Increased platelet and leukocyte activation as contributing mechanisms for thrombosis in essential thrombocythemia and correlation with the JAK2 mutational status

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Background and Objetives. The mechanisms accounting for the increased risk of thrombosis in patients with essential thrombocythemia (ET) are not well known. The aim of the present study was to ascertain the role of platelet and leukocyte activation in the thrombosis of ET.

Design and Methods. The activation status of platelets and leukocytes was assessed by flow cytometry studies in 49 patients with ET (22 with previous thrombosis and 27 without a history of thrombosis) and in a group of age- and sex-matched healthy individuals. The assessment included platelet P-selectin expression (measured both at baseline and after stimulation with ADP, thrombin, arachidonic acid (AA), and collagen), platelet-neutrophil and platelet-monocyte complexes, determination of CD11b in the neutrophils and monocytes, and expression of tissue factor in the monocytes (mTF). The JAK2 V617F mutation was studied and correlated with platelet and leukocyte activation.

Results. As compared with controls, ET patients had significantly higher values of baseline P-selectin and thrombin- and AA-induced platelet P-selectin expression, as well as higher platelet-neutrophil and platelet-monocyte complexes, neutrophil CD11b expression and baseline mTF expression. Platelet P-selectin, monocyte CD11b, and lipopolysaccharide-induced mTF expression was significantly higher in ET patients with a history of thrombosis than in patients without thrombosis. Patients with the JAK2 V617F mutation or thrombosis showed higher baseline and AA-induced platelet P-selectin expression than did those without thrombosis.

Interpretation and Conclusions. These results would support a role for platelet and monocyte activation in the thrombosis of ET. In these patients, the presence of the JAK2 V617F mutation is associated with higher platelet activation.

Key words: essential thrombocythemia, thrombosis, platelet activation, leukocyte activation, JAK2 mutation.

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Essential thrombocythemia (ET) is a myeloproliferative disorder associated with an increased tendency to thrombosis.[1,2] Since its treatment is primarily aimed at preventing the appearance of such a complication,[1] identification of patients at higher risk of thrombosis is important. The pathogenesis of the thrombosis in ET is not well known. Thrombocytosis and the morphological, biochemical and functional abnormalities of the platelets are considered possible mechanisms,[3] but no clear correlation has been found between such abnormalities and the occurrence of thrombosis. Based on the presence of an activated neutrophil phenotype, it has been suggested that leukocytes have a role in the thrombosis of ET.[4] Platelets and leukocytes interact and modulate each other's functions. In this sense, an increase in circulating platelet-leukocyte complexes has been observed in subjects with unstable angina, myocardial infarction and stroke.[5,6] Such an increase has also been found in the myeloproliferative disorders, suggesting a role in the thrombosis of ET.[7] Tissue factor (TF), a glyco-protein synthesized by monocytes and considered the main initiator of blood coagulation and thrombus formation,[8,9] has also been implicated in the thrombosis of several conditions,[9] but information on its role in the thrombosis of ET is scarce.

Recently, a gain-of-function mutation exchanging valine to phenylalanine at position 617 (V617F) of the Janus kinase 2 (JAK2) protein has been identified in most patients with polycythemia vera and half of those with ET or idiopathic myelofibrosis.[10-13] In one study, patients with chronic myeloproliferative disorders harboring the JAK2 V617F mutation had a higher rate of thrombotic complications than those with wild-type JAK2.[13] The aim of the present study was to determine the role of platelet and leukocyte activation in the thrombosis of ET. For such purpose, the activation status of platelets and leukocytes was assessed by flow cytometry studies in patients with and without thrombosis. Additionally, the JAK2 V617F mutation status was studied and correlated with the results of platelet and leukocyte activation.

