Research Article

EVALUATION OF NEUTROPHIL FUNCTION, OPSONISING CAPACITY AND LYMPHOCYTE PROLIFERATION FOR RISK OF DEVELOPING ISCHEMIC HEART DISEASE IN TYPE 2 DIABETES MELLITUS PATIENTS

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ABSTRACT

Background: Ischaemic heart disease (IHD) is a leading cause of mortality in type 2 diabetes mellitus (DM) patients. Elevated lipid profile levels may not always be associated with IHD. Increased reactive oxygen production, decreased chemotaxis, altered opsonization in DM patients alters phagocytosis. T and B lymphocyte proliferations are also found to be impaired.

Objectives: Phagocytic index (PI), serum opsonization, T and B cell proliferation have been studied to evaluate their role as risk markers for developing type 2 DM mediated cardiac events.

Methods: The patients were classified into three groups. Blood glucose, glycated haemoglobin (HbAlc) and ECG changes were taken into consideration when grouping. All the recruited patients had normal lipid profile and were not taking any lipid lowering drugs. The study cohort were grouped as Group 1 (DM-IHD) consisting of 33 patients with uncontrolled diabetes and ECG changes, Group 2 (DM) consisting of 51 patients with uncontrolled diabetes, without any ECG changes, both groups receiving antihypertensives and antidiabetic therapy. Group 3 (control) consisted of 30 healthy subjects with no history of DM or IHD and with normal fasting blood glucose levels and normal HBA1c values. Phagocytic index, opsonization with serum (WBO), IgG and C3 as well as T and B cell proliferation were assessed.

Results: Both the study groups were compared to each other and the control. PI NO (without serum opsonisation) was decreased in group 1 & 2. Group 1 showed decreased PI (WBO, C3, IgG). In group 2 forty patients had higher PI (WBO, C3, IgG), remaining 11 displayed values similar to group 2 indicating risk of developing IHD. T and B cell proliferation rate in groups 1 and 2 were decreased.

Conclusions: PI can be used as a marker to predict IHD events in DM patients whose lipid values cannot predict IHD and can be used as part of an improved strategy for preventing atherosclerosis.

Keywords: Ischaemic heart disease, Lymphocyte proliferation, Opsonisation, Phagocytic index, Type 2 Diabetes mellitus.

INTRODUCTION

Coronary artery disease (CAD) is a leading cause of mortality in patients with Diabetes Mellitus (DM)[1]. The various reported causes of atherosclerosis and CAD are smooth muscle cell proliferation induced by insulin stimulation that increases collagen synthesis within the vascular wall; increased accumulation of low density lipoproteins (LDL)[2] and its oxidized products[3], premature senescence in vascular cells due to phenotypic changes in the vascular smooth muscle cell proliferation and adhesion molecule expression due to increased reactive oxygen species (ROS)[4]. The other reasons being decreased resorption of atherosclerotic plaques due to phagocytic dysfunction[5].

To prevent cardiovascular events in diabetic patients there is a need for simple markers that could identify patients with a risk of developing myocardial ischaemia. One of the commonly used predictor for coronary heart is the lipid profile[6]. Lipid profile may not always be elevated levels in DM led CAD patients indicating that not all DM patients with CAD develop an altered lipid profile. Thus lipid profile evaluation cannot always be used to predict cardiac events[7]. Investigation into the role of other factors such as altered neutrophil phagocytic capacity, role of opsonins complement 3 (C3) and Immunoglobulin G (IgG) on phagocytic capacity and proliferation capacity of the T and B cells of the immune system may be helpful.

Findings also suggest that polymorphonuclear leucocytes (PMNLs) from DM patients have an increased reactive oxygen metabolite production that may cause cell and tissue damage, opsonisation impairment (C3 and IgG)[8], decreased chemotaxis and decreased PMNLs phagocytic activity[9] which contributes to increased progression of atherosclerosis leading to CAD[10]. During opsonisation antigens are bound by antibody and/or complement molecules enhancing phagocytosis, important opsonins being IgG and C3[11]. Several lines of evidence suggest that PMNLs, which represent a major mechanism of innate immunity and inflammation which play a pivotal role in human atherosclerosis are altered in DM led CAD[12]. Peripheral PMNLs along with pro-inflammatory cytokines are found to be increased in the circulation in coronary artery diseases [13]. Patients with DM also display a significant increment in the basal levels of calcium in PMNLs and this is found to be associated with marked impairment in their phagocytosis[14].

T and B lymphocyte proliferation rates are found to be impaired in DM[15]. Common hypertensive drugs cause elevation in cell calcium, which reduces cell proliferation, mainly B cell proliferation along with PMNLs phagocytic function[16]. More over atherosclerosis being a chronic inflammatory disorder of the vessel wall, defective immune responses are known to influence disease progression[17]. There is currently a great deal of interest in the role of immune system in vascular pathology. It is to be hoped that a better understanding of how immune response contributes to atherogenesis will suggest new targets for therapeutic intervention in cardiovascular disease.

