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Comparison of Platelet CD Markers between Normal Individuals and Untreated Patients with Type 2 Diabetes Mellitus

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Abstract

Objective: Micro and macro vascular complications are associated with diabetes mellitus. Purpose of this study was to evaluate and compare the platelet CD markers between normal individuals and untreated patients with type 2 diabetes mellitus using flow cytometry.

Methods: A total of 107 individuals [32 normal and 75 diabetics] were recruited in this study. Complete blood counts, random and fasting blood sugar, hemoglobin A1c, lipid profile and platelets flow cytometry were determined of all blood samples.

Results: In normal individuals and in patients with untreated type 2 diabetes mellitus, comparison of platelet markers CD41 and CD61 at rest and after activation with ADP did not show any statistical difference in their mean % fluorescence positivity. Number of CD63 and CD62p positive platelets increased with a p value of 0.001 in normal individuals and in patients with untreated type 2 diabetes after activation with ADP compared with their resting states.

Conclusion: Upon platelet activation with ADP, positivity of CD63 and CD62p was increased in normal individuals. CD63 and CD62p positivity was also increased in patients platelets after activation with ADP. Increase in the mean % positivity of CD63 and CD62p was however more in diabetics as compared with normal individuals.

Keywords: Diabetes mellitus; Platelets; CD markers

Introduction

Diabetes mellitus is associated with micro and macro vascular complications. Microvascular complications i.e. retinopathy, nephropathy and neuropathy result in increased morbidity in patients with diabetes mellitus [1]. Diabetes mellitus is a major risk factor for cardiovascular diseases [2]. Chronic hyperglycemia is not the only cause of these complications; these ischemic events are also associated with platelet hyper activation, abnormal activation of coagulation proteins, abnormal endothelial function and hypo-fibrinolysis [3,4]. More than 75% of patients with diabetes die due to cardiovascular complications [5].

Diabetic thrombocytopathy refers to differences in platelet functions between diabetic and non-diabetic individuals. A number of studies have documented several platelet function defects in patients with diabetes mellitus. Platelets hyper aggregation in response to glucose was recognized in 1965 [6]. Enhanced platelet aggregation in response to ADP, thrombin, collagen, arachidonic acid and epinephrine is seen in patients with diabetes mellitus as compared to non-diabetic individuals [7]. Under *in vitro* conditions these platelets after stimulation with platelet agonists show reduced threshold for platelet aggregation. Increased platelet aggregation is more apparent in patients with diabetes associated with macro vascular disease. Platelets from diabetic subjects show an increased adhesiveness and increased spontaneous aggregation as well as aggregation on extra cellular matrix [8,9].

CD61 and CD41 are glycoproteins present on platelets and megakaryocytes. These glycoproteins act as receptors for fibrinogen, von Willebrand factor [vWf], fibronectin and vitronectin [10] CD62p is a glycoprotein of 140 kDa present in α-granules of resting platelets. It is translocated to the plasma membrane after activation. Expression of CD62p on the surface of circulating platelets indicates *in vivo* activation of platelets [11] CD63 a lysosomal membrane protein detected on the surface of activated platelets after release reaction normally not found on non-stimulated platelets [12].

Vericel et al. [13] found that platelets at rest show increased activity in patients with type 1 and type 2 diabetes patients. They further reported that hyper active platelets were detectable in metabolically controlled patients with diabetes without cardiovascular complications.

Eibl et al. [14] found that platelets of patients with type 2 diabetes mellitus show increased expression of platelet activation markers CD31, CD36, CD49b, CD62P and CD63 as compared to normal individuals. Newly diagnosed type 1 diabetic patients also show increased expression of activation dependent platelets surface glycoprotein receptors. Increased CD62 positive platelets were also found in normal first-degree relatives of type 1 diabetic patients [15].

One major cause of atherosclerosis and thrombosis in diabetes is enhanced platelet activation, aggregation and increased expression of CD63 and CD62. Enhanced platelet activation and aggregation leads to the development of vascular complications in diabetes in the early stage of the disease. Flow cytometric detection of platelets with increased surface expression indicates enhanced platelet activation a hallmark of thrombosis [16].

The purpose of this study was to compare the platelet CD markers between normal individuals and untreated patients with type 2 diabetes mellitus by flow cytometry. It was postulated that platelets in patients with untreated type 2 diabetes mellitus circulate in the blood in hyperactive state and are causative of thrombotic complications so frequently encountered in these patients.