Design and Methods

Patients and diagnostic criteria

Fifty-three patients diagnosed with ET according to the criteria of the Polycythemia Vera Study Group[14] at the Hospital Clínic of Barcelona between 1990 and 2003 were studied. Forty-nine age- and sex-matched healthy individuals served as controls. Thrombosis was defined as a history of major or minor occlusive vascular events. Major events[2] included: (i) central nervous system (CNS) vascular complications, including transient ischemic attacks (TIA), stroke, and retinal vessel thrombosis; (ii) cardiac vascular complications (angina pectoris and myocardial infarction) documented by electrocardiographic and enzymatic findings; (iii) intra-abdominal vascular complications, including Budd-Chiari syndrome and splenic-portal thrombosis,[15] and (iv) peripheral thrombosis, such as intermittent claudication of the legs, peripheral arterial thrombosis, deep venous thrombosis, and pulmonary thromboembolism. Minor events were: (i) erythromelalgia and (ii) superficial thrombophlebitis of the extremities. Microvascular arterial disturbances (acroparesthesias, tinnitus, dizziness, headache or visual complaints) were not considered thrombotic events.

To exclude underlying prothrombotic disorders, all subjects were screened for antithrombin, protein C activity, total and free protein S, plasminogen activity, activated protein C resistance, factor V Leiden mutation, prothrombin gene G20210A mutation, lupus anticoagulant and anticardiolipin antibodies as described elsewhere.[15]

Samples and reagents

Blood was obtained from an antecubital vein through a 21-gauge butterfly needle with a light tourniquet. After the first 5 mL of blood had been discarded, 4.5 mL of blood were collected into a citrate-containing tube (0.129 M, 3.8%, Vacutainer system, Becton Dickinson, San Jose, CA, USA). All studies were started within ten minutes after sample collection. Phosphate-buffered saline (PBS) was used as universal diluent (pH 7.4, Roche, USA). PE-Cy5 mouse anti-CD42b directed against the platelet glycoprotein Ib was used to identify platelet and leukocyte-platelet complexes and FITC-conjugated mouse anti-human CD62 monoclonal antibody was used to detect platelet P-selectin expression. PE-conjugated mouse anti-human CD11b monoclonal antibody was used for neutrophil and monocyte CD11b expression. Monocytes were identified with FITC-conjugated anti-CD14 monoclonal antibody. Monocyte tissue factor expression was determined by PE-conjugated anti-CD142 monoclonal antibody. Anti-CD42b, anti-CD62, anti-CD11b, anti-CD14 and anti-CD142 were purchased from Becton Dickinson (San Josè, CA, USA). Irrelevant subclass-matched monoclonal antibodies served as negative controls. Electronic compensation was used to remove spectral overlap and aligned daily with CaliBrite beads (Becton Dickinson). The DNA stain, DRAQ-5 (BioStatus, Shepshed, UK), was used to distinguish intact nucleated cells from non-nucleated cells, damaged nucleated cells and cell aggregates. The human thrombin, the tetrapeptide glycyl-L-prolyl-L-arginyl-L-proline (GPRP) and the lipopolysaccharide (LPS, from E. coli, strain 026: B6) were obtained from Sigma Chemical (St Louis, MO, USA). Adenosine diphosphate (ADP) and collagen were purchased from Arkray (Aggrepack, Japan) and arachidonic acid (AA) from Menarini Diagnostica (Milan, Italy).

Platelet P-selectin determination by flow cytometry

For the platelet P-selectin assay, a whole blood flow cytometry method was used as described by Shattil et al.[16] and following the recommendations of the European Working Group on Cell Analysis.[17] To minimize artificial platelet activation, no washing, centrifugation or fixation steps were performed. Thus, 5 µL of whole blood were aliquoted in a polypropylene tube containing 50 µL of PBS solution, anti-CD42b-PE and anti-CD62P-FITC. For the activation assay, ADP (final concentration 10 µM), thrombin (0.1 U/mL) plus GPRP (2.5 mM), collagen (10 µg/mL) or AA (0.1 mM) were added to diluted whole blood with saturating monoclonal antibodies. After 15 minutes of incubation at room temperature, samples were resuspended in 1 mL of PBS before analysis.

Flow cytometry data were collected on a FACScan (BDIS, San Josè, CA, USA). Samples were analyzed in a side scatter and the fluorescence data obtained in the logarithmic mode. Single platelet populations were identified in whole blood by their characteristic side scatter and CD42b positivity. Ten thousand events were collected from each sample. Baseline and agonist-induced activation of platelet membrane P-selectin expression was measured in arbitrary units of mean fluorescence intensity (MFI) and as the percentage of positive platelets compared with a negative control. Platelet P-selectin expression was quantified by converting MFI values into molecules of equivalent soluble fluorochrome (MESF) units using standardized fluorescent beads (Quantum™ FITC and Quantum™ PE Medium Level, BangsLabs, USA).

