Original Article

Flow cytometry based chronologic analysis of surface glycoproteins of resting and stimulated platelets in normal individuals

Muhammad Saboor[1], M. Moinuddin[2], Samina Ilyas[3]

ABSTRACT

Objective: To evaluate the effect of timed incubation on the activation dependent glycoproteins on the surface of unfixed platelets after stimulation with adenosine diphosphate (ADP).

Methodology: A total of 32 normal subjects were recruited in this study. CBC, Hb A1C, fasting and random blood sugar, urea, creatinine, cholesterol, triglycerides, high density as well as low density lipoproteins were determined. Flow cytometric analysis of platelets was done using FACSCalibur (BD Bioscience). Two sets of CD markers i.e. platelets CD41& CD61 and CD63 & CD 62p were studied during resting state and after stimulation with ADP.

Results: Mean± SD values of CD41 and CD61 surface markers of resting and stimulated platelets showed no statistical difference; p value being 0.198 and 0.486 respectively. Comparison of CD41 and CD61 markers at 20 minutes Vs 2 hrs, 20 minutes Vs 3 hrs and 2 hrs Vs 3 hrs didn't show any significant difference after stimulation with ADP. This indicates that these markers remain stable and they are not affected by incubation for up to 3 hours. Activation of platelets with ADP resulted in an increase in the number of CD63 and CD62p positive platelets with a p value of 0.001. Activity of CD63 remained high while that of CD62p progressively decreased after incubation for 2 and 3 hours.

Conclusion: CD63 is an ideal marker to evaluate functional status of the platelets while CD62p positivity, though it increases after activation, cannot be used alone as a marker of platelet activation because its positivity decreases with the passage of time. Changes in CD62p positivity when considered in conjunction with increased CD63 positivity lend support to the presence of increased number of activated platelets in the peripheral blood.

KEY WORDS: Platelets, Flow cytometry, CD63, CD62p, Incubation.

Pak J Med Sci July - September 2012 Vol. 28 No. 4 625-629

How to cite this article:

Saboor M, Moinuddin M, Ilyas S. Flow cytometry based chronologic analysis of surface glycoproteins of resting and stimulated platelets in normal individuals. Pak J Med Sci 2012;28(4):625-629

1. Muhammad Saboor, M.Sc. (Hematology), M.Phil (Hematology),
2. Dr. M. Moinuddin, FRCP (C), FRCP (E),
3. Samina Ilyas, M.Sc. (Hematology)

1-3: Baqai Institute of Hematology,
Baqai Medical University,
Karachi, Pakistan.

Correspondence:

Muhammad Saboor, M.Sc., M.Phil (Hematology),
Assistant Professor,
Baqai Institute of Hematology,
Baqai Medical University,
Karachi, Pakistan.
Email: mujhgan_16@hotmail.com

* Received for Publication: April 20, 2012
* Revision Received: May 16, 2012
* Revision accepted: May 18, 2012

INTRODUCTION

Platelets play a central role in hemostasis and thrombosis.[1,2] They are also instrumental in the development of atherothrombosis.[3-5] A large number of platelet function tests are now available for clinical and research purposes. These include platelet aggregation studies, platelet count before and after stimulation, adhesion to polymer beads, quantification of high shear platelet adhesion and aggregation, urinary 11-dehydrothromboxane B_2, thromboxane generation and platelets flow cytometry. Platelet function tests are primarily of

value in the diagnosis and management of bleeding disorders, they are of secondary importance in thrombo-embolic disorders.[6] These procedures not only evaluate platelet functions, they also help establish the diagnosis of various inherited platelet disorders.

Each assay has its pros and cons. Light transmission aggregometry is considered the "gold standard" for platelet function studies. Large volume of the sample, immediate processing and preparation, variations in the results, prolonged processing time, need for normal controls and non availability of technical expertise are some of the limitations of light transmission aggregometry.[7-9]

Flow cytometry is superior to other methods by virtue of the smaller blood sample, simple processing and minimum manipulation of platelets during processing.[10] Activated and resting state of platelets can also be studied simultaneously with this technique.

