Original Article

Accelerated Platelet Activation in Asian Indians with Diabetes and Coronary Artery Disease - The Chennai Urban Population Study (CUPS-13)

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Abstract

Aim : To assess platelet activation in south Indian type 2 diabetic subjects with and without CAD.

Methods : Four groups of subjects were studied; Group 1 comprised of non-diabetic subjects without coronary artery disease (CAD) (n = 30). Type 2 diabetic subjects without CAD formed Group 2 (n = 30); Group 3 comprised of type 2 diabetic subjects with CAD (n = 30) and Group 4 consisted of non- diabetic subjects with CAD (n=14). CAD was diagnosed based on coronary angiographic evidence of severe double or triple vessel disease. Platelet activation was tested after an overnight fast in blood obtained from a bleeding wound at 1 minute post-incision (wound-induced activation) as well as venous blood stimulated in vitro with collagen, using whole blood flow cytometry. In subjects with CAD, aspirin was withdrawn for 7 days and nitrates for 24 hours.

Results : Collagen induced GP IIb / IIIa binding was significantly higher among diabetic subjects with (28.10 ± 19.89; $p<0.05$) and without CAD (21.02 ± 19.62; $p<0.05$) and non-diabetic subjects with CAD (23.89 ± 15.65; $p<0.05$) compared to non-diabetic subjects without CAD (11.69 ± 13.69). Regression analysis showed collagen induced GP IIb / IIIa binding to be significantly associated with CAD [odds ratio (OR): 1.029, $p = 0.025$] and diabetes (OR: 1.037, $p = 0.007$).

Conclusion : Increased platelet activation is seen in urban south Indians with diabetes and CAD. ©

Asian Indians have been shown to have much high rates of premature coronary artery disease (CAD) compared to Europeans[1] and Chinese.[2] While part of this is explained by increased insulin resistance[3] and high rates of diabetes,[4] these factors do not fully explain the excess CAD risk in this ethnic group. There are very few studies in Asian Indians on platelet activation,[5] which is an important pathogenic event in predisposition to CAD[6] and it is also associated with diabetes.[7] Studies in western populations have shown platelet activation to be higher in diabetic subjects compared to non-diabetic subjects.[8] However, there have been virtually no studies on Asian Indian diabetic subjects, which is significant as Asian Indians have greater insulin resistance than Europeans despite being lean.[9] They also have a greater susceptibility to both CAD and diabetes.[10,11] We therefore studied platelet activation, [P-selectin expression and GP IIb / IIIa binding-anti-ligand induced binding sites-1 binding (LIBS-1)] in the whole blood using flowcytometric technique and compared the same in Asian Indians with and without diabetes and CAD and this is the first such study to our knowledge in this population.

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Material And Methods

The following age and sex-matched groups of subjects were studied.

Group 1 composed of 30 non-diabetic subjects without CAD, selected from an ongoing population based study - the Chennai Urban Population Study (CUPS), the details of which are published elsewhere.[12] The inclusion criteria were: normal glucose tolerance, absence of angina, myocardial infarction or history of any vascular disease and a normal resting 12 lead ECG.

Groups 2 and 3 consisted of type 2 diabetic subjects selected from the Dr. Mohan's Diabetes Specialities Centre, a specialized centre for diabetes at Chennai (formerly Madras). Diagnosis of type 2 diabetes was

based on the WHO consulting group criteria[13] i.e. fasting plasma glucose ≥ 126 mg / dl and / or 2 hr post glucose ≥ 200 mg / dl.

Group 2 included 30 type 2 diabetic subjects without clinically evident CAD. All subjects in this group denied any history of angina, myocardial infarction or any vascular disease and had normal resting ECGs.

Group 3 comprised of 30 type 2 diabetic patients with CAD who were diagnosed based on coronary angiographic evidence of significant (> 70%) stenosis of two or more coronary arteries.

Group 4 consisted of 14 non-diabetic patients with CAD (i.e. normal glucose tolerance) diagnosed angiographically using similar criteria as described for Group 3.

Age and sex matched subjects for Group 3 and 4 were recruited with the help of a cardiologist (SA).

Institutional ethical committee approval was obtained for the study and informed consent was obtained from all study subjects.