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Material and Methods

A total of 107 individuals [32 normal and 75 diabetics] were recruited in this study. Criteria followed for normal individuals were no history of illness and anti-platelet drugs intake. Criteria for inclusion in the study for patients with diabetes mellitus were: untreated patients type 2 diabetes mellitus, no history of drug intake particularly the antidiabetic or anti-platelet drugs, no evidence of diabetic complications and non-smokers. Patients with type 2 diabetes mellitus were selected from Baqai University Hospital and Baqai Institute of Diabetes and Endocrinology Nazimabad. This study was approved by the ethics committee of Baqai Medical University, Karachi. Written informed consent was taken from all individuals.

All individuals were initially tested for random blood glucose level for exclusion or inclusion in the study. Whole blood samples were collected after overnight fasting by venipuncture technique under minimal tourniquet pressure. Blood was collected in vacutainers [BD Biosciences]. 3 ml of blood was collected in EDTA tube for CBC and HbA1c; 2.7 ml of blood was added to sodium citrate tube for platelet flow cytometry while 5 ml of blood was collected in a gel tube for biochemistry. Flow chart of the study design is shown below.

Complete blood counts of all samples were determined by automated cell analyzer. Fasting blood glucose level, urea, creatinine and lipid profile were determined using automated biochemistry analyzer [Hitachi 900 Roche].

Flow cytometric analysis of platelets was done within one hour after the collection of blood samples. Immediately after blood collection, the citrated tube was centrifuged for 10 minutes at 2000 rpm. Platelet rich plasma was separated into another polystyrene tube. Polystyrene test tubes were labeled as tubes 1, 2, 3, 4 and 5.10 µl of IgG isotype, anti CD41, anti CD63, anti CD61 and anti CD63 [BD Biosciences USA] were added to the tubes as follows:

Tube 1: IgG isotype control. Tube 2: Anti CD41 FITC and anti CD63 PE. Tube 3: Anti CD61 PE and anti CD 62P FITC. Tube 4: Anti CD41 FITC and anti CD63 PE. Tube 5: Anti CD61 PE and anti CD 62P FITC.

Tubes 2 and 3 were considered as resting platelets while tubes 4 and 5 were activated platelets. 50 µl of platelet rich plasma was added to each tube. 25 µl of ADP was added to tube 4 and 5. Each tube was incubated in the dark for 20 minutes. After incubation, 1 ml of phosphate buffer saline was added to each tube for dilution. The tubes were analyzed using flow cytometer FACSCalibur [BD Biosciences]. Forward scatter and side scatter signals were acquired with log amplification. A region was drawn on platelet population in the scatter plot. CD41, CD61, CD62P and CD63 expression was analyzed on the gated population. Results were recorded as % of the positive gated platelets.

Student t test was used for comparison of inter-group variables while independent t test was used for the comparison of groups using SPSS version 16 (Figure 1).

Results

Mean age of the normal individuals was 45 ± 6 years while the in diabetics it was 51 ± 8. Normal group comprised of 15 male and 17 females, while diabetic group included 28 men and 47 females. Mean fasting blood sugar of normal subjects was 81.27 ± 7.94 mg/dl while that of diabetic group was 140.54 ± 9.63 mg/dl. Comparison of both groups showed statistically significant difference [p<0.001]. Random blood