Diagnosis of inherited thrombocytopathies i.e. Bernard-Soulier's syndrome, Glanzmann's thrombasthenia and storage pool disease has become easy through flow cytometry.[11] Conditions like acute coronary syndromes, acute cerebrovascular ischemia, peripheral vascular disease, diabetes mellitus and pre-eclampsia can be serially studied with flow cytometry by detecting activated platelets, platelet derived microparticles and leukocyte-platelet aggregates in the circulating blood. It is also used for monitoring platelet functions before and during angioplasty and cardiopulmonary bypass.[11,12] Flow cytometric identification of circulating monocyte-platelet aggregates can also serve as a sensitive marker for in vivo platelet activation.[13] This technique can also be used to evaluate the therapeutic response of various antiplatelet drugs.[14]

CD61, also known as gpIIIa, is a 110 kDa glycoprotein that belongs to integrin family of proteins. CD41 is a glycoprotein composed of 2 chains i.e. GPIIbα of 120 kDa and GPIIbβ of 23 kDa linked by disulfide bond. Both these receptors are present on platelets and megakaryocytes. CD41/CD61 antigen forms the GPIIb/IIIa complex which acts as a receptor for fibrinogen, von Willebrand factor (vWf), fibronectin and vitronectin.[15]

CD62p also known as P-selectin, GMP 140 is a glycoprotein of 140 kDa present in α-granules of resting platelets and is translocated to the plasma membrane after activation. P-selectin expression on circulating platelets indicates in vivo activation of platelets.[9]

CD63 is a 53 kDa lysosomal membrane protein detected on the surface of activated platelets after release reaction normally not found on non stimulated platelets. CD63 modulates platelet spreading and platelet tyrosine phosphorylation on immobilized fibrinogen. CD63 is used as a marker of in vivo platelet activation.[16]

The purpose of this study was to evaluate the effect of incubation on the expression of activation dependent glycoproteins on the surface of unfixed platelets after stimulation with ADP.

METHODOLOGY

A total of 32 normal subjects i.e. 15 male and 17 female were enrolled in this study. Questionnaire proforma was completed and individuals fulfilling the inclusion criteria were accepted for the study. Informed written consent was taken from all subjects. Selection criteria included; non smoker healthy normal individuals with no history of any illness or drug intake particularly the anti-platelet drugs during the past six weeks. This study was approved by the ethics committee of Baqai Medical University, Karachi.

Collection of Blood Samples: Blood samples were collected from the antecubital vein under minimal tourniquet pressure to avoid spontaneous activation of platelets. Blood was collected in vacutainers (BD), 3 ml of blood was collected in EDTA tubes for CBC & HbA1C; 2.7 ml of blood was added to sodium citrate tube for platelet flow cytometry while 5 ml of blood was collected in gel tube for biochemical studies.

Complete Blood Counts (CBC) and Hemoglobin A_1C: CBC of all samples were determined using automated cell analyzer. This included hemoglobin estimation, red blood cell count, total leukocyte count, platelet count, packed cell volume (PCV), mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration% (MCHC) and red cell distribution width (RDW). Hb A_1C was determined using micro lab 200.

Biochemical Investigations: Random and fasting blood sugar, urea, creatinine, cholesterol, triglycerides, high density and low density lipoproteins were determined using automated biochemistry analyzer (Hitachi 902 Roche).

Flow Cytometry: Four monoclonal antibodies were used i.e. Anti CD41 FITC, anti CD63 PE, Anti CD61 PE and anti CD 62P FITC (BD Biosciences USA) were used for this study. An IgG isotype matched control was also used to study nonspecific binding with platelets. Flow cytometric analysis of platelets

Table-I: Setting up the tubes as per study design.

Contents	*Tube*								
	1	2	3	4	5	6	7	8	9
PRP	+	+	+	+	+	+	+	+	+
IgG isotype	+	—	—	—	—	—	—	—	—
Anti CD41	—	+	—	+	—	+	—	+	—
Anti CD 63	—	+	—	+	—	+	—	+	—
Anti CD 61	—	—	+	—	+	—	+	—	+
Anti CD 62p	—	—	+	—	+	—	+	—	+
ADP	—	—	—	+	+	+	+	+	+

was done immediately after blood collection. Citrated tube was centrifuged for 5 minutes at 1600 rpm. Supernatant was collected into a separate polystyrene tube as platelet rich plasma (PRP).

10 µl of PRP was added to nine tubes labeled from 1 to 9 as shown in Table-I. Tube 1 was used for IgG isotype control. Anti CD41 FITC and anti CD63PE were added to tubes 2, 4, 6 and 8 while anti CD61PE and anti CD62p FITC were added to tubes 3, 5, 7 and 9. Tubes 2 and 3 were treated as resting, non stimulated platelets (ADP was not added to these tubes). 25 µl of ADP (Helena) was added to tubes 4, 5,6,7,8 and 9. Tubes 1, 2, 3, 4 and 5 were incubated for 20 minutes, tubes 6 and 7 were incubated for 2 hours while tubes 8 and 9 were incubated for 3 hours. After incubation, 1 ml of phosphate buffer saline (PBS) was added to each tube for dilution. All tubes were analyzed for % fluorescence positive platelets by FACS Calibur (BD Bioscience).