Determination of platelet-leukocyte complexes

Platelet-neutrophil (PNC) and platelet-monocyte (PMC) complexes were measured by flow cytometry using an adapted no-lyse, no-wash method.[15] Briefly, 125 µL of a PBS-diluted blood sample containing 0.5×10^6 leukocytes were incubated for 15 minutes at room temperature with anti-CD42b-PE, anti-CD14-FITC and DRAQ-5. Before analysis, 125 µL of PBS solution were added to the tube. Neutrophils were selected in a forward versus side scatter dot plot and monocytes were selected in a FL-1 versus side scatter dot plot by gating CD14 positive cells. After gating neutrophil and monocyte populations, PNC and PMC were identified as those events expressing the platelet marker CD42b, selected in a FL-2 versus side scatter dot plot. Results were expressed as a percentage of the neutrophils or the monocytes, respectively.

Determination of CD11b in neutrophils and monocytes and tissue factor in monocytes by flow cytometry

Neutrophil and monocyte membrane CD11b expression was measured as previously described.[15] Monocyte membrane tissue factor (mTF) expression was measured as described[15] and adapted from the method by Amirkhosravi et al.[18] A blood sample containing 0.5×10^6

leukocytes was diluted in PBS buffer. For the baseline assay, 125 μL of the sample dilution were labeled during 15 minutes at room temperature with anti-CD14-FITC, anti-CD11b-PE and DRAQ-5 for the CD11b determination or anti-CD14-FITC, CD142-PE, and DRAQ-5 for the mTF. For the activation assay, two aliquots were incubated with LPS (10 μg/mL at 37°C for 1 hour) and subsequently stained for 15 minutes with anti-CD14-FITC, anti-CD11b-PE and DRAQ-5 or anti-CD14-FITC, anti-CD142-PE and DRAQ-5 for the CD11b and mTF determination, respectively. An acquisition threshold was set in the red fluorescence channel (FL-3) to exclude erythrocytes. Sample acquisition was performed at a low-medium rate (150 events/second). Nucleated cells were labeled with DRAQ-5 and measured through a 670 LP filter (FL-3). Flow cytometric acquisition and analysis of at least 2,500 monocytes were performed. A side scatter *versus* FL-3 dot plot was used to distinguish nucleated cells from erythrocytes and debris, and in this first dot plot a region was created in order to exclude leukocyte aggregates from the analysis. Neutrophils were selected in a forward *versus* side scatter dot plot. CD11b histograms were then obtained from a second gate, selecting CD11b positive events in a FL-2 versus side scatter dot plot. Monocytes were selected in a FL-1 versus side scatter dot plot by gating CD14 positive cells. For the measurement of CD11b and TF, FL-2 histograms were obtained from gated monocytes. CD11b was measured in MFI arbitrary units and tissue factor also in MFI units and as the percentage of CD14 positive cells staining positive for CD142. The positive delineator was determined by gating 1% background staining on the isotype control fluorescence. As for platelet P-selectin, the expression of neutrophil and monocyte CD11b and monocyte tissue factor was also quantified in MESF units.

Allele-specific polymerase chain reaction (PCR) for JAK2 V617F mutation

Genomic DNA was extracted from blood samples using a QIAamp DNA Mini kit (Qiagen, Germany). PCR was used to amplify the portion of the JAK2 region that acquires the JAK2 V617F mutation. An amplicon product of 360 bp was generated using primers conjugated with fluorescent dyes JAK2F: (6-FAM) GGTTTCCTCAGAACGTTGATGG and JAK2R: (5-HEX) CCTAGCTGTGATCCTGAAACTGAAT and then digested with the BsaXI restriction enzyme (New England Biolabs, Hitchin, UK). Digested products were detected in an ABI 310 sequence detector (Applied Biosystems, Foster City, USA) using the Genescan software. Samples harboring the *JAK2* V617F mutation no longer have the BsaXI restriction site.

