Assessment of platelet activation in myeloproliferative disorders with complementary techniques

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Bleeding and thrombosis in myeloproliferative disorders (MPD) are common events, sometimes both are present in the same patient during the course of the disease. Platelet activation in patients with MPD is often suggested. The present study analyses the presence of circulating activated platelets, using simultaneously flow cytometry and aggregometric studies in MPD. We studied 28 patients: 13 with polycythaemia vera, seven with essential thrombocythaemia, and eight chronic myeloid leukaemia. We performed functional tests, aggregation and adenosine triphosphate (ATP) release and flow cytometric assays (mepacrine staining and platelet activation markers CD62, CD63 and fibrinogen binding (B-FG)). Twenty-one MPD samples (75%) had reduced aggregation and ATP release. Acquired δ-SPD was detected in 11 of 28 MPD patients (39%), and we found no association between reduced mepacrine labelling and abnormal ATP release. High levels of activation markers were obtained: CD62 in 19 of 28 patients (68%), CD63 in 13 of 28 patients (46%) and B-FG in 19 of 28 patients (68%). The most prevalent abnormality was a reduced aggregation and ATP release. The lack of association between ATP release and mepacrine labelling suggests that other mechanisms, besides the deficit of intraplatelet ATP/adenosine diphosphate, might occur. High levels of activation markers were also observed. We conclude that both tests are complementary and necessary to understand the functional status of platelets in MPD. *Blood Coagul Fibrinolysis* 15:235–240 © 2004 Lippincott Williams & Wilkins.

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Introduction

Bleeding and thrombosis in myeloproliferative disorders (MPD) are common events; sometimes both are present in the same patient during the course of the disease [1]. Thrombosis is a major problem in polycythaemia vera (PV) and essential thrombocythaemia (ET); nevertheless, the risk of thrombosis in chronic myeloid leukaemia (CML) is very low even with thrombocytosis [2]. Thrombosis has been attributed to platelet activation or platelet abnormalities as a consequence of clonal megakaryocyte proliferation [3]. This view is supported by the observation that most patients with MPD have elevated plasma levels of platelet alpha-granule proteins [4,5] and an acquired dense granule storage pool defect [6]. However, this defect of MPD platelets may be due to an intrinsic abnormality of storage organelles and/or alterations in the secretion mechanism rather than to platelet activation in the circulation [7]. Interestingly, Jensen *et al.* [8] demonstrated in whole blood flow cytometric assays that an increased alpha-granule secretion of non-stimulated platelets occurs in MPD; but the response of platelets – in terms of alpha and lysosomal secretion and activation of the glycoprotein (GP) IIb–IIIa complex – was reduced in patients following stimulation with a strong agonist, and to a lesser extent with a weak agonist. Such observations suggested that, in MPD, an intrinsic cellular defect in receptor-mediated granule secretion and redistribution of membrane GPs might occur.

Also, platelet aggregation studies using whole blood or platelet-rich plasma (PRP) have revealed a tendency towards spontaneous platelet aggregation, suggesting increased *in vivo* activation [9] and impaired platelet aggregation on platelet agonist [adenosine diphosphate (ADP), adrenaline (ADR)] stimulation [10,11].

The aim of the present study was to analyse the presence of circulating activated platelets, using simultaneously whole blood flow cytometric and aggregometric studies in MPD patients.

Design and methods

Blood collection

Venous blood was collected by clean venipuncture and separated into different tubes to perform all the studies at same time.

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Sample preparation for functional studies