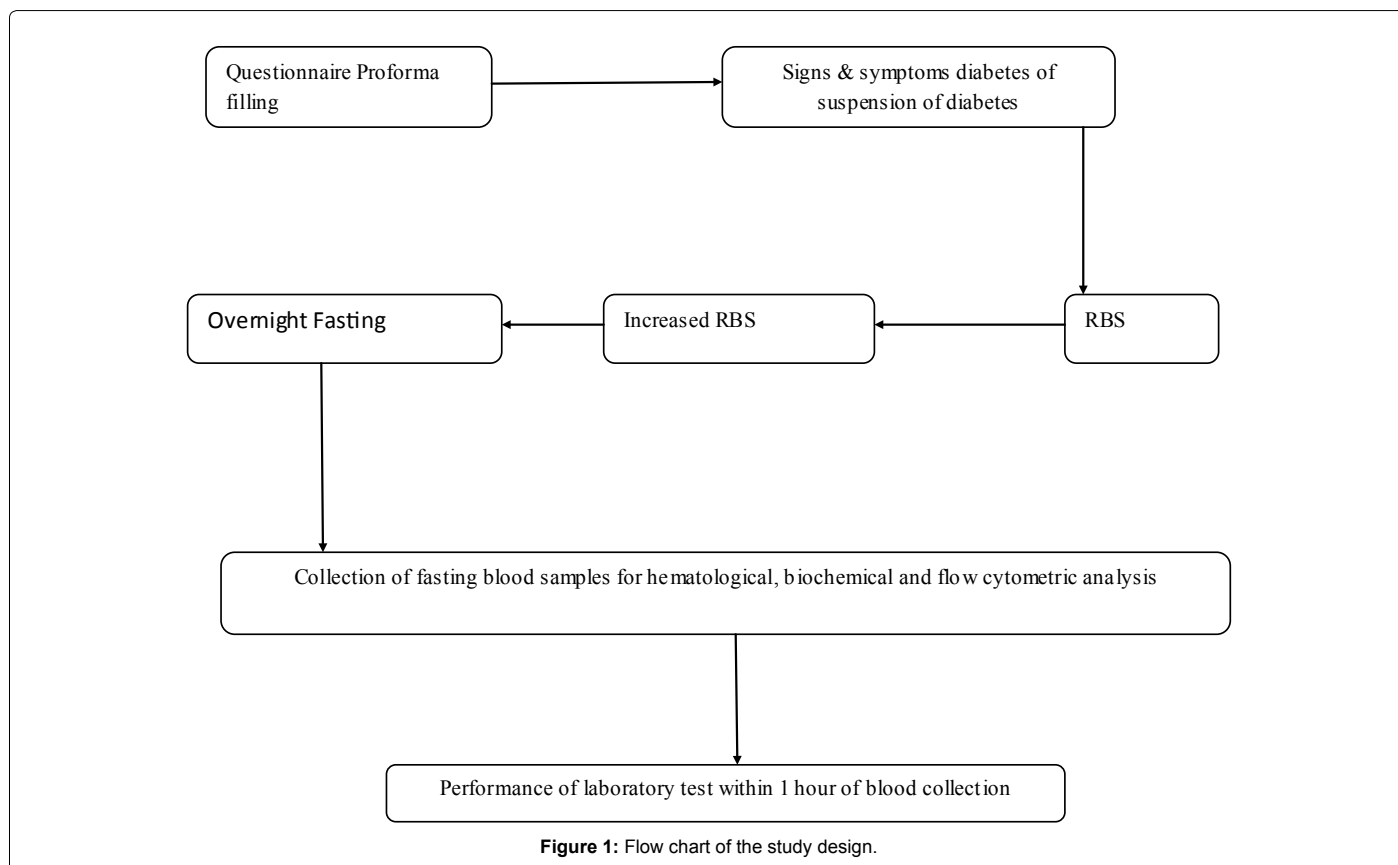**Figure 1:** Flow chart of the study design.

sugar also showed significant difference [p<0.001]. Concentration of HbA1c in normal group was 4.14 ± 0.61% that was significantly lower [p<0.001] as compared to diabetic group i.e. 7.43 ± 0.41%. Comparison of lipid profile of normal and diabetic subjects showed statistically significant difference [p=0.001] in serum cholesterol, triglycerides and high density lipoprotein. Results of biochemical parameters are shown in table 1.

In normal subjects, mean ± SD % values of CD41 and CD61 of resting and activated platelets showed no statistically significant difference; p value being 0.198 and 0.486 respectively. Results of CD63 and CD62p positivity of activated platelets were however significantly different. Upon activation with ADP, the number of CD63 and CD62p positive platelets increased significantly with a p value of 0.001.

In patients with untreated type 2 diabetes mellitus, comparison of platelet markers CD 41 and CD61 at rest and after activation with ADP did not show any statistical difference in their % fluorescence positivity [p=0.749, 0.870 respectively]. However, CD62 and CD62p showed significant increase in their surface expression after activation with ADP as compared to their resting state as shown in table 2.