Forward light scatter (FS) and side light scatter (SS) were displayed on logarithmic scales. Platelets were identified by binding with FITC (fluorescein isothiocyanate) and PE (phycoerythrin) labeled anti CD41 and anti CD61 antibodies. Antibodies labeled platelets were analyzed for 10,000 platelet events using flow cytometer. Results were expressed as mean fluorescence percentage of positive platelets.

Statistical Analysis: Descriptive statistics was used for mean± SD. Paired t-test was used to analyze the differenc using Statistical Package for the Social Sciences (SPSS) version 16.

RESULTS

Only normal individuals with normal platelet count and biochemical parameters were included in this study in order to eliminate any possibility of interference by variations in the test sample. Consolidated results of complete blood counts and biochemical findings are given in Table-II.

Mean± SD values of CD41 of resting and activated platelets were 88.71±4.79 Vs 90.21±4.81 respectively; there was no statistically significant difference; p value being 0.198. Similarly CD61 positivity during resting and activated states was 89.53±5.69 Vs 90.40 ± 5.2 showing no statistically significant difference (p=0.486). Comparison of CD41 and CD61 at 20 minutes Vs 2 hrs, 20 minutes Vs 3 hrs and 2 hrs Vs 3 hrs didn't show any significant difference after platelets stimulation with ADP as shown in table III. Results of CD63 and CD62p positivity of stimulated platelets were however significantly different. Upon activation with ADP for 20 minutes, the number of CD63 positive platelets increased from 0.95±0.30 to 58.60±9.68 with a p value of 0.001. CD62p positivity that was 0.78 ± 0.28 in resting platelets increased to 53.87 ± 9.93 with a p value of 0.001 after incubation with ADP. Table-III compares mean % fluorescence positive platelets of CD41, CD61, CD63 and CD62p after 20 minutes, 2 hours and 3 hours of incubation.

Table-II: Results of complete blood counts and biochemical parameters, Data is shown as Mean ± SD.

Parameters		*Normal Male*	*Normal Female*
Hb	g/dl	14.21 ± 1.04	12.52 ± 0.68
PCV	%	41.92 ± 3.25	36.32 ± 2.06
RBC	M/µl	4.79 ± 0.36	4.25 ± 0.32
MCV	fl	87.16 ± 4.49	85.74 ± 5.84
MCH	pg	29.45 ± 1.79	28.73 ± 3.27
MCHC	%	33.80 ± 1.01	31.92 ± 1.01
RDW	%	14.12 ± 0.91	14.28 ± 1.18
TLC	X 10^3/µl	6.67 ± 1.69	6.69 ± 1.61
Platelet count	X 10^3/µl	261.20 ± 52.88	263.24 ± 67.82
RBS	mg/dl	110.73 ± 8.93	114.0 ± 7.59
FBS	mg/dl	81.27±7.94	82.94±8.09
Hb A1c	%	4.14 ± 0.61	4.35 ± 0.58
Urea	mg/dl	18 ± 5	19 ± 7
Creatinine	mg/dl	0.7 ± 0.2	0.8 ± 0.2
Cholesterol	mg/dl	164 ± 18	158 ± 12
Triglyceride	mg/dl	85 ± 14	93 ± 10
HDL	mg/dl	49 ± 09	51 ± 11
LDL	mg/dl	102 ± 05	107 ± 07

Hb= Hemoglobin, PCV= Packed Cell Volume, MCV= Mean Cell Volume, MCH= Mean Cell Hemoglobin, MCHC= Mean Cell Hemoglobin Concentration, RDW= Red Cell Distribution Width, TLC= Total Leukocyte count, RBS= Random blood sugar, FBS= Fasting blood sugar, HDL= High density lipoproteins, LDL= Low density lipoproteins.

DISCUSSION

Platelet membrane has a large number of glycoproteins that are essential for their normal functioning. Some of the glycoproteins are present in the resting state as well as after stimulation e.g.

Table-III: Comparison of flow cytometric analysis of platelets at different intervals.