Statistical methods

The SPSS 10.0 statistical package (SPSS Inc., Chicago, IL, USA) was used. The results of platelet-leukocyte complexes and platelet P-selectin, neutrophil and monocyte CD11b and tissue factor expression were expressed as the mean and 95% confidence intervals. Differences between groups were analyzed with one-way ANOVA followed by post-hoc analysis using the Student-Newman-Keuls test. The χ^2 test was used to compare categorical variables and the Mann-Whitney U test to compare continuous variables between the groups. All p values were two-sided. p values <0.05 were considered statistically significant.

Results

Clinical and hematologic data and JAK2 V617F mutational status

No patient had protein C, protein S, plasminogen or antithrombin deficiency. Four patients with thrombosis were excluded from the study because they were demonstrated to have a thrombophilic factor (two cases each of prothrombin gene G20210A mutation carriership and antiphospholipid antibodies). Therefore, 49 patients (26 males and 23 females, median age 68 years, range: 28-91) were finally analyzed. The median plasma concentrations of natural anticoagulants did not differ in the two ET groups (*data not shown*). In three patients the diagnosis of ET and the thrombosis were simultaneous, in two the thrombosis preceded the diagnosis of ET, whereas in the remaining 17 it was a complication of a known ET. Table 1 summarizes the vascular events registered in 22 ET patients. In 19 patients it was a major complication and it affected the arterial circulation in the same number of cases. The median interval between ET diagnosis and blood sampling was 86 months (range: 16-231) for patients with thrombosis and 59 months (range: 11-210) for patients without thrombosis (p not significant). The median interval between thrombosis and blood sampling was 61 months (range: 4-154). No patient was studied during the acute phase of the thrombosis. At the time of study, patients were receiving different therapies (Table 2). Controls had higher hemoglobin concentrations and, leukocyte, neutrophil and monocyte counts than had patients, probably related to the use of hydroxyurea, whereas patients had significantly higher platelet counts.

The JAK2 mutation was detected in 21 out of 50 assessable patients (42%). At diagnosis, ET patients with the JAK2 mutation had higher hemoglobin levels than those without the mutation (140 g/L, 95% CI: 134-146 vs 131 g/L, 95% CI: 128-135, p=0.01). Age, sex, disease duration and proportion of patients with a history of thrombosis were not different depending on the presence or not of the mutation.

Baseline and agonist-induced platelet P-selectin expression

As shown in Table 3, in unstimulated samples, ET patients with thrombosis had a significantly higher percentage of platelets expressing P-selectin than had patients without thrombosis and controls (p=0.0001 for both comparisons). Thrombin-induced stimulation increased the percentage of platelets expressing P-selectin in ET patients (p=0.001, Table 3). Following AA activation, ET patients with thrombosis had a significantly higher mean percentage of platelet P-selectin expression than had patients without thrombosis and controls (p=0.0001, Table 3). AA-induced platelet P-selectin expression in MESF units was significantly higher in ET patients than in the controls (p=0.0001, *data not shown*), but no differences were observed between patients with

Table 1. Occlusive vascular events in 22 patients with essential thrombocythemia.

	Number of patients (%)*
Erythromelalgia	6 (27)
Transient ischemic attack	6 (27)
Stroke	4 (18)
Myocardial infarction	4 (18)
Intermittent claudication	3 (14)
Deep vein thrombosis	3 (14)
Superficial thrombophlebitis	2 (9)
Budd-Chiari syndrome	2 (9)

Some patients had more than one type of event: erythromelalgia and myocardial infarction (n=2), deep venous thrombosis, pulmonary thromboembolism and intermittent claudication (n=1), deep venous thrombosis and pulmonary thromboembolism (n=1), myocardial infarction and intermittent claudication (n=1), recurrent superficial thrombophlebitis and deep venous thrombosis (n=1), superficial thrombophlebitis and transient ischemic attack (TIA) (n=1), and recurrent TIA and stroke (n=1).

and without thrombosis. No differences in P-selectin expression between patients and controls were observed following stimulation with ADP or collagen (Table 3).

Platelet-leukocyte complexes

ET patients had higher percentages of circulating PNC and PMC (Table 4, *p*=0.0001) but no difference was found between patients with and without thrombosis. A positive correlation was seen between P-selectin expression and both PNC (R=0.529, *p*=0.01) and PMC percentages (R=0.470, *p*=0.01).