Washout for aspirin therapy and nitrates

As this study deals with platelet activation, washout of anti-platelet drugs is necessary. All the patients with CAD subjects for the study who were using anti-platelet drugs were on aspirin alone. Aspirin is known for its antiplatelet aggregatory effect through its irreversible inhibition of platelet cyclooxygenase.[14] Since the lifespan of platelets is 5-7 days, we opted for 7 days wash out for aspirin. All diabetic and non-diabetic subjects with CAD who were on aspirin therapy, consented for the wash out period of 7 days before collecting blood for platelet activation studies. The pharmacokinetics of nitrates suggests that the half-life of the nitrates is 5 hours. Hence we decided on a 24-hour washout for nitrates. All subjects with CAD on nitrates consented for 24 hours washout.

Anthropometric measurements

Physical examination included height and weight measurements and the body mass index (BMI) was calculated.

Biochemical parameters

A fasting blood sample was taken for biochemical studies. Biochemical analyses were done on Hitachi - 912 Autoanalyser (Hitachi, Mannheim, Germany) using kits supplied by Roche Diagnostics, (Mannheim, Germany). Fasting plasma glucose (glucose oxidase-peroxidase method), serum cholesterol (cholesterol oxidase-peroxidase-amidopyrine method), serum triglycerides (glycerol phosphate oxidase-peroxidase-amidopyrine method) and HDL cholesterol (after precipitation of LDL and chylomicrons using phosphotungstic acid) were measured. Low-density lipoprotein (LDL) cholesterol was calculated using the Friedewald formula.[15] Glycated hemoglobin (HbA1C) was estimated by high-pressure liquid chromatography using the Variant machine (Bio-Rad, Hercules, Calif., USA).

Platelet activation studies

Whole venous blood, collected in 3.8% sodium citrate, was added to tubes containing Walsh's buffer, a saturating solution of the anti-CD42b antibody and saturating solutions of one of the two antibodies for activation detection: AC1.2 PE for detection of P-selection expression (an indication of platelet secretion); and biotinylated Anti-LIBS-1, specific for GP IIb / IIIa (ligand) binding. Negative and positive control tubes were prepared for each subject, as previously noted [5] and additional tubes were prepared using 100 $mg.ml^{-1}$ of equine collagen, Type 1. A modified bleeding time test was performed. After inflating a sphygmomanometer to 40 mm Hg for 30-60 seconds, an incision was made using a Surgicutt R lancet, using moderate pressure. The incision was allowed to bleed for 1 minute, at which time a sample was taken using a heparinized capillary tube to collect all blood present at the incision site. Following the 1-minute sample, the incision was bandaged with steristrips. Whole blood collected in heparinized capillary tubes was added immediately to similar tubes with one of the two activation antibodies. All tubes were mixed gently and incubated for 15 min with labeled antibody. The reaction was then stopped and the platelets fixed by the addition of 450 ml of 0.25% paraformaldehyde. Flow cytometric acquisition [Becton Dickinson Immunocytometry Systems, Belgium] and analysis was performed within 6 h by a trained operator blinded to subjects status. Acquisition was limited to include only particles with the characteristic properties of platelets and stained for the SZ2-FITC (or SZ2-PE) antibody. Analytical markers were used to determine the percentage of activated platelets.

Statistical Analysis

One-way ANOVA or students "t" test as appropriate was used to compare groups for continuous variables. Chi-square test or Fisher's Exact test as appropriate was used to compare proportions. Regression analysis was done to determine the association of platelet activation with diabetes and CAD. All analysis was done using Windows based SPSS statistical package (Version 10.0, Chicago) and p values <0.05 were taken as the level of significance.

RESULTS

Table 1 shows the characteristics of the study population. Serum cholesterol and triglycerides were significantly higher among the non-diabetic subjects with CAD compared to non-diabetic subjects without CAD.

Table 2 presents the results of platelet activation in study groups. Both wound induced and collagen induced P-selectin expression were higher in subjects

Table 1 : Clinical and biochemical characteristics of the study population

Parameters	Non-diabetic subjects without CAD (n = 30)	Type 2 diabetic subjects without CAD (n = 30)	Type 2 diabetic subjects with CAD (n = 30)	Non-diabetic subjects with CAD (n = 14)
Age (Yrs)	55 ± 8	55 ± 7	55 ± 8	55 ± 13
Body mass index (Kg/m^2)	23.8 ± 3.4	25.4 ± 3.3	25.3 ± 3.0	25.9 ± 4.5
Systolic blood pressure (mm Hg)	126 ± 17	129 ±11	131 ± 12	130 ± 19
Diastolic blood pressure (mm Hg)	80 ± 8	84 ± 4*	84 ± 6*	88 ± 13*
Fasting plasma glucose (mg/dl)	95 ± 11	167 ± 40 *	173 ± 56 *	99 ± 16 #@
HbA1c (%)	5.4 ± 0.4	7.7 ± 1.4 *	8.5 ± 1.7 *	5.5 ± 0.6# @
Serum cholesterol (mg/dl)	154 ± 46	185 ± 31*	192 ± 39*	196 ± 35 *
Serum triglycerides (mg/dl)	124 ± 53	150 ± 69	192 ± 62*	154 ± 69 *
HDL cholesterol (mg/dl)	42 ± 12	43 ± 8	39 ± 8	42 ± 12
LDL cholesterol (mg/dl)	119 ± 31	115 ± 23	119 ± 31	115 ± 46