We have studied the phagocytic function of neutrophils, serum opsonisation capacity, T and B cell proliferation in DM and DM Ischaemic heart disease (IHD) patients in order to assess these markers in predicting CAD events in DM patients.

PATIENTS AND METHODS

This prospective cohort study included 84 type 2 DM patients, with normal lipid profile and 30 healthy age and gender matched controls. The duration of the diabetes ranged from 3 to 7 years. Patients visiting on an outpatient basis to Tanvi Medical Center and Yenepoya Hospital, Mangalore, Karnataka, India were recruited for the study after obtaining their consent. The study was conducted after obtaining approval by the Yenepoya University Ethics Committee.

Patients were sampled after fasting overnight, before taking any medication, after 10 – 15 minutes of resting in upright position. All the patients underwent a complete cardiac examination followed by

12 lead ECG and echocardiography. Following this blood samples were collected. Exclusion criteria were known malignancies, surgeries in previous month, other existing chronic and acute diseases, severe hypertension, stroke, left bundle branch block, atrial fibrillation, significant valvular disease, ventricular dysfunction, significant diabetic nephropathy and renal failure.

The patients were classified into three groups. Blood glucose, glycated haemoglobin (HbAlc) and ECG changes were taken into consideration when grouping. All the recruited patients had normal lipid profile and were not taking any lipid lowering drugs.

Group 1 (DM-IHD) consisted of 33 patients with uncontrolled diabetes, with ECG changes (ST segment elevation or depression and T wave inversion) receiving antihypertensives and antidiabetic therapy.

Group 2 (DM) consisted of 51 patients with uncontrolled diabetes, without any ECG changes, receiving antihypertensives and antidiabetic therapy.

Group 3 consisted of 30 healthy subjects with no history of DM or IHD and with normal fasting blood glucose levels and normal HBA1c values. This group served as control.

Chemicals and reagents

Concanavalin A(Con A), Lipopolysaccharide(LPS), 3-(4,5-dimethylthiazol 2-yl)-,5 diphenyltetrazolium bromide(MTT), SDS.

Sample Collection and Data Collection:

Venous blood (four ml) was collected from the patients in appropriate vaccutainers (with and without anticoagulants). Samples were immediately transferred to the laboratory and processed within 6 hours. Opsonization capacity, phagocytic index, T and B cell proliferation was measured through laboratory experiments. Blood glucose, HbAlc[18] and lipid profile (Total Cholesterol, LDL Cholesterol and Triglycerides) assays were performed in all patients.

Separation of neutrophils for evaluation of phagocytic capacity

PMNLs were isolated by a method modified from Boyum[19]. Venous blood samples from the subjects was drawn into heparinized syringes and settled by gravity in 6% dextran (mol. wt. 70,000; Hi media chemicals, India) in normal saline (1:3). The leucocyte-rich plasma was withdrawn and centrifuged at 160g for 10 minutes. The pellet was re-suspended in Eagle's minimal medium; 6 ml of the cell suspension was carefully layered onto 3 ml of Ficoll-Paque (Hi media chemicals, India) and centrifuged at 160 g for 35 minutes. Mononuclear cells of the interface were removed. Residual erythrocytes in the pellet were lysed with ice-cold ammonium chloride (0.87% in sterile water). After centrifugation at 160 g for 5 minutes, PMNLs were washed twice with Hanks balanced salt solution (HBSS). Number of neutrophils was adjusted to a final suspension such that 1ml contains 5×10^6 PMNLs.

Neutrophil Phagocytosis and Serum Opsonisation

For phagocytosis to take place and to study the opsonisation capacity, to the separated PMNLs suspended in phosphate buffered saline (PBS) and Bovine serum albumin (BSA) mixture add bacteria opsonised with complete serum, serum without IgG, serum without C3. Incubate at 37°C for 30 minutes. Two smears were prepared and stained with Lieshman's stain and the numbers of bacteria engulfed by 200 neutrophils[20] were counted under a 100x microscope for phagocytosis.

Preparation of the bacteria was done as follows. *Staphylococcus aureus* grown in nutrient agar under standard conditions and the suspension was washed in HBSS at pH 7.2 containing 0.1M mercaptoethanol, then diluted in HBSS and adjusted to the required quantity. PMNLs and Bacteria ratio of 1:9 was used to study opsonisation and phagocytic activity. The stock was stored for all experiments[21]. Opsonisation by IgG of the organism was done by inactivation of Complement C3 as explained by Jan Kallman et al[22]. Opsonisation of the organism with C3 of the serum was done with salt chelating of IgG[23]. Autologous serum was used in all experiments. *S. aureus* cells were centrifuged at 1200 rpm washed with PBS and then incubated with 500 µl of whole serum for evaluation of PI with serum opsonised bacteria (PI - WBO), 500 µl serum without IgG[23] for evaluation of PI with C3 opsonised bacteria (PI- C3) and 500 µl serum without C3[22] for evaluation of PI with IgG opsonised bacteria (PI-IgG). Non opsonised bacteria (PI-NO) were also used for evaluation of PI. The bacterial solution was then incubated at 37°C for 45 minutes with shaking. The cells were then centrifuged, pellets obtained were washed and reconstituted in PBS.