Time	*CD41*		*CD61*		*CD63*		*CD62p*	
	Mean positive cells	*p value*	*Mean positive cells*	*p value*	*Mean positive cells*	*p value*	*Mean positive cells*	*p value*
20 min Vs	90.25 ± 4.52 Vs	0.526	89.75 ± 4.31 Vs	0.748	58.60 ± 9.68 Vs	0.166	53.87 ± 9.93 Vs	0.001
2 hrs	89.43 ±4.5		89.37 ± 5.0		60.68 ± 5.0		43.96 ±10.78	
20 min Vs	90.25 ± 4.52 Vs	0.773	89.75 ± 4.31 Vs	0.635	58.60 ± 9.68 Vs	0.154	53.87 ± 9.93 Vs	0.001
3 hrs	90.56 ± 4.20		90.21 ± 4.62		61.60 ± 4.4		35.64 ± 9.7	
2 hrsVs	89.43 ± 4.5 Vs	0.325	89.37 ± 5.0 Vs	0.483	60.68 ± 5.0 Vs	0.690	43.96 ±10.78Vs	0.001
3 hrs	90.56 ± 4.2		90.21 ± 4.62		61.60 ± 4.4		35.64 ± 9.7	

Paired t-test was used to analyze the difference.

CD41, CD42, and CD61. Fibrinogen receptors, CD62p and CD63 are neoepitopes that appear only on the surface of activated platelets (activated state).[9,16]

In this study CD41 and CD61 remained unaffected after 20 minutes of incubation and after platelet activation with ADP (p=0.198 and 0.486 respectively). Comparison of CD41 at different intervals did not show any significant statistical change as shown in table-III. Similarly incubation of PRP after stimulation with ADP did not show any statistical difference as shown in table-III. These findings suggest that CD41 and CD61 are not affected by either incubation or by stimulation with ADP. In vitro activation of platelet rich plasma with ADP caused increased number of CD63 positive platelets and no statistical difference was found after 2 (p=0.166) and 3 hours (p=0.154) of incubation as shown in Table-III. Persistent increase in the number of CD63 positive platelets reflects the stability of this molecule after activation. This finding is in agreement with that of Buggle et al.[17] In vivo increase in CD63 was also reported by several authors in various clinical conditions characterized by persistent platelet activation. Peripheral artery disease, myocardial infarction, congestive heart failure, acute cerebral infarction, atherosclerotic ischemic stroke and non-embolic ischemic stroke are some of the common clinical conditions that are frequently associated with increased CD63 activity in vivo.[18-23]

In our study incubation of PRP with ADP resulted in an initial increase in CD62p positivity (p=0.001); this rise was however transient. When ADP mixed PRP was incubated for 2 and 3 hours, the mean fluorescence percent positivity of CD62p progressively and significantly (p=0.001) decreased as shown in Table-III. Reduction in CD62p positivity was greater after 3 hours than after 2 hours of incubation. Although CD62p positivity decreased during incubation, its values remained higher than the base line values after 3 hours of incubation (0.78 ± 0.28 Vs 35.64 ± 9.7). Buggle et al[17] also found reduction in the CD62p positive platelets at different intervals in stroke patients. Matzdorff et al[24] also reported decreased number of CD62p positive platelets in their in vitro experiments.

It is well documented that activated de-granulated platelets in vivo very rapidly lose their surface CD62p molecules. However these platelets remain in circulation and effectively perform their normal functions.[25] Although increased number of CD62p is found in conditions associated with chronic activation of platelets, this parameter alone cannot be used as a marker for the activation status of the platelets. However, together with CD63 values, it can be reliably used for evaluating the state of platelet activation.

Grant Support: Baqai Institute of Hematology, Baqai Medical University, Karachi.

REFERENCE

1. Mylotte D, Foley D, Kenny D. Platelet function testing: methods of assessment and clinical utility. Cardiovasc Hematol Agents Med Chem 2011;9(1):14-24.
2. Pakala R, Waksman R. Currently available methods for platelet function analysis: advantages and disadvantages. Cardiovasc Revasc Med 2011;12(5):312–322.
3. Jennings LK. Mechanisms of platelet activation: need for new strategies to protect against platelet-mediated atherothrombosis. Thromb Haemost 2009;102:248–257.
4. Puddu P, Muscari A, Puddu GM, Cravero E, Giannoni C, Zoli M. The complexity of platelet metabolism and its contribution to atherothrombosis. Acta Cardiol 2009;64:157–165.
5. Gawaz M, Langer H, May AE. Platelets in inflammation and atherogenesis. J Clin Invest 2005;115:3378–3384.
6. Collet JP, Montalescot G. Platelet function testing and implications for clinical practice. J Cardiovasc Pharmacol Ther 2009;14:157–169.
7. Gurbel PA, Becker RC, Mann KG, Steinhubl SR, Michelson AD. Platelet function monitoring in patients with coronary artery disease. J Am Coll Cardiol 2007;50:1822–1834.