Leukocyte CD11b expression

The results of neutrophil and monocyte CD11b expression are shown in Table 4. Significant differences were detected among the three groups in the baseline CD11b leukocyte expression. Patients with thrombosis showed higher monocyte baseline CD11b expression than did patients without thrombosis and controls (*p*=0.0001 for all comparisons, Figure 1). Although ET patients had a significantly higher neutrophil CD11b expression than had the controls, the expression was not significantly higher in patients with thrombosis. Following LPS activation, an increase in CD11b expression was seen in the three groups, with ET patients showing the highest values (Table 4).

Monocyte tissue factor expression

When expressed in MESF units, mTF expression was higher in the two ET groups both at baseline and following LPS activation (Table 5). LPS activation increased mTF expression in all groups, but the percentage of positive monocytes was higher in patients with thrombosis than in those without thrombosis and in the controls (*p*=0.0001 in all cases, Figure 2).

Correlation between JAK2 mutation and platelet and leukocyte activation

The results of platelet and leukocyte activation in ET patients according to JAK2 mutational status are shown in Table 6. As can be seen, the median percentage of platelets expressing P-selectin both at baseline and after stimulation with AA were significantly higher in patients with the mutation than in those with the wild-type allele

Table 2. Treatment administered to 49 patients with essential thrombocythemia (ET).

	ET without thrombosis (n=27)	ET with thrombosis (n=22)
No therapy	3 (11%)	1 (5%)
Cytolytic drug	23 (85%)	21 (95%)
Hydroxyurea	13 (48%)	14 (64%)
Anagrelide	3 (11%)	4 (18%)
^{32}Phosphorus	7 (26%)	3 (14%)
Aspirin (100 mg)	4 (15%)	8 (36%)
Ticlopidine	1 (4%)	4 (18%)
Acenocoumarol	3 (11%)	4 (18%)

Data presented are number of patients (percentages are given in parentheses). There were no significant differences between the two groups using the χ^2 test.

Table 3. Percentages of platelets expressing P-selectin at baseline and after agonist-induced activation in healthy controls and ET patients.

	Controls (n=49)	ET without thrombosis (n=27)	ET with thrombosis (n=22)	p value*
Baseline	3.1 (2.7-3.5)	4.7 (3.8-5.5)	6.3 (5.4-7.1)	0.0001°
ADP	51 (48-55)	57 (51-62)	56 (50-63)	NS
Thrombin	38 (33-46)	55 (46-64)	54 (46-63)	0.001
Collagen	9 (8-11)	9 (8-11)	10 (8-12)	NS
Arachidonic acid	37 (30-45)	63 (53-73)	81 (73-89)	0.0001°

*Values given as mean and 95% confidence intervals (in parentheses); NS: not significant; ADP: adenosine diphosphate; *one-way ANOVA test followed by post-hoc analysis using the Student-Newman-Keuls test for paired comparisons; the p values refer to the comparisons between controls and ET patients with or without thrombosis; °significant difference among the three groups.*

Table 4. Platelet-leukocyte complexes and neutrophil and monocyte CD11b expression in healthy controls and ET patients.

	Controls (n=49)	ET without thrombosis (n=27)	ET with thrombosis (n=22)	p value*
PNC	17 (13-22)	56 (52-63)	60 (53-67)	0.0001
PMC	33 (28-41)	81 (75-88)	85 (78-91)	0.0001
Baseline CD11b				
Neutrophil	32 (27-37)	84 (63-106)	93 (70-115)	0.0001
Monocyte	62 (53-72)	128 (111-146)	157 (138-177)	0.0001°
LPS-induced CD11b				
Neutrophil	178 (161-187)	280 (248-312)	303 (259-348)	0.0001
Monocyte	162 (157-189)	244 (215-272)	263 (230-295)	0.0001

*PNC (platelet-neutrophil complexes) and PMC (platelet-monocyte complexes) are given as percentages and CD11b in MESF (molecules of equivalent soluble fluorochrome) units ×10³; Values given as mean and 95% confidence intervals (in parentheses); LPS: lipopolysaccharide; *one-way ANOVA test followed by post-hoc analysis using the Student-Newman-Keuls test for paired comparisons; the p values refer to the comparisons between controls and ET patients with or without thrombosis; °significant difference among the three groups.*