Venous blood was anticoagulated with 3.80% sodium citrate in the ratio 9 : 1 and was centrifuged at $300 \times g$ for 10 min. The platelet-rich supernatant was removed and the remainder centrifuged once more at $1300 \times g$ for 15 min to obtain platelet-poor plasma (PPP). The platelet count of PRP was adjusted to 300×10^9/l using PPP. Platelet aggregation in PRP was studied by the Born optical method [12]. All manipulations were completed within 4 h of collection. We considered normal results when: (i) biphasic aggregation with 10 μmol/l ADR (Diagnostica Stago, Asnieres, France), 2.5 μmol/l and 5 μmol/l ADP (Sigma Chemical Co, St Louis, Missouri, USA) was recorded; and (ii) irreversible aggregation following a lag phase with 1 μg/ml and 8 μg/ml collagen (Horm, München, Germany) and 0.5 mmol/l arachidonic acid (Sigma Chemical Co.) were observed. Results were considered reduced under the following circumstances: (i) when only primary aggregation occurred with ADR or ADP; (ii) when the lag phase for collagen was prolonged three times greater than normal; or (iii) when the amplitude of aggregation in response to any agonist was less than 20%. Adenosine triphosphate (ATP) release from dense granules was measured by the luciferin-luciferase assay [12]; 450 μl PRP at 300×10^9/l were placed in a cuvette and stirred in a lumi-aggregometer (Chrono-log Corporation, Havertown, Pennsylvania, USA) with 25 μl of 10 μg/ml luciferin-luciferase (Sigma Chemical Co.). The reaction was started by the addition of the agonist, after which the aggregation and luminescence were recorded. When aggregation was completed, a 1.81 μmol/l final concentration ATP standard (Sigma Chemical Co.) was added and the peak height recorded. ATP release was calculated by comparison of the peak luminescence recorded from the patient sample against the ATP standard [13].

Sample preparation for flow cytometric studies

The method of whole blood flow cytometric analysis was based on the methods described by Shattil *et al.* [14] and Warkentin *et al.* [15] under conditions that minimized platelet activation. Briefly, 4.50 ml blood was collected into polypropylene tubes containing 0.50 ml ethylenediamine tetraacetic acid (EDTA) 2% plus para-formaldehyde (PFA) 1%. The use of PFA avoids the GP IIb–IIIa complex disruption, inhibits the degrading enzymes present within platelet that might remove surface antigens, and gives a good stability of the stained cells [16]. Within 5 min of collection, 5 μl blood were transferred into polystyrene tube containing 40 μl phosphate-buffered saline (PBS) (pH 7.4) and 5 μl saturating concentration of fluorescein isothiocyanate-conjugate (FITC) or R-phycoerythrin-conjugate (PE) monoclonal antibody. After 30 min incubation at room temperature in the dark, 950 μl PBS were added and the tubes immediately processed for flow cytometry. The study was performed in a FACScan (Becton-Dickinson, San Jose, California, USA). The excitation wavelength was set at 488 nm. The FACScan was used in standard configuration with a 530 nm bandpass filter. In initial experiments, forward angle light scatter (FSCH) was used to distinguish platelet from red blood cells and white blood cells; in our experiments at least 98% of the particles within this population bound CD42b, the antibody to GP Ib. Light scatter and fluorescence data were obtained at a logarithmic setting. FACScan data were analysed with CELL-QUEST analysis software. The monoclonal antibody panel for analysis included the following: CD42b-PE; CD62-FITC, and CD63-FITC (Immunotech, Marseille, France) [17,18].

In order to determinate the percentage of positive platelets, a negative control sample was stained with an isotype-matched non-specific monoclonal antibody. Platelet-bound fibrinogen (B-FG) was detected with rabbit polyclonal anti-human fibrinogen coupled to FITC (Dako Ltd, Glostrup, Denmark) [19]. To determinate the percentage of positive platelets a sample was stained with a rabbit polyclonal FITC-conjugated negative control (Dako).

Mepacrine staining

Quinacrine mustard (Sigma Chemical Co.) was used as fluorescent marker of dense granules [20]. Whole blood was collected into plastic tubes containing 0.50 ml EDTA 2%, then blood (5 μl) was transferred into a polystyrene tube containing 40 μl PBS (pH 7.4) to incubate with mepacrine at 0.1 mmol/l or buffer during 30 min. The stained platelets were re-suspended in 950 μl PBS buffer and kept in the dark until analysed. In order to find out the percentage of positive platelets, a sample without mepacrine was used to measure autofluorescence as negative control.

Patients and control subjects

Control blood was collected from 50 healthy volunteers (30 women and 20 men, age range 20–65 years, mean age 55 years) who had taken neither aspirin nor other anti-inflammatory drugs within the previous 10 days. Those samples were used to establish normal ranges in functional and cytometric studies.

Patients with MPD ($n = 28$) were studied: 13 with PV, seven with ET, and eight with CML. Diagnosis was determined by standard methods including examination of the peripheral blood film and bone marrow, chromosomal, blood and plasma volume studies, and neutrophil alkaline phosphatase score (see Table 1).