8. Harrison P. Platelet function analysis. Blood Rev 2005;1:111–123.
9. Matzdorff A. Platelet function tests and flow cytometry to monitor antiplatelet therapy. Semin Thromb Hemost 2005;31(4):393-399.
10. Kenneth A Ault. The clinical utility of flow cytometry in the study of platelets. Seminars in hematology 2001;38(2)160-168.
11. Linden MD, Frelinger AL, Barbard MR, Przyklenk K, Furman MI, Michelson AD. Application of flow cytometry to platelet disorders. Semin Thromb Hemost 2004;30(5):501-511.
12. Harrison P. Assessment of platelet function in the laboratory. Hamostaseologie 2009;29(1):25-31.
13. Michelson AD, Barnard MR, Krueger LA, Valeri CR, Furman MI. Circulating monocyte–platelet aggregates are a more sensitive marker of in vivo platelet activation than platelet surface P-selectin: studies in baboons, human coronary intervention, and human acute myocardial infarction. Circulation 2001;104:1533–1537.
14. Sbrana S, Della Pina F, Rizza A, Buffa M, De Filippis R, Gianetti J. Relationships between optical aggregometry (type born) and flow cytometry in evaluating ADP-induced platelet activation. Cytometry B Clin Cytom 2008;74(1):30-39.
15. Cox D. Methods for monitoring platelet function. Am Heart J 1998;135:S160-S169.
16. Michelson AD, Furman MI. Laboratory markers of platelet activation and their clinical significance. Curr Opin Hematol 1999;6(5):342-348.
17. Buggle F, Mayer H, Marquardt GL, Ruf A, Mansmann U, Winter R. Course of platelet activation markers after ischemic stroke. Stroke 2002;33:2570-2574.
18. van der Zee PM, Biro E, Ko Y, de Winter RJ, Hack CE, Sturk A, et al. P-selectin- and CD63-exposing platelet microparticles reflect platelet activation in peripheral arterial disease and myocardial infarction. Clin Chem 2006;52(4):657-664.
19. Tan KT, Tayebjee MH, Lynd C, Blann AD, Lip GY. Platelet microparticles and soluble P selectin in peripheral artery disease: relationship to extent of disease and platelet activation markers. Ann Med 2005;37(1):61-66.
20. Chang I, Choudhury A, Patel J, Lip GY. Soluble, platelet-bound and total P-selectin as indices of platelet activation in congestive heart failure. Ann Med 2009;41(1):45-51.
21. Belniak-Legiec E, Stelmasiak Z. Blood platelet activation markers in patients with acute cerebral infarction during the earliest stage of the disease--evaluation using flow cytometry methods. Neurol Neurochir Pol 2000;34(5):853-864.
22. Cha JK, Jo WS, Shin HC, Bae HR, Ho JM, Kim JW. Increased platelet CD63 and P-selectin expression persist in atherosclerotic ischemic stroke. Platelets 2004;15(1):3-7.
23. Tsai NW, Chang WN, Shaw CF, Jan CR, Chang HW, Huang CR, et al. Levels and value of platelet activation markers in different subtypes of acute non-cardio-embolic ischemic stroke. Thromb Res 2009;124(2):213-218.
24. Matzdroff AC, Kuhnel G, Kemkes-Matthes B, Pralle H, Voss R, Fareed J. Effect of glycoprotein IIb/IIIa inhibitors on CD62p expression, platelet aggregates, and microparticles in vitro. J Lab Clin Med 2000;135:247-255.
25. Michelson AD, Barnard MR, Hechtman HB, MacGregor H, Connolly RJ, Loscalzo J, et al. In vivo tracking of platelets: circulating degranulated platelets rapidly lose surface p-selectin but continue to circulate and function. Proc Natl Acad Sci USA 1996;93:11877–11882.

Authors Contribution:

Muhammad Saboor carried out the research work, statistical analysis and writing of manuscript.
Dr. M. Moinuddin supervised, reviewed and finalized the manuscript for publication..
Samina Ilyas worked as co-researcher and co-author.