Comparison of mean % fluorescence positivity of CD41 and CD61 in resting and ADP activated platelets showed no statistical difference in normal subjects and diabetics. However, comparison of CD63 and CD62p positive platelets of normal subjects and patients with untreated type 2 diabetes mellitus in resting and activated state showed significant difference p value being 0.001.

Discussion

Platelet flow cytometry is an emerging tool in diagnostic and therapeutic hematology. It is eminently suited to assess the expression of platelet surface receptors both qualitatively as well as quantitatively. It can thus serve as a useful parameter for *in vivo* expression of platelet activation and thus fore-warn the risk of thromboembolism which is inherent in patients with untreated type 2 diabetes mellitus [12].

This study showed that platelet CD makers 41 and 61 when evaluated in resting platelets as well as activated state did not show any change in their % fluorescence positivity. Furthermore these are highly stable markers are not affected by activation with ADP. This finding

correlates well our earlier findings [17]. In contrast to CD 41 and 61, CD 63 and 62p are up regulated upon activation with platelet agonists. Activation of platelets with ADP showed increased expression of CD63 and CD62p in normal as well as in patients with untreated type 2 diabetes mellitus. However, the response was different between the two groups. Significant increased positivity of CD63 and CD62p indicates that platelets of patients with diabetes mellitus are more responsive to agonists and are more prone to activation after exposure to the agonists. This observation constitutes an important and first step in the evaluation of the state of platelet reactivity in disorders associated with thrombo-atheromatus complications, type 2 diabetes mellitus being the prototype of such disorders. This finding shows that platelets of patients with diabetes are in activated state as compared to normal subjects. Our findings are consistent with that of Matzdroff et al. [11] and Ostermann et al. [18] Matzdroff et al. [11] reported that activation of platelets with agonists like ADP or epinephrine results in increased expression of CD62 and CD63 on the surface of the platelets. Nomura et al. [19] used CD62 for the identification of platelet functional status in diabetes.

Tschoepe et al. [15] showed that platelets with increased surface glycoprotein expression continue to circulate in the blood before consumption during platelet aggregation. It is a well-established fact that patients with diabetes mellitus have large platelets in their blood with enhanced aggregation potential [20]. Activated platelets in patients with diabetes suggest increased consumption and as well as production of platelets [21]. Young platelets are more reactive and become easily activated *in vivo* [22].

Glycation of platelet membrane proteins leads to changes in the protein structure, conformation and alterations in membrane lipids [23]. Altered structure of lipids in the platelet membrane causes increased expression of CD62, CD63, fibrinogen receptors and von Willebrand factor receptors [24]. Increase in the surface expression of these receptors results in the increased tendency of ligand binding [25].

In the light of the above results and discussion it is concluded that; CD41 and CD61 markers do not show any change after treatment with ADP. These markers therefore are of no value in the diagnosis and treatment of various thromboembolic episodes. Increased CD63 and CD62p expression in newly diagnosed untreated patients with diabetes is the hallmark of *in vivo* platelet activation and can be used as marker of activated platelets.

Parameters		Normal	Diabetics	p value
FBS	mg/dl	81.27 ± 7.94	153.54 ± 9.63	0.001
RBS	mg/dl	110.73 ± 8.93	198.79 ± 26.72	0.001
HbA1c	%	4.14 ± 0.61	7.43 ± 0.41	0.001
Urea	mg/dl	18 ± 5	21 ± 7	0.132
Creatinine	mg/dl	0.7 ± 0.2	0.8 ± 0.2	0.115
Cholesterol	mg/dl	164 ± 18	176.45 ± 20.85	0.09
Triglycerides	mg/dl	85 ± 14	154.87 ± 23.89	0.001
HDL	mg/dl	49 ± 09	42.86 ± 11.58	0.001
LDL	mg/dl	102 ± 05	110 ± 12	0.123

Table 1: Biochemical parameters in normal subjects and in patients with untreated type 2 diabetes mellitus.

Markers	Resting platelets	Activated platelets	p value
CD41	89.58 ± 4.61	89.33 ± 4.95	0.749
CD61	90.38 ± 4.83	90.25 ± 4.81	0.870
CD63	36.32 ± 5.97	85.26 ± 5.63	0.001
CD62P	32.09 ± 5.08	83.81 ± 5.12	0.001

Paired *t*-test was used for comparison

Table 2: Comparison of resting and activated platelets of patients with diabetes.

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