers of endothelial cell activation

Since ET patients had higher levels of CD41/CD62E-positive microparticles, which suggests endothelial activation, we investigated the activation status of endothelium by measuring plasma levels of soluble E-selectin, mature vWF and propeptide.

No significant differences in the plasma levels of E-selectin were observed between ET patients (20 ng/mL, range, 5-40) and controls (14 ng/mL; range, 5-41; p=0.52). The removal of microparticles from plasma by centrifugation did not affect the levels of E-selectin (*data not shown*), indicating that most of the soluble E-selectin was not bound to these microparticles. Furthermore, no correlation was found between soluble E-selectin levels and the number of CD41/CD62E-positive microparticles (*data not shown*).

ET patients had significantly higher concentrations of mature vWF in plasma than did controls (median 50 *vs.* 35 nM, p=0.045) but similar concentrations of propeptide (7 *vs.* 5 nM, p=0.07). The mature vWF and propeptide pattern was, therefore, significantly different in patients and controls, resulting in a higher mature vWF: propeptide ratio, a pattern previously shown to indicate a state of chronic endothelial activation[25] (Figure 4). No correlation was found between mature vWF, propeptide and CD41/CD62E-positive microparticles.

Tissue factor-positive microparticles

TF-positive microparticles accounted for less than 1% of all microparticles in both patients and controls. TF was expressed on microparticles also expressing platelet markers and CD62E. The number of microparticles carrying TF was significantly higher (p=0.036) in ET patients (median 1.8×10^6/L) than in controls (median 0.9×10^6/L). No correlation was found between a history of throm-

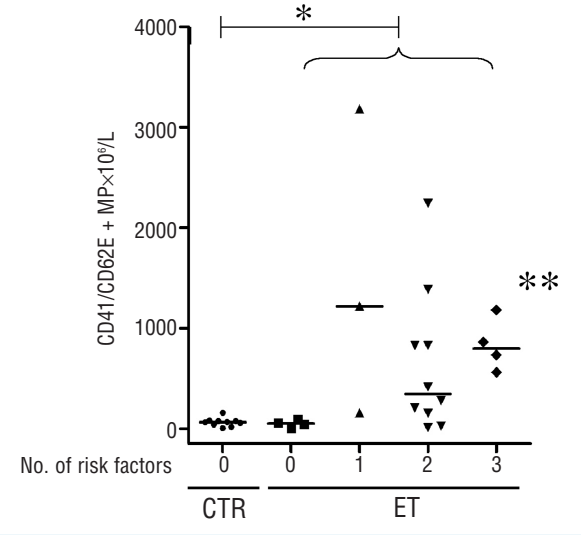

Figure 3. CD41/CD62E-positive microparticles and risk factors for thrombosis. Dotplot of the number and the median of CD41/CD62E-positive microparticles in controls and in ET patients categorized for number of risk factors (age older than 60, previous thrombotic event, platelets > 1000x10^9/L, presence of a cardiovascular risk factor:[26] 0=no risk factor, 1=one risk factor, 2= two risk factors, etc. *ET patients have significantly more CD41/CD62E-positive microparticles than controls (p=0.01), and **ET patients with one or more risk factors have higher numbers of CD41/ CD62E-positive microparticles compared to ET patients without risk factors (p=0.034).

