SHORT COMMUNICATION

Effect of Clopidogrel on Platelet CD Markers in Normal Individuals and in Patients with Untreated Type 2 Diabetes Mellitus

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Abstract

The objective of this study was to evaluate the effect of clopidogrel response in patients with untreated type 2 diabetes mellitus as compared with normal individuals. One hundred and seven subjects i.e. 32 normal and 75 patients with untreated type 2 diabetes mellitus were enrolled in this study. In the first step, normal subjects as well as diabetic patients were selected and tested for various laboratory parameters and platelets flow cytometry. In the second step, an antiplatelet drug (clopidogrel) was administered for 10 days to each individual enrolled in the study. After 10 days blood samples were collected for platelets flow cytometry. CD41 and CD61 did not show any change after the administration of clopidogrel in resting and activated platelets. CD63 and CD62p positivity was increased in normal and in diabetic patients' platelets after activation with ADP before clopidogrel. It was decreased in normal resting and ADP stimulated platelets after clopidogrel treatment. CD63 and CD62p positivity in resting and ADP stimulated patients' platelets was also decreased after clopidogrel treatment. The change was, however, not as marked as in normal subjects.

Key Words: *Platelets. Flow cytometry. CD63. CD62p. Diabetes mellitus. Clopidogrel.*

Diabetes mellitus is a major risk factor for cardiovascular diseases.[1] Microvascular complications i.e. retinopathy, nephropathy and neuropathy result in increased morbidity in patients with diabetes mellitus.[2]

Clopidogrel is a prodrug that is absorbed from the intestine and metabolised by oxidative pathways of hepatic cytochrome P450 enzymes i.e. CYP2C19, CYP3A4/5, CYP2B6, CYP2C9 and CYP1A2.[3] These enzymes oxidize clopidogrel to 2-oxo-clopidogrel which is further metabolised to an active thiol compound. This short lived active metabolite binds with platelet $P2Y_{12}$ receptors through disulfide bonds between the reactive thiol group and the two cysteine residues (cys17 and cys270) of extracellular domains of $P2Y_{12}$ receptor. Binding of clopidogrel to the $P2Y_{12}$ receptors is selective and irreversible.[4] Because of the irreversible binding, effect of clopidogrel persists for the lifespan of the affected platelets.

This study was undertaken with the primary object of determining platelet reactivity in patients with untreated type 2 diabetes mellitus and to study the effect of clopidogrel administration on the state of *in vivo* platelet activation in these patients. It was extended to include the effect of clopidogrel on reactive platelet CD markers after *in vitro* pre-treatment with platelet agonists. It was postulated that platelets in patients with type 2 diabetes mellitus circulate in the blood in hyperactive state and predispose to thrombotic complications so frequently encountered in these patients.

A total of 107, i.e. 32 normal individuals and 75 patients with untreated type 2 diabetes mellitus were enrolled in this study. Criteria for normal individuals were; healthy individuals with no history of any illness during the past 6 weeks, no history of drug intake particularly anti-platelet agent and no history of smoking. Normal individuals were selected from the hospital and laboratory staff. Inclusion criteria for patients were untreated type 2 diabetes mellitus with no history of drug intake particularly the anti-diabetic or anti-platelet drug, no evidence of diabetic complications and non-smokers. Diabetic patients with a history of drug intake for diabetes, with cardiovascular diseases, macro or micro vascular complications or smokers were excluded from the study.

Patients with untreated type 2 diabetes mellitus were selected from Baqai Institute of Diabetes and Endocrinology (BIDE) and Baqai University Hospital, Nazimabad. Informed written consent was taken from all subjects. This study was conducted from March 2010 to July 2012 and was approved by the ethics committee of Baqai Medical University, Karachi.

This study was carried out in two steps. In the first step normal as well as diabetic patients were selected and tested for various laboratory parameters and platelets flow cytometry. Initially 120 individuals were enrolled in the study of which 13 individuals were excluded due to non-compliance (n=10) and abnormal laboratory findings in 'normal' subjects (n=3). A total of 107 individuals i.e. 32 normal subjects (male 15, female 17) and 75 (male 28, female 47) patients with type 2 diabetes mellitus were enrolled in the study. Mean age

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of the normal subjects was 51 ± 12 years while that of the diabetic group was 56 ± 16 years.

Blood samples were collected from the antecubital vein under minimal tourniquet pressure to avoid spontaneous activation of platelets. Blood was collected in vacutainers (BD Biosciences). Three ml of blood was collected in EDTA tubes for CBC and HbA_1C, 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 of all samples were determined using automated cell analyzer. HbA_1C was determined using micro lab 200. Fasting and random blood sugar, urea, creatinine, cholesterol, triglycerides, high density and low density lipoproteins were determined using automated biochemistry analyzer (Hitachi 902 Roche).

The citrated blood sample was analyzed for percentage fluorescence positive platelets for anti-CD41, 61, 62p and 63 by FACS Calibur (BD Biosciences). 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-CD42 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.

After collection of the first blood sample, clopidogrel was administrated to each individual (75 mg/day) for 10 days in the second step of the study. Second blood samples were collected after the administration of clopidogrel for flow cytometry. Samples were analyzed according to the same procedure. Data were analyzed using the Statistical Package for Social Sciences (SPSS) version 16.0. Paired t-test was used for the comparison of parameters. A p-value of < 0.05 was considered statistically significant.

