An acquired form of Bernard Soulier syndrome associated with acute myeloid leukemia

Khalil A. Aziz, *PhD.*

ABSTRACT

Objective: To investigate glycoprotein-1b (GP-1b) expression on platelets from patients with acute myeloid leukemia (AML).

Methods: Purified platelets, obtained from AML-patients and normal control subjects, were examined for surface membrane GP1b-expression by flow cytometry and GP1b-mediated aggregation responses by aggregometry. The level of elastase in plasma from patients and controls was measured by enzymed-linked immunosorbent assay. The whole of this work was carried out at the University of Liverpool, Liverpool, United Kingdom during the period of 1994-2001.

Results: Platelets from the majority of AML-patients showed reduced GP1b-expression and reduced GP1b-mediated aggregation responses. Reduction in platelet GP1b-expression was associated with increased plasma elastase levels.

Conclusion: The present study suggests that elastase, released from leukemic blasts, degrades platelet GP1b, resulting in dysfunctional circulating platelets in AML-patients. These results could explain the bleeding disorders observed in these patients.

Saudi Med J 2005; 26 (7): 1095-1098

Polymorphonuclear leukocytes (PMN, neutrophils) play an important role in inflammation, while platelets play an equally important role in the process of hemostasis.[1] In the past, the 2 processes of inflammation and hemostasis were viewed to occur independently of each other and, therefore, the function of each cell-type was examined in isolation. However, the observations of close proximity of PMN and platelets in histo-pathological samples from patients with the adult respiratory distress syndrome suggested the possibility that these 2 cell types might interact in-vivo.[2,3] Such observations stimulated many researchers to investigate the interaction of these cells in-vitro.

We were amongst the first to provide direct evidence for the interaction of PMN and platelets.[4-11] Thus, we showed that platelets enhanced various PMN function including oxidant production and enzyme degranulation, while PMN were shown to, reciprocally, induce the proteolytic loss of platelet glycoprotein-Ib (GP-Ib).[5,11] The enzyme was identified as elastase released from PMN primary granules.[11]

Glycoprotein-Ib is an important cell membrane receptor that mediates the initial binding of platelets to exposed subendothelial matrix of damaged blood vessels. Such binding results in platelet activation and aggregation leading to the formation of a hemostatic plug that prevents blood loss from the damaged blood vessel. Congenital deficiency of platelet GP-Ib, as seen in patients with Bernard Soulier syndrome (BSS), is associated with life threatening bleeding episodes.

Our in-vitro study showing elastase, released from stimulated-PMN, causes the degradation of platelet GP-Ib led us to postulate that elevation of this enzyme in-vivo could, similarly, lead to the

From the Department of Immunology, Birmingham Heartlands Hospital, Bordesley Green East, Birmingham, *United Kingdom.*

Received 18th January 2005. Accepted for publication in final form 30th April 2005.

Address correspondence and reprint request to: Dr. Khalil A. Aziz, Senior Clinical Scientist, Regional Department of Immunology, Birmingham Heartlands Hospital, Bordesley Green East, Birmingham B9 5SS, *United Kingdom.* (Formerly affiliated to the University of Liverpool, Liverpool, United Kingdom). Tel. +1 (121) 4240185. Fax. +1 (121) 4243129. E-mail: khalilazizh@yahoo.co.uk

degradation of platelet GP-Ib; producing an acquired form of BS-like platelets.

Elevation of elastase enzyme in-vivo has been demonstrated in patients with acute myeloid leukemia (AML).[12] These patients suffer from life threatening bleeding episodes, particularly during the remission induction therapy, and the bleeding patterns reflect that of platelet disorders, rather than coagulation factors deficiency. This led us, therefore, to postulate that the high levels of elastase in AML-patients could result in the degradation of platelet GP-Ib in-vivo.

In the present study, we have therefore examined GP-Ib expression on platelets from patients with AML.

Methods.

Isolation of platelets from whole blood. Fresh citrated normal and patient venous blood (12.9 mM tri-sodium citrate) was centrifuged for 10 minutes at 200g and platelet-rich plasma (PRP) removed. To separate platelets from plasma, PRP was centrifuged for 30 minutes at 500 g, at room temperature, on a discontinuous albumin density gradient consisting of 340 mg/ml and 250 mg/ml bovine serum albumin (BSA) (Sigma Chemical Co.) solutions in citrated Hanks balanced salt solution (HBSS) (Flow Laboratories, Scotland). Platelets were collected from the interface between the two albumin solutions and washed once in citrated HBSS. The cells were gently resuspended in 0.5 ml of citrated-HBSS, counted and finally diluted to the desired concentration with RPMI-medium (Northumbria Biological Ltd).