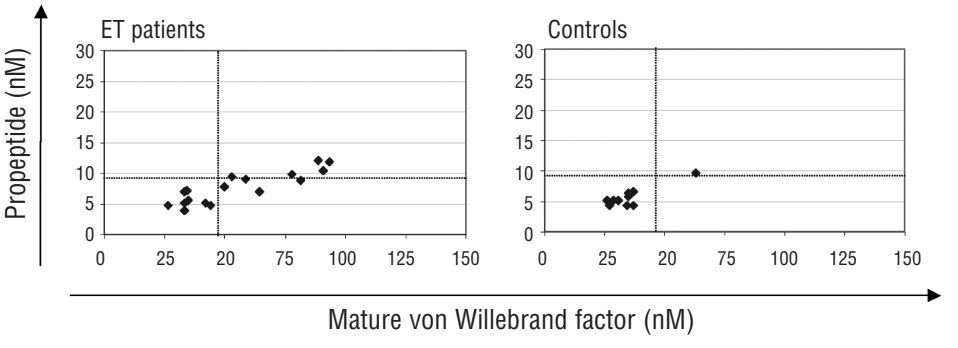

Figure 4. Relation between mature vWF and propeptide levels in patients with ET and controls. Dotted lines represent the upper limit of the 95% confidence interval of mature vWF and propeptide levels of the control group. ET patients have higher levels of mature vWF (p=0.045), and similar levels of propeptide as compared to levels in controls.

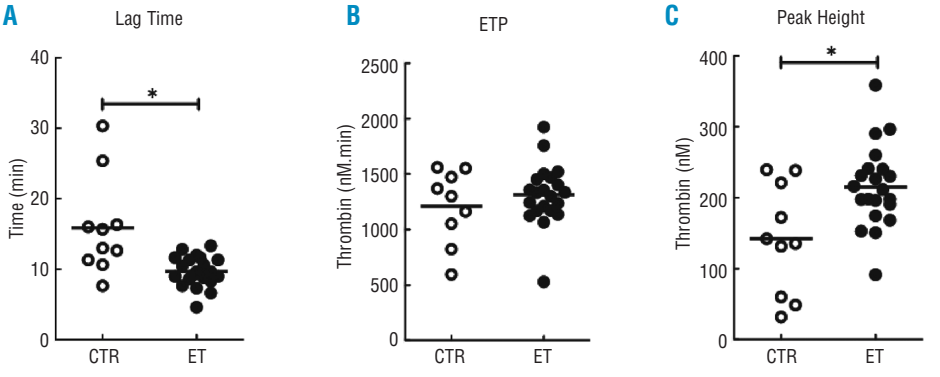

Figure 5. Thrombin generation in the absence of exogenous TF and phospholipids in platelet–poor, microparticle-rich plasma. The results of the parameters lag time (A), endogenous thrombin potential (ETP) (B) and peak height (C) are shown for ET patients (●) and controls (CTR) (○). Lines represent the median value. ET patients have a significantly shorter lag time and a higher peak height compared to controls. *$p<0.05$.

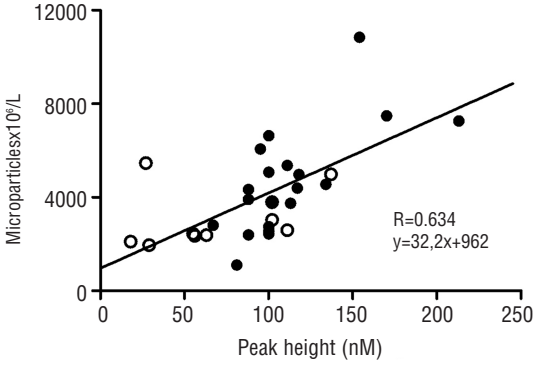

Figure 6. Correlation between the peak height of thrombin generation in the absence of exogenous TF and phospholipids, and the total number of microparticles. Data are shown for microparticle-rich plasma of ET patients (●) and controls (○). R=0.634, $p<0.001$.

boembolic events or positive risk score for thrombosis and TF-positive microparticles.