Platelets flow cytometry is a simple and reliable test for the evaluation of therapeutic efficacy of anti-platelet drugs. Compared with normal subjects, resting platelets in diabetic patients after clopidogrel administration show increased expression of CD63 ($p < 0.001$) as illustrated in Table I. Similarly, CD62p showed increased positivity ($p < 0.001$) in diabetic patients as compared with normal individuals. CD41 and CD61 activity, however, did not show any significant difference between normal individuals and patients with diabetes as shown in Table I. Activation of platelets, using ADP as agonist, in normal subjects and in patients with diabetes did not show any difference in the positivity of CD41 and CD61 after clopidogrel administration as shown in Table II. This observation suggests that CD41 and CD61 are the platelet markers not affected by clopidogrel after activation with ADP and their expression remains unchanged. Platelets of normal subjects had decreased CD63 and CD62p percentage fluorescent positivity after clopidogrel administration as compared with diabetic patients as shown in Table II. These findings suggest that ADP activated platelets in diabetic patients have increased reactivity even after the administration of anti-platelet drug. Patients with untreated type 2 diabetes showed low response to clopidogrel. CD63 and CD62p expression after stimulation with ADP identify clopidogrel non-responsiveness; this correlates well with increased expression of these receptors.[5]

This study provides an avenue to assess the effect of varying doses of clopidogrel on suppressing platelet activation. In patients with persistent thrombotic complications despite continued administration of clopidogrel may be benefitted if an effective dose is administered which may be determined by titrating the dose and offering a customized dose of clopidogrel as prophylactic antithrombotic therapy.

Table I: Comparison of platelet CD markers at resting state in normal individuals and in patients with diabetes mellitus.

Variables	Normal (n=32)	Diabetics (n=75)
CD 41		
Pre-treatment	89.06 ± 4.06	89.95 ± 4.89
Post-treatment	90.27 ± 3.97	89.80 ± 4.06
p-value	0.09	0.880
CD61		
Pre-treatment	90.13 ± 4.45	90.53 ± 5.04
Post-treatment	90.03 ± 4.55	90.85 ± 4.39
p-value	0.925	0.774
CD63		
Pre-treatment	33.31 ± 5.93	38.27 ± 5.17
Post-treatment	13.79 ± 6.24	12.59 ± 4.11
p-value	< 0.001	< 0.001
CD62p		
Pre-treatment	29.17 ± 4.87	33. 78 ± 4.39
Post-treatment	10.68 ± 5.82	12.59 ± 4.11
p-value	< 0.001	< 0.001

Paired t-test was used for comparison.

Table II: Comparison of platelets CD markers after activation of platelets with ADP in normal subjects and in diabetics.

variables	Normal (n=32)	Diabetics (n=75)
CD 41		
Pre-treatment	89.65 ± 5.58	89.27 ± 4.62
Post-treatment	90.37 ± 4.20	91.87 ± 4.23
p-value	0.613	0.07
CD61		
Pre-treatment	91.44 ± 4.92	89.65 ± 4.72
Post-treatment	90.00 ± 4.45	90.25 ± 4.65
p-value	0.290	0.525
CD63		
Pre-treatment	84.89 ± 5.50	85.48 ± 5.69
Post-treatment	25.34 ± 8.32	20.14 ± 3.71
p-value	< 0.001	< 0.001
CD62p		
Pre-treatment	83.68 ± 5.37	83.72 ± 5.09
Post-treatment	22.65 ± 5.47	16.70 ± 3.24
p-value	< 0.001	< 0.001

Paired t-test was used for comparison.

$P2Y_{12}$ receptors are up-regulated in patients with diabetes mellitus.[6] This leads to decreased response to clopidogrel in patients with diabetes mellitus as compared to non-diabetics.[6] Antiplatelet effect of clopidogrel varies among patients with diabetes. Those with enhanced platelet reactivity are at increased risk of atherothrombosis.[7] Accelerated procoagulant activities has been associated with an increased risk of ischemic events and may explain as to why these events occur despite pharmacological blockade of specific platelet targets.[8]

It has been observed that clopidogrel pharmacodynamic response varies significantly. Based upon their response, patients are classified as responders, low responders, poor responders, non-responders or clopidogrel-resistant.[9] Sub-optimal clopidogrel response is seen in some patients with diabetes mellitus.[7] Mechanism of impaired clopidogrel response in these patients is unclear. Several hypotheses have been put forward to explain variations in response to clopidogrel. Decreased circulating active metabolites of clopidogrel, genetic differences in $P2Y_{12}$ receptors, increased expression of $P2Y_{12}$ receptors, increased circulating ADP and upregulation of other platelet activation pathways, poor glycemic control, and non-compliance are some of the causes of clopidogrel non-responsiveness.

In the light of the above results and discussion it is concluded that; CD41 and CD61 do not show any change after treatment with ADP and after the administration of clopidogrel. CD63 and CD62p positivity in normal resting and ADP stimulated platelets decreases significantly after clopidogrel treatment. CD63 and CD62p positivity in resting and ADP stimulated platelets in the patients also decreases after clopidogrel treatment. CD63 and CD62p positivity in resting platelets in the patients does not decrease as much as in normal subjects after clopidogrel treatment. It is, however, lower than before clopidogrel treatment in both groups.

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