(Figure 3A and 3B). Moreover, baseline, thrombin- and AA-induced platelet P-selectin expression in MESF units were significantly higher in ET patients with the mutation than in those without (*data not shown*). Higher values of platelet-leukocyte complexes and leukocyte CD11b and mTF expression were observed in ET patients with the JAK2 mutation when compared with those with the wild-type, but the difference did not reach statistical sig-
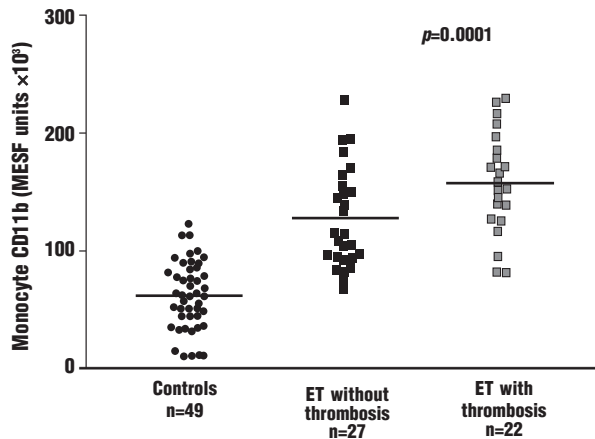
Figure 1. Baseline monocyte CD11b expression from the three groups in molecules of equivalent soluble fluorochrome (MESF) units. The horizontal lines represent the median values.
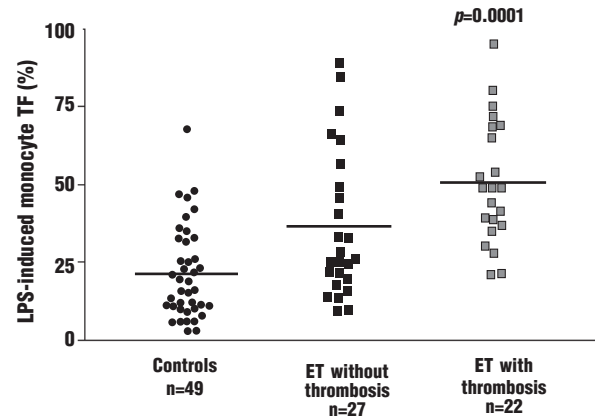

Figure 2. Percentage of monocytes expressing tissue factor (TF) after lipopolysaccharide (LPS) activation in the three groups in study. The horizontal lines represent the median values.

Table 5. Baseline and LPS-induced monocyte tissue factor expression in healthy controls and ET patients.

	Controls (n=49)	ET without thrombosis (n=27)	ET with thrombosis (n=22)	p value*
Baseline				
%	7 (4-8)	10 (6-14)	9 (7-12)	NS
MESF	9 (8-10)	15 (14-17)	14 (13-15)	0.0001
Lipopolysaccharide				
%	21 (16-26)	36 (27-46)	51 (42-60)	0.0001°
MESF	10 (10-11)	18 (16-20)	19 (17-22)	0.0001

Values given as mean and 95% confidence intervals (in parentheses) and expressed as percentage of positive monocytes and as intensity of the expression in MESF (molecules of equivalent soluble fluorochrome) units ×10³; NS: not significant; *one-way ANOVA test followed by post-hoc analysis using the Student-Newman-Keuls test for paired comparisons; the p values refer to the comparisons between controls and ET patients with or without thrombosis; °significant difference among the three groups.

Table 6. Comparison of platelet and leukocyte activation parameters of ET patients with and without the JAK2 V617F mutation.