Platelet aggregation. Aggregations studies were performed at 37°C by the turbidimetric method[13] with 5 x 10^7 platelets in a final volume of 1 ml using an aggregometer (Accuteck, Litteborough) coupled to a chart recorder. The cells were initially suspended in 0.2 ml of RPMI-medium. Prior to aggregation measurements, 0.66 of pre-warmed citrated HBBS and 0.1 ml of normal plasma (source of vWF) were added to the cell suspensions. Platelet aggregation was initiated by the addition of 40 µL of 20 mg/ml ristocetin solution. The optical density changes resulting from aggregation were recorded at the appropriate sensitivity setting and chart speed.

Analysis of platelet surface GP-Ib by flow cytometry. Platelets were fixed with paraformaldehyde for one hour. Following fixation, platelets were then washed with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) and stained with anti-GP-Ib monoclonal antibody (AN51), or class specific control (Dako) at 4°C for 30 minutes. Following washing, platelets were again washed with BSA/PBS and incubated with a second layer antibody (fluorescein isothiocyanate (FITC) labeled goat anti-mouse immunoglobulin for 30 minutes at 4°C. After washing, the cells were finally suspended in fluorescein activated cell sorter (FACS) buffer (Becton Dickinson) and fluorescence measured by flow cytometry with Becton Dickinson FACS analyser using the standard recommended procedure.

Measurement of plasma elastase levels. The levels of elastase (complexed to α-1 proteinase-inhibitor) were measured by a commercial enzyme-linked immunosorbent assay.

Results.

Surface expression of GP-Ib on platelets from patients with AML. Platelets from 10 patients with AML were examined for GP-Ib

Table 1 - Data of patients with acute myeloid leukemia.

Patient No.	Age/ Gender	FAB classification	WBC x 10^9/L	Blast (%)	Platelets x 10^9/L	Elastase µg/L	GP1b negative platelets (%)
1	68/M	M1	166	78	35	377	(90)
2	83/M	M1	63	70	100	485	(33)
3	64/M	M2	97	>90	16	214	(30)
4	66/M	M1	24	>90	112	56	(10)
5	50/M	M4	132	>90	50	153	(30)
6	30/F	M3	50	>90	18	383	(46)
7	44/F	M4	27	56	12	209	(70)
8	54/M	M3	3	0	35	841	(9)
9	78/M	M1	9	66	28	29	(76)
10	52/F	M1	8	84	26	ND	(47)

Normal values: Elastase; 22 ±10 µg/L, (%) GPIb-negative platelets 4.4 ± 3.2 (mean ± SD of 10 normal individuals).
ND; not done, WBC - white blood cell count, FAB - French-American British

expression by flow cytometry. The classification and the hematological data of these patients are shown in **Table 1**. Two distinct populations of platelets were observed in 8 of the 10 AML-patients examined. One population corresponded to platelets with a normal expression of GP-Ib, while the other corresponded to platelets that were completely devoid of GP-Ib expression. **Table 1** shows the number of GP-Ib-negative platelet populations from the 10 AML-patients used in the present study. For comparison, we examined GP-Ib expression on platelets from patients with myelodysplastic syndrome (MDS). Unlike the AML-patients, MDS-patients showed one single platelet population that was similar to that observed in normal control subjects.

In addition, we examined platelets from 2 AML-patients after achieving remission. Prior to treatment, both patients had 2 platelet populations. Treatment resulted in the disappearance of the GP-Ib-negative population, and the single platelet population observed was comparable to that of the normal control subjects.

Measurement of ristocetin induced platelet aggregation. Platelets from 2 AML-patients were tested for their ability to aggregate in response to ristocetin. Compared with the control, platelets devoid of platelet GP-Ib failed to aggregate, while platelets with a moderate GP-Ib expression (from patient 2 in **Table 1**) showed only a moderate aggregation response. It appears therefore that reduction in platelet GP-Ib expression is paralleled by reduction in ristocetin induced platelet aggregation.

Measurement of elastase levels in plasma from AML-patients. **Table 1** shows the levels of elastase in the ten AML-patients. Elastase levels were elevated in 9 out of the 10 patients examined, with concentration ranging between 56 μg/L and 841 μg/L.

Discussion.

In the present study, we have demonstrated that a proportion of platelets from the majority of AML-patients lacked the surface membrane receptor, GP-Ib. In-vivo, GP-Ib mediates platelet adhesion to exposed subendothelial matrix of damaged blood vessels, via von-Willebrand factor (vWF), resulting in platelet activation and aggregation leading to the formation of a hemostatic plug that prevent blood loss. Platelet aggregation via GP-Ib can be demonstrated in-vitro using ristocetin in the presence of vWF.[14] As expected, reduction in GP-Ib expression on platelets from AML-patients was associated with reduction in ristocetin-induced platelet aggregation.

We have previously shown in-vitro that elastase, released from stimulated-PMN, induced degradation of platelet GP-Ib and reduction in ristocetin-induced platelet-aggregation.[11] In the present study, we have shown that high levels of elastase in AML-patients were associated with the presence of GP-Ib negative platelet population. Myeloid leukemic blasts contain large amount of elastase and release of this enzyme in-vivo would be expected to lead to degradation of platelet GP-Ib, and thus would explain the presence of the GP-Ib negative platelets in AML-patients.