*p <0.05 compared to non-diabetic subjects without CAD; #p<0.05 compared to type 2 diabetes without CAD; @p<0.05 compared to type 2 diabetes with CAD

Table 2 : Platelet activation parameters in the study group

Parameters	Non-diabetic subjects without CAD (n = 30)	Type 2 diabetic subjects without CAD (n = 30)	Type 2 diabetic subjects with CAD (n = 30)	Non-diabetic subjects with CAD (n = 14)
P selectin expression (W)	52.66 ± 16.77	58.41 ± 24.15	57.52 ± 20.86	58.21 ± 18.25
GP IIb/IIIa binding (W)	13.44 ± 10.25	17.76 ± 13.24	17.18 ± 11.01	18.58 ± 5.56
P selectin expression (C)	64.22 ± 23.45	68.54 ± 29.65	69.12 ± 25.76	73.16 ± 21.61
GP IIb/IIIa binding (C)	11.69 ± 13.69	21.02 ± 19.62*	28.10 ± 18.89*	23.89 ± 15.65*

W - Wound induced; C - Collagen induced; * p <0.05 compared to non-diabetic subjects without CAD

Table 3 : Association of collagen induced GP IIb/IIIa binding with CAD and diabetes - Regression Analysis

	OR	95% CI	p value
Dependent variable: CAD			
GPIIb/IIIa - Collagen induced			
Unadjusted	1.029	1.004 - 1.054	0.025
Adjusted for HbA1c	1.026	1.000 – 1.053	0.050
Dependent variable: Diabetes			
GP IIb/IIIa - collagen induced			
Unadjusted	1.037	1.009 – 1.066	0.007
Adjusted for CAD	1.030	1.001 – 1.059	0.040

with diabetes both with and without CAD and in non-diabetic with CAD compared to non-diabetic subjects without CAD. However, the difference did not reach statistical significance probably due to the small sample size.

Collagen induced GP IIb/IIIa binding was significantly higher among diabetic subjects with and without CAD (p<0.05) and non-diabetic subjects with CAD (p<0.05) compared to non-diabetic subjects without CAD.

Fig. 1 shows the mean values of collagen induced GP IIb/IIIa binding levels in relation to HbA_1c. There was a significant increase in collagen induced GP IIb/IIIa binding levels with increase in tertiles of HbA1c (ANOVA p=0.026).

Table 3 shows the results of the regression analysis which showed collagen induced GP IIb/IIIa binding to be significantly associated with CAD [odd ratio (OR): 1.029, p=0.025]. This association persisted even after adjusting for HbA1c (OR: 1.026, p=0.050).

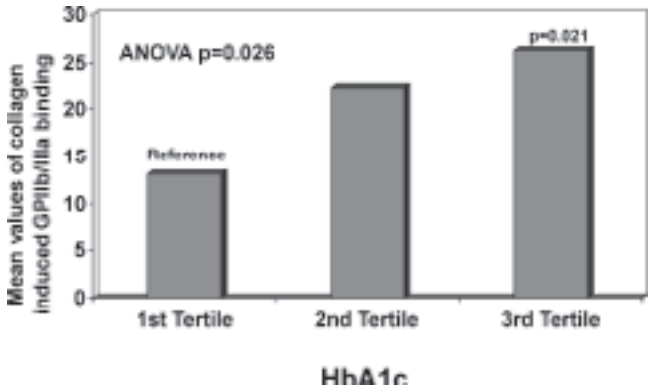

Fig. 1 : Mean values of collagen induced GP IIb/IIIa binding in relation to HbA1c

Similarly, collagen induced GPIIb/IIIa binding (unadjusted: OR: 1.037, p=0.007; adjusted: OR: 1.030, p=0.040) also showed a significant association with diabetes even after adding CAD as an independent variable into the regression equation.