	Wild-type JAK2 (n=29)	JAK2 V617F (n=21)	p value
Platelet P-selectin, %			
Baseline	4.7 (4-5)	6.4 (5-7)	0.006
ADP	54 (49-59)	61 (54-67)	NS
Thrombin	56 (49-64)	53 (41-62)	NS
Collagen	9 (7-11)	10 (9-12)	NS
Arachidonic acid	63 (53-73)	80 (72-87)	0.03
Platelet-neutrophil complexes, %	57 (51-62)	59 (52-66)	NS
Platelet-monocyte complexes, %	82 (77-88)	84 (77-90)	NS
Baseline neutrophil CD11b, MESF	81 (63-99)	92 (65-118)	NS
Baseline monocyte CD11b, MESF	135 (118-151)	148 (125-171)	NS
Baseline monocyte TF, %	8 (5-11)	9 (6-11)	NS
LPS-induced monocyte TF, %	41 (33-49)	43 (32-55)	NS

Values given as mean and 95% confidence intervals (in parentheses) and expressed as percentage or as intensity of the expression in MESF (molecules of equivalent soluble fluorochrome) units ×10³; NS: not significant; ADP: adenosine diphosphate; LPS: lipopolysaccharide; TF: tissue factor.

nificance (Table 6). Following LPS activation, neutrophil CD11b expression was higher in patients with the mutation (310 MESF×10³, 95% CI: 263-358) than in those without (267 MESF×10³, 95% CI: 241-294) and in the controls (178 MESF×10³, 95% CI: 161-187) (*p*=0.0001 for all comparisons). In addition, LPS-induced mTF expression in MESF units was significantly higher in ET patients with the mutation (19×10³, 95% CI: 17-22) than in those without (17×10³, 95% CI: 15-19) and in the controls (10×10³, 95% CI: 9-11) (*p*=0.0001, for all comparisons). No influence of treatment modality, type of thrombosis or presence of recurrent thrombosis was noted for any of the parameters analyzed in the study (i.e., the platelet and leukocyte activation parameters and the JAK2 mutational status).

Discussion

P-selectin (CD62P) is constitutively expressed on the internal membrane of the α granules of the platelets and is translocated to the platelet surface upon stimulation.[19] It may be involved in platelet-platelet and shear-induced platelet aggregation[19] and in the genesis of leukocyte-derived microparticles containing active tissue factor that enhance fibrin deposition.[20] In the present study, the platelets of ET patients with thrombosis showed an increased P-selectin membrane expression at baseline and after AA-induced activation. This is in keeping with the results of a recent study in patients with myeloproliferative disorders and arterial thrombosis[21] and supports a role for platelet activation in the genesis of thrombosis in ET. This could be explained by the clinical and experimental evidence of increased cyclo-oxygenase platelet activity and thromboxane A2 generation in ET.[3,22,23] In the present study, a significant increase in P-selectin expression was observed in ET patients following thrombin stimulation. In this sense, Jensen *et al.*[24] found decreased MESF P-selectin expression following platelet activation induced by either ADP or thrombin receptor activating peptide (TRAP) in both ET and polycythemia vera, a disparity that might reflect differences in patient selection or in the flow cytometry methods used. In the present study, patients with ET had higher percentages of circulating PNC and PMC than the controls. Besides, a positive
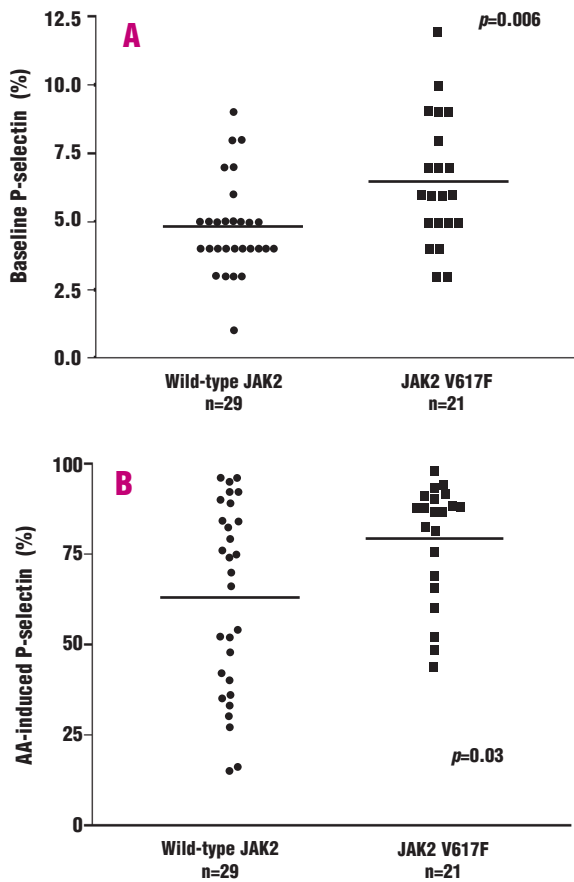
Figure 3. Flow cytometry quantification of platelet P-selectin expression at baseline (**A**) and following arachidonic acid (AA) activation (**B**) in ET patients based on the mutational status of JAK2. The horizontal lines represent the median values.