The diagnosis of CML was confirmed by the Phi chromosome on cytogenetic analysis. PV and ET were diagnosed by the criteria described elsewhere [21].

Aggregation and ATP release studies or flow cytometric assays were performed on patients' samples ($n = 28$), and one or two healthy controls were done simultaneously.

Statistical analysis

Univariate analysis for association of categorical factors between patients and controls, and between patient groups was performed using Fischer's exact test. Continuous factors were compared using Student's t test. Analysis of correlation was performed using Spearman's rank correlation coefficient. A general two-sided significance level of 5% was applied.

Results

Functional studies

The first wave with ADR was absent in 4 of 28 patients (14%) of MPD, meanwhile the second wave and ATP release were greatly reduced or absent in 16 of 28 patients (57%). With ADP the second wave of aggregation and ATP release was either absent or reduced in 23 of 28 patients (82%). The aggregation response to arachidonic acid was abnormal in eight of 28 patients (28%) and ATP release was reduced in 11 of 28 patients (39%). Aggregation and ATP release with 1 μg/ml collagen was reduced in eight of 28 patients (28%) (see Table 2). Increasing the dose to 8 μg/ml restored aggregation response in all the subgroups but without ATP release (data not shown). The proportion of patients with abnormal ATP release values was significantly higher in ET and PV patients (considered together) than in CML patients (Fischer's exact test, one-tail $P = 0.009$).

Flow cytometric studies

Mepacrine labelling

In normal controls ($n = 50$) the observed range was $75 \pm 15\%$ (mean $\pm$ 2 standard deviations). There was no statistical difference between MPD and normal controls means (Student's t test, $P = 0.071$). The mean of ET and PV together was lower than normal controls (Student's t test, $P = 0.012$). Eleven of 28 patients (39%) with MPD had abnormal mepacrine labelling and absence or markedly reduced ATP release and second wave of aggregation. However, we did not find an association between reduced mepacrine labelling and abnormal ATP release (Fischer's exact test, one-tail $P = 0.191$). When ET and PV patients (considered together) were compared with CML patients, no sig-

Table 1. Clinical data of patients with myeloproliferative disorders

Patient	Age (years)	Sex	Platelet-rich plasma (platelet count) (10^9/l)	White blood cells (10^9/l)	Haematocrit (%)	Haemoglobin (g/dl)
ET 1	54	Female	1000	64.00	47	14.8
ET 2	30	Female	3300	232.00	39	12.8
ET 3	45	Female	1000	66.00	36	11.9
ET 4	70	Female	690	69.00	37	11.6
ET 5	20	Male	2000	52.00	40	12.2
ET 6	66	Female	4000	95.00	38	11.9
ET 7	67	Female	2000	75.00	39	12.0
PV 1	69	Male	455	48.00	42	13.4
PV 2	59	Male	510	109.00	62	17.5
PV 3	36	Male	690	116.00	44	13.7
PV 4	50	Male	515	84.00	51	15.2
PV 5	58	Male	600	164.00	57	11.6
PV 6	71	Female	750	56.00	58	17.1
PV 7	61	Female	450	62.00	50	15.9
PV 8	64	Female	860	286.00	47	10.7
PV 9	45	Female	666	54.00	42	12.5
PV 10	49	Female	447	55.00	41	13.0
PV 11	52	Male	413	37.00	51	15.3
PV 12	65	Female	395	95.00	48	8.3
PV 13	76	Male	865	68.00	44	14.6
CML 1	34	Male	800	93.00	38	12.0
CML 2	30	Male	458	85.00	35	11.1
CML 3	41	Male	313	70.00	40	12.5
CML 4	35	Male	430	242.00	40	12.7
CML 5	76	Male	236	85.00	33	10.5
CML 6	62	Female	1000	125.00	35	11.2
CML 7	51	Male	1072	93.00	41	13.0
CML 8	45	Female	372	554.00	34	10.4
Mean of MPD (range)	52 (20–76)	14 female/14 male	–	–	–	–
Mean of controls (range)	55 (20–65)	30 female/20 male	460 (330–720)	68.00 (49.00–91.00)	43 (35–47)	14.5 (11.8–15.7)

CML, chronic myeloid leukaemia; ET, essential thrombocythaemia; MPD, myeloproliferative disorders; PV, polycythaemia vera.