Discussion

This study shows that collagen induced GP IIb/IIIa binding seems to be associated with CAD as well as diabetes in Asian Indians and that collagen induced GP IIb/IIIa binding is strongly associated with glycemic control. These findings are of interest as this study, for the first time, using specific flowcytometric techniques demonstrates that platelet activation is associated with CAD as well as diabetes in this high-risk population.[10,11]

Although the prevalence of premature CAD is very high among Indians, the exact causes of the excess CAD rates have not been clearly identified. Hence there is a need to search for newer risk factors. Platelet hyperactivity has been shown to be associated with diabetes, myocardial infarction, stroke, emotional stress, smoking, unstable angina, dyslipidemia and angioplasty.[16,17] Earlier studies by us on platelet activation factors like sCD40L and sP-selectin indicated increased platelet activation among diabetic subjects compared to normals.[18] This increase could probably be a reflection of all the metabolic derangements inclusive of increased lipids among the diabetic subjects. However, most of these studies have used methods like platelet aggregation, radioimmunoassay (RIA) or enzyme linked immunosorbent assay (ELISA) for detecting platelet activation. These measures have some limitations, as artefactual in vitro platelet activation could occur due to methodological issues. Whole blood flow cytometry has minimized these problems yielding a method to assess platelet activation in the physiological milieu of the blood. In this study we have used flow cytometry to assess platelet activation dependent increase in P-Selectin and ligand induced conformational changes in the GP IIb / IIIa complex using specific antibodies. GP IIb / IIIa receptor has recently gained a lot of interest in the field of cardiology and inhibitors against these receptors are used in treating CAD.[19] The CURE study showed that clopidogrel while prevents expression of GP IIb / IIIa receptor,[20] which is beneficial in subjects with acute coronary syndrome.[21]

In this study we used collagen as a stimulus to activate platelets. In addition, we also used bleeding time or wound induced platelet activation and assessed GP IIb / IIIa binding. The wound-induced model has the advantage of assessing the platelet activation in response to an in vivo vascular injury. We found that both wound and collagen induced GP IIb / IIIa binding were increased in diabetic compared to non-diabetic subjects. This is in agreement with earlier findings, which have shown platelet aggregation and adhesion to be higher among diabetic subjects compared to normals.[17] The relation of collagen induced GP IIb / IIIa binding with diabetes was not affected by the presence of CAD, as observed from the regression analysis, indicating that diabetes per se also alters platelet activation. This is in agreement with a recent review, which suggests that diabetes is a CAD risk equivalent.[22] Moreover, these alterations have been shown to be influenced by blood glucose levels as also shown in the study in relationship to HbA_1c levels.

Collagen induced GP IIb / IIIa binding levels were higher among subjects with CAD. This finding is of importance as earlier studies have shown increased GP IIb / IIIa binding levels in Asian Indians.[5] Thus GP IIb / IIIa binding may be associated with CAD in Indians. Though HbA_1c showed an association with collagen induced platelet activation, this did not affect its association with CAD as observed in the regression analysis.

This study has several limitations. Firstly, the sample size is rather small, particularly the non-diabetics with CAD. However it is to be noted that these studies are extremely labour intensive and expensive and hence had to be done on small numbers. Secondly, not all platelet activation measures showed an association with diabetes or CAD. This could again be an effect of the small sample size as most measures of platelet activation were higher in the subjects with diabetes and / or CAD compared to controls. Thirdly, we were unable to measure plasma and platelet concentrations of nitric oxide and lipoxins which would have added more information on vasodilatation and monocyte adherence, as these processes are also involved in atherosclerosis. Finally, a cause and effect relation between platelet activation and diabetes or CAD cannot be established due to the cross sectional nature of the study. Further, we were unable to assess the role of interventions that could reduce platelet activation. This indicates the need of future prospective studies in order to establish the significance of lowering platelet activation with respect to the reduction of cardiovascular events.

In conclusion the study results indicate that platelet activation is increased in urban South Indians with both diabetes and CAD.

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Late Dr. Jerome Markovitz, Associate Professor of Medicine and Cardiology at the University of Alabama, Birmingham, USA was responsible for setting up and standardizing the platelet activation assay at the Madras Diabetes Research Foundation and supported the project until his untimely demise in September 2002. We dedicate this work to him. Later Dr. MD Gross, who took over from Dr. Jerome Markovitz, helped with the project. This is the 13th publication from the Chennai Urban Population Study (CUPS).

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