correlation was found between P-selectin percentage, platelet count, and circulating PNC and PMC, in agreement with results recently reported in patients with myeloproliferative disorders.[7,15,25] Enhanced platelet-leukocyte interaction is known to facilitate leukocyte adhesion, activation and tissue factor transfer between monocytes and platelets.[8,9,26,27]

Integrin CD11b is the α_M subunit of the CD11b/CD18 ($\alpha_M \beta_2$, Mac-1) heterodimer responsible for firm attachment of the leukocytes to endothelium and platelets.[28] Upon activation, the leukocytes upregulate membrane CD11b expression promoting their adherence to the endothelium, phagocytosis, homotypic aggregation, adhesion-dependent respiratory burst, and degranulation.[26,28] In addition, the phagocytes, especially monocytes, are involved in the process of fibrin deposition and modulate the activation of blood coagulation through the release of TF, proteases and reactive oxygen species.[27] Baseline monocyte CD11b expression was significantly higher in ET patients with thrombosis. Increased baseline neutrophil CD11b expression was also observed in ET patients, although those with thrombosis did not have a higher expression. It is possible that the increase in leukocyte and platelet activation contributes to the local lesion of the endothelium and/or the progression of the coagu-

lation reactions by engagement of the adhesion receptors and release of proteases or cytokines. Data on mTF expression in ET are scarce.[29] In our study, ET patients had higher mTF expression than had the controls, and patients with thrombosis had a higher percentage of monocytes with LPS-induced TF expression. Since the monocyte is considered a key cell in the coagulation process,[27] our data suggest that increased mTF expression can be important in the pathogenesis of the thrombosis in ET. It could be hypothesized that up-regulation of the phagocyte CD11b antigen could be due to the clonal hematopoietic proliferation of ET. In this sense, mTF overexpression would reflect both cell activation status and CD11b up-regulation. Enhanced monocyte-platelet interaction would be a second possibility.[9,19,20]

The frequency of the JAK2 V617F mutation observed in our patients was similar to that reported by other authors.[10-13,30-32] The lack of association between the *JAK2* mutation and the incidence of thrombotic events is in agreement with the findings of two recent studies in ET patients.[31,32] A significant association between the JAK2 mutation and increased baseline and thrombin- or AA-induced platelet P-selectin expression was found in the present study. This observation would suggest a possible JAK2-mediated effect on platelet P-selectin expression and is consistent with the presence of JAK2 in human platelets and its subsequent phosphorylation on challenge with thrombin.[33] The current evidence of the role of thrombopoietin in platelet activation and its enhanced response after agonist stimulation via the JAK2 pathway signaling[34] could explain in part our findings. Besides, ET patients with the JAK2 mutation showed higher expression of neutrophil CD11b and mTF in MESF units after LPS stimulation, which would be consistent with the involvement of JAK2 in LPS-induced leukocyte activation.[35]

Although in our study no differences in P-selectin, CD11b or mTF expression were seen depending on the use of hydroxyurea, a lower incidence of arterial thrombosis has recently been reported in ET patients treated with hydroxyurea plus low-dose aspirin as compared to in those receiving anagrelide plus low-dose aspirin.[36] Whether increased P-selectin, CD11b or mTF could identify ET patients at higher thrombotic risk remains to be confirmed in prospective studies.

In conclusion, ET patients with previous thrombosis show a marked increase in platelet and especially monocyte activation, whereas the presence of the JAK2 mutation is associated with higher platelet activation in these patients. Such alterations might play a role in the pathogenesis of the thrombosis of ET but further studies are required to ascertain whether such findings could identify ET patients at greater thrombotic risk.

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