Table 2. Functional and flow cytometric studies

Patient	Platelet aggregation (ADR, ADP, COL AA)	ATP R	Mepacrine (%)	CD62 (%)	CD63 (%)	B-FG (%)
ET 1	A, A, A, N	A	30	13	9	33
ET 2	NA, A, A, A	A	58	3	5	16
ET 3	A, A, N, N	A	90	14	6	23
ET 4	A, N, A, N	A	60	14	11	44
ET 5	A, A, A, N	A	34	15	17	20
ET 6	NA, A, N, N	A	50	16	4	15
ET 7	A, A, N, N	A	90	7	7	22
PV 1	A, A, N, N	A	83	30	11	33
PV 2	N, A, N, N	N	71	17	15	21
PV 3	A, A, N, N	A	29	21	10	25
PV 4	A, A, N, A	A	40	15	6	11
PV 5	A, A, N, A	A	95	14	4	12
PV 6	N, A, N, N	N	65	7	6	9
PV 7	A, A, N, A	A	50	8	8	10
PV 8	NA, A, A, A	A	67	12	8	23
PV 9	N, A, N, A	A	97	27	21	30
PV 10	A, A, N, N	A	30	19	7	22
PV 11	A, A, N, N	A	62	2	6	16
PV 12	A, A, N, N	A	90	24	11	19
PV 13	A, A, N, A	A	74	18	15	21
CML 1	N, A, N, N	N	78	5	6	9
CML 2	A, A, A, N	A	94	8	6	8
CML 3	N, N, N, N	N	55	6	15	20
CML 4	N, N, N, N	N	88	18	2	19
CML 5	N, N, A, A	A	90	9	5	6
CML 6	A, A, A, N	A	50	21	9	18
CML 7	N, A, N, N	N	79	5	5	6
CML 8	NA, N, N, N	N	85	4	4	5
Normal control (mean ± standard deviation) ($n = 50$)	N, N, N, N	N	75 ± 15	8 ± 2	5 ± 1	11 ± 2

ET, essential thrombocythaemia; PV, polycythaemia vera; CML, chronic granulocytic leukaemia; ATP R, reduced ATP release; adrenaline, 10 μmol/l; ADP, 2.5 μmol/l adenosine diphosphate; collagen, 1 μg/ml; AA, 0.5 mmol/l arachidonic acid; B-FG, binding of fibrinogen; A, abnormal or very reduced; aggregation response; NA, no aggregation response; N, normal aggregation response.

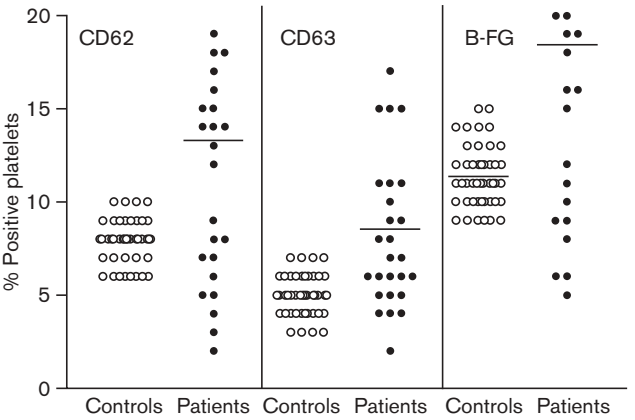
nificant difference was observed (Fischer's exact test, one-tail $P = 0.419$).

Activation markers

Compared with controls, the mean percentage of CD62, CD63 and B-FG positivity was significantly increased in unstimulated platelets from patients with MPD (Student's t test: 13.3 ± 7.4 versus 7.9 ± 1.1, $P < 0.0001$; 8.5 ± 4.5 versus 5.0 ± 1.0, $P < 0.000$; 18.4 ± 9.2 versus 11.3 ± 1.6, $P < 0.0001$, respectively), indicating alpha granule secretion and activation of the fibrinogen-binding sites on the GP IIb–IIIa complex (Fig. 1). A good correlation was found between the B-FG with the expression of CD62 or CD63 (Spearman's correlation: $r = 0.59$, $P = 0.0008$ and $r = 0.66$, $P = 0.0001$, respectively).