Thrombin generation

Thrombin generation triggered with 1 pM of TF and 4 μM of phopholipids was increased in microparticle-rich plasma from ET patients as indicated by increased peak height [ET *vs.* controls: 411 nM (95%CI: 358-465) and 279 nM (95%CI: 196-361), respectively, p=0.01[. No differences were found in lag time and endogenous thrombin potential, suggesting attenuated inhibition rather than altered activation or stimulation under these conditions. Since plasma-, possibly microparticle-derived TF may contribute to the initiation of coagulation, the thrombin generation assay was repeated in the absence of additional TF and only 4 μM of phospholipids were added. Again, the peak height was higher for ET patients (325 nM; 95%CI: 289-360) than for controls (171 nM; 95%CI: 94-249; p=0.001). Furthermore, the lag times were on average 4 min shorter for ET patients (12.0 min; 95%CI: 10.7-13.4) than for controls (15.8 min; 95%CI: 12.0-19.7; p=0.04). This latter observation is suggestive of the presence of more TF in plasma, and indeed maybe on microparticles, from ET patients than from controls. To further characterize the procoagulant potential of plasma and microparticles from patients with ET, the thrombin generation assay was performed in the absence of both TF and phospholipids (Figure 5). Using these conditions, ET patients had a shorter lag time (9.7 min; 95%CI: 8.7-10.7 versus 15.9 min; 95%CI: 10.9-20.9; p=0.001), and an increased peak height (215 nM; 95%CI: 189-241 versus 142 nM; 95%CI: 87-189; p=0.038), indicating endogenous presence of procoagulant phospholipids and TF, possibly provided by microparticles. Indeed, a negative correlation was found between the total number of microparticles and lag time (R= -0.379, p=0.039) and a positive correlation between the total numer of microparticles and peak height (R=0.634, $p<0.001$) (Figure 6). Finally, after removal of microparticles by centrifugation there was no activation of thrombin generation (*data not shown*), which is compatible with the absence of microparticle-derived TF and phospholipids.

Discussion

This is the first extensive analysis of microparticles and their cellular origin in patients with ET. Like patients with other thromboembolic diseases, ET patients show higher levels of platelet-derived microparticles than healthy subjects.[4,5,27] This is not necessarily attributable to higher numbers of platelets because neither in ET patients nor in controls platelet did microparticle numbers correlate with platelet numbers. This lack of correlation was also observed in an earlier study,[28] suggesting that microparticle formation may be a regulated rather than a constitutive process. In spite of the large proportion of platelet-derived microparticles in ET patients, the actual number of these microparticles with markers of platelet activation (CD62P and CD63) was not increased. This could be because most ET patients were on anti-thrombotic drugs at the time of blood sampling, which may have affected markers of platelet activation; aspirin inhibits the expression of CD62P and CD63 on platelets.[29]

Remarkably, half of the ET patients showed a large increase in CD62E-positive microparticles. These CD62E-positive microparticles were not normal endothelial microparticles since they co-expressed CD41, a platelet marker, a finding that we did not observe in other conditions characterized by endothelial perturbation, including diabetes mellitus[20] and renal failure (*data not shown*). Chirinos *et al.*[8] described marked elevations of CD62E-positive microparticles in patients with venous thromboembolism, but co-expression with platelet mark-

ers was not investigated. CD62E, or E-selectin, is an adhesion molecule that mediates contact between endothelial cells and other cells, including platelets. Normal resting endothelial cells do not express E-selectin, but a soluble form of this molecule is released from activated cells.[30] The presence of CD62E-positive microparticles suggest endothelial activation, a finding substantiated by the higher levels of mature vWF in ET. The observation that this was not accompanied by a rise in propeptide levels suggests that the endothelial activation is chronic rather than acute in nature.[25]