In-vivo, released elastase is normally inhibited by the plasma α-1 antiproteinase (α-1 AP) inhibitor and, to some extent, α-2 macroglobulin.[15] However, it is anticipated that release of large amounts of elastase enzyme from leukemic blasts would result in saturation of α-1 AP in the local vicinity and excess enzyme would then be free to act on platelet GP-Ib. This would be expected to occur in microcirculations that exist in organs such as the spleen and lungs. Indeed, our previous in-vitro study, showing that elastase inhibitors failed to prevent platelet GP-Ib degradation under stirring conditions, would support this hypothesis.[11]

Bleeding in AML-patients is common, particularly in patients with promyelocytic leukemia (M3) and during remission induction therapy. Such bleeding has previously been attributed to reduction in coagulation factors.[16] The present findings would offer an additional explanation for the bleeding disorders associated with AML, and open the way for the use of anti-proteinase therapy in AML-patients. Anti-proteinase therapy has been successfully and safely used in open-heart surgery. However, before proceeding to clinical trials, further work would clearly be needed, in particular, to asses the contribution, if any, of leukemic blast fragments to the GPIb-negative platelet population.

In conclusion, the present study suggests that elastase, released from leukemic blasts, lead to degradation of platelet GP-Ib; producing dysfunctional platelets resembling those observed in patients with BSS.

Acknowledgment. I would like to thank Dr. H. Legget for supplying the clinical data and Dr. M. Zuzel for his suggestions and encouragement.

References

1. Cramer EB. Cell biology of phagocyte migration from bone marrow, out of the blood stream and across epithelium. In: Gallin JI, Goldstein IM, Snyderman R, editors. Inflammation: Basic Principals and clinical correlates. New York (NY): Raven Press. p. 341-351.
2. Backofen M, Weibel ER. Structural alterations of lung parenchyma in adult respiratory distress syndrome. ***Clin Chest Med*** 1982; 3: 35-41.
3. Stimler NP, Hugli TE, Bloor CM. Pulmonary injury induced by C3a and C5a anaphylatoxins. ***A J Path*** 1980; 100: 327-348.

4. Aziz KA, Cawley JC, Zuzel M. Platelet-neutrophil interaction: readily released α-granule platelet factor 4 (PF4) primes neutrophils by facilitating translocation and secretion of myeloperoxidase. ***Blood*** 1994; 84: 35.
5. Aziz KA, Cawley JC, Zuzel M. Platelets prime PMN via released PF4: mechanism of priming and synergy with GM-CSF. ***Br J Haematol*** 1995; 91: 846-853.
6. Davey PC, Zuzel M, Kamaguti AS, Hunt JA, Aziz KA. Activation-dependent proteolytic degradation of polymorphonuclear CD11b. ***Br J Haematol*** 2001; 111: 934-942.
7. Aziz KA. CD44 mediates polymorphonuclear leukocyte motility on hyaluronan. ***Saudi Med J*** 2003; 24: 447-451.
8. Aziz KA. Regulation of polymorphonuclear leukocyte function by platelets. ***Saudi Med J*** 2001; 22: 526-530.
9. Aziz KA, Cawely JC, Treweeke AT, Zuzel M. Sequential potentiation and inhibition of PMN reactivity by maximally stimulated platelets. ***J Leukoc Biol*** 1997; 61: 322-328.
10. Treweeke AT, Aziz KA, Zuzel M. The role of G-CSF in mature neutrophil function is not related to GM-CSF-type cell priming. ***J Leukoc Biol*** 1994; 55: 612-615.
11. Aziz KA, Kamaguti AS, Zuzel M. Degradation of platelet glycoprotein 1b by elastase released from GM-CSF treated neutrophils. ***Br J Haematol*** 1995; 91: 46-54.
12. Egbring R, Schmidt W, Fuchs G, Havemann K. Demonstration of granulocytic protease in plasma of patients with acute leukaemia and septicaemia with coagulation defects. ***Blood*** 1977; 49: 219-230.
13. Mustard JF, Hegardt TB, Rowsell HC, MacMillan RL. Effect of adenosine nucleotides on platelet aggregation and clotting time. ***J Lab Clin Med*** 1964; 64: 548-559.
14. Jenkins CSP, Phillips DR, Clemetson KJ, Meyer D, Larrieu MJ, Luscher EF. Platelet membrane glycoproteins implicated in ristocetin-induced aggregation: studies of the proteins on platelets from patients with Bernard-Soulier syndrome and von Willebrand's disease. ***J Clin Invest*** 1976; 57: 112-124.
15. Ohlsson K, Ohlsson I. Neutral protease of human granulocytes. Interaction between human granulocyte elastase and plasma protease inhibitors. ***Scand J Clin Lab Invest*** 1974; 34: 349-356.
16. Tallman MS, Kwann HC. Reassessing the haemostatic disorder associated with acute promyelocytic leukaemia. ***Blood*** 1992; 79: 543-553.