High levels, over the cut-off value, of activation markers were obtained with: CD62 in 17 of 28 patients (61%), CD63 in 14 of 28 patients (50%) and B-FG in 19 of 28 patients (68%) of MPD (see Table 2). Taking ET and PV together, we found that the percentage of patients with positive platelets for CD62 or B-FG exceeded the cut-off value was significantly high compared with CML (Fischer's exact test: one-tail $P = 0.022$ and $P = 0.044$, respectively). There was no difference with CD63.

Fig. 1



Data depicting the individual percentages of CD62-positive, CD63-positive and binding of fibrinogen (B-FG)-positive platelets. Horizontal lines indicate means. Solid circles, patients; open circles, controls.

Discussion

Thrombo-haemorrhagic complications are the main

cause of morbidity and mortality in MPD, affecting about two-thirds of patients [1].

In the present study we have evaluated platelet aggregation response using PRP and the presence of activated platelet in whole blood samples by flow cytometry in a cohort of 28 patients with MPD. Eight of them were CML, and they are included in the classification of MPD by the World Health Organization [22,23].

The pattern of *in vitro* platelet function was not suggestive of one specific platelet defect because a lack of aggregation response to one agent was often associated with little or no response to another aggregation-inducing agonist. The characteristic pattern of absence of ADR-induced platelet aggregation, first wave, was seldom found. However, all MPD samples had reduced aggregation and ATP release with at least two agonists, especially ADP and ADR. This was the more prevalent abnormality among all the assays performed, including cytometric assays. Besides, ATP release was significantly reduced when ET and PV were together compared with CML.

Mepacrine uptake was reduced in only 39% (11 of 28 patients). However, we found no association between reduced mepacrine labelling and abnormal ATP release, suggesting that not only a deficit of intraplatelet ATP/ADP occurs. The abnormalities in the receptor-mediated granule secretions would be engaged in MPD.

Flow cytometry gives single-cell multiparameter correlated data with high statistical precision, allowing for the detection of rare events such as a small portion of platelets binding a certain ligand or exposing a specific receptor [24,25]. Resting platelets do not bind fibrinogen; upon activation, a structural change of the GP IIb–IIIa complex results in exposure of the fibrinogen-binding sites [19].

There is ample evidence that the platelet activation may continuously be abnormal in a significant proportion of patients with MPD [26–28]. However, Cahill *et al*. failed to show any significant platelet activation as assessed by markers of alpha and lysosomal degranulation [29]. In our group of MPD, using whole blood for the minimal manipulation of platelets, we have demonstrated an increase in membrane expression of alpha and lysosomal granule secretion or activation of the fibrinogen-binding sites on the GP IIb–IIIa complex.

Comparison of B-FG with CD62p or CD63 expression on platelets demonstrated a good correlation, suggesting that the activation of the fibrinogen-binding sites on the GP IIb–IIIa complex and degranulation occurred simultaneously.

Jensen *et al*., using whole blood flow cytometry, had demonstrated an increased membrane expression of activation markers on non-stimulated platelets of MPD. However, when they performed stimulation assays with a strong agonist (TRAP) or a weak agonist (ADP) the response of platelets in terms of the activation markers were reduced in these patients. They postulated that an intrinsic cellular defect in receptor-mediated granule secretion and redistribution of membrane glycoproteins might occur [8].

As we described earlier, aggregometric studies in our MPD patients suggest a hyporeactivity pattern as the more prevalent defect. The average of activation marker levels was increased in MPD. Interestingly, there is a group of patients with both abnormalities (hyporeactivity and high levels of CD62, CD63 or B-FG over the cut-off). Although, we performed this study with different combination assays (aggregonometry and flow cytometry), these findings correlated with Jensen *et al*.'s results.

The proportion of patients with abnormal ATP release values, reduced mepacrine labelling and increased levels of activation markers was significantly high when ET and PV were together compared against CML. In fact, this agrees with CML having no evident risk of thrombosis [30].

Flow cytometry is very useful but platelet function cannot be easily evaluated by this method and it is rather expensive. Besides, standardization is easier with simple platelet aggregation assays than with flow cytometry.

Finally, both techniques are complementary and would suggest the presence of a hyporeactive platelet population not only as a secondary process to activation and degranulation.

Further studies investigating platelet parameters during the clinical course of MPD and a major number of patients may help to elucidate this point and find the interrelation between platelet functional status and bleeding or thromboembolic events.

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