However, these CD62E-positive microparticles co-express CD41, a platelet marker. CD62E was not observed on platelets from controls or ET patients (*data not shown*). An explanation for this double positivity could be an interaction between platelets (or platelet fragments) and endothelial cells resulting in cellular activation and generation of microparticles of bilineage origin. Circulating microparticles with characteristics of two distinct cell populations have been described, and substantiated by confocal immunofluorescence microscopy.[10] Membrane transfer from microparticles to cells resulting in expression of cell lineage-unrelated receptors is also a recognized phenomenon.[31,32] In these cases microparticles express antibodies of both original cell types, thereby showing that they are the result of direct or indirect cell-cell contact, in this case endothelial cell/platelet activation. It is also conceivable that microparticles acquire CD62E during their formation via expression by the microparticle source, the activated platelet. Passive adherence of CD62E was considered as an alternative explanation. However, we consider this unlikely since there was no relation between microparticle CD62E expression and soluble E-selectin levels and removal of the microparticles from plasma by centrifugation did not affect soluble E-selectin levels, indicating a very low quantity of CD62E on microparticles as compared to soluble E-selectin in the plasma of patients as well as of controls. A low quantity of CD62E on microparticles as compared to soluble E-selectin in the plasma was shown previously for septic patients.[33]

Increased numbers of CD41/CD62E-positive microparticles may be of pathophysiological significance since they appear to be related to risk factors for thrombosis in ET. A relation between these microparticles and thrombosis was not apparent in our present study, but the study was not designed to detect such a relation, and had a limited sample size and short follow-up.

Higher numbers of CD66acde and CD66b-positive microparticles in ET are likely to be related to granulocyte activation in this condition.[18] The level of TF-positive microparticles was increased in ET patients, but such microparticles accounted for less than 1% of all microparticles in this study, and it is unclear whether this difference is clinically relevant. In this respect, the absence of a correlation between TF-positive microparticles and clinical parameters, such as a history of thromboembolic events or a positive risk score for thrombosis, may be primarily due to a lack of power. TF expression on platelets has been related to the presence of a *JAK2*^{V617F} mutation,[34] but a correlation was not observed in our study between TF-positive microparticles and the presence of the *JAK2*^{V617F} mutation.

A limitation of this study is that the ages of the control subjects and patients differed significantly. However, we did not find a correlation between older age and microparticles in our study, and such a correlation has not been described in other studies.[35]

The thrombin generation measurements showed an increased peak height for assays with 1 pM of TF and 4 μM of phospholipids, which is compatible with a hypercoagulable state in ET patients.[36] The shorter lag times observed for ET patients whose assays were conducted without additional TF and phospholipids provide evidence for the presence of a procoagulant factor, possibly on the membrane of microparticles, in the plasma of these patients. We suggest that the differences found in thrombin generation are due to microparticles, since removal of phospholipids abolished thrombin generation. This result is compatible with the observation by Pereira *et al*.[37] that thrombin generation is largely dependent on the number of microparticles in plasma, the only available phospholipid source in such a system. We found a correlation between peak height and the total number of microparticles, as well as for lag time and microparticles under these conditions, suggesting that phospholipids from microparticles and intrinsic or extrinsic (TF) coagulation activators in plasma and/or on the membrane of microparticles do indeed account for the observed differences that were abolished after removal of microparticles by centrifugation. In the light of the previously described evidence of the presence of non-microparticle bound functional TF in human plasma,[38] future studies should address the question of whether a particular form of plasma TF contributes to the observed differences between microparticle-rich ET plasma and normal plasma.

In conclusion, ET patients have higher numbers of microparticles expressing platelet and endothelial markers, suggesting ongoing endothelial activation. This is confirmed by a signature of chronic endothelial activation given by an elevated level of mature vWF in the presence of a relatively low level of propeptide. Microparticles from ET patients are associated with increased thrombin generation, shortened lag time and increased peak height. CD41/CD62E-positive microparticles are elevated only in ET patients with risk factors for thrombosis. These findings suggest a role for microparticles in thrombosis in ET and this deserves further prospective studies.

Authorship and Disclosures

MCT: collection/analysis of clinical and experimental data, flow cytometry, writing of the paper; MvS: collection/analysis of experimental data, flow cytometry, writing of the paper; MM: thrombin generation experiments, discussion of the data; HS: analysis and interpretation of thrombin generation data; HtC: discussion of the data; AL, WT: study design, data analysis, writing of the paper; AF: study design, data analysis, clinical care of the patients.

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