Phagocytic capacity of the neutrophils was evaluated as Phagocytic index, calculated as follows: Phagocytic Capacity that is number of bacteria/Neutrophil, Phagocytic % which is %age of Neutrophils having phagocytic capacity and the Phagocytic Index which is the Phagocytic Capacity times the Phagocytic % divided by 100. Phagocytic Index reveals the phagocytic function of the PMNLs.

T and B lymphocyte proliferation in response to mitogens in 48 hours lymphocyte culture:

Lymphocytes were separated by commercially available lymphocyte separation media (Hisep from Hi media, India) based on the principle of density gradient.

In order to study the T and B lymphocyte proliferation two specific mitogens Con A and LPS were used respectively. Cell proliferation assays were performed with 20µg Con A /ml and 100µg LPS/ml added to the cell cultures of lymphocytes at the beginning of the incubation period[24]. 5×10^5 lymphocytes were obtained and re-suspended in 200µL of RPMI-1640 medium mixed with 10% fetal calf serum, containing 5.6mM glucose, 2.0mM glutamine, and antibiotics (streptomycin 100 units/ml and penicillin 200 units/ml). Cells were then incubated in the 96-well plate at 37° C with 5% CO_2 for 48 hours.

MTT assay for cell proliferation

Cell proliferation was measured by the 3-(4, 5-dimethylthiazol2-yl)-2,5diphenyltetrazolium bromide (MTT) assay. At the end of 44 hours of incubation of lymphocytes, 15µL MTT reagent (5mg/ml) was added. After 4 hours of incubation at 37°C, 150µL of 2% SDS solution was added into each well to dissolve the tetrazolium crystals. Following overnight incubation, the absorbance at 570nm was recorded in a microplate reader which is proportional to the rate of lymphocyte proliferation in vitro[25].

Statistical Analysis

The data were analyzed by using Graph Pad Prism for Windows. The significance of differences was calculated by using one-way analysis of variance (ANOVA) followed by Tukey Kramer procedure for multiple comparisons. P value < 0.0001 was considered highly significant (hs) and P < 0.001 was considered significant (sig).

Table 1: Showing study groups with parameters HBA1c, FBS

Groups	No. of patients	HBA1c %	Fasting glucose mg/dl
Group 1- Uncontrolled DM with ECG changes	33	5.9±0.82	105.7±20.2
Group 2- Uncontrolled DM without ECG changes	51	11.37±2.63	219.49± 64.6
Group 3 - Healthy controls	30	4.8±0.7	110.09±17.67

All the results are expressed as Mean ±SD

HBA1c – Haemoglobin A1c, FBS – Fasting Blood Sugar

Table 2: Showing study groups with parameters Phagocytic Index, T Cell and B Cell Proliferation

Groups	PI (NO)	PI (WBO)	PI (C3O)	PI (IgG O)	T cell proliferation	B cell proliferation
Group 1- Uncontrolled DM with ECG changes	0.36 ±0.14	1.03±0.246	0.64±0.15	0.5±0.12	0.16±0.019	0.079±0.007
Group 2- Uncontrolled DM without ECG changes	.33±0.16	**a**- 2.36 ±0.63 (40) **b**-1.06 ±0.23 (11)	**a**- 1.19±0.27 (40) **b**- 0.65±0.14 (11)	**a**- 1.30±0.47 (40) **b**- 0.4±0.02 (11)	0.155±0.021	0.083±0.007
Group 3-Healthy controls	1.17 ±0.41	6.04±1.26	2.93±1.15	2.68±1.02	0.91±0.25	0.13±0.04

All the results are expressed as Mean ±SD

PI – Phagocytic index, NO – Non Opsonised, WBO- Serum Opsonised, C3 O – Complement C3 Opsonised, IgG O – Immunoglobulin G Opsonised

RESULTS

The results are depicted as mean ± SD. Fasting blood glucose and HBA1c levels in the groups are given in Table 1 and PI, T and B cell proliferation in Table II. The statistical analysis is given in the graphs. The results of the study groups (Groups 1 and 2) were compared with the control group also the study groups were compared to each other. All patients had normal lipid profile (Total Cholesterol 183±26.8, LDL Cholesterol 110.4±16.8 and Triglycerides 167.3 ± 48.2).

Phagocytic index of the neutrophils without the influence of opsonins PI (NO)

In groups 1 and 2 PI (NO) was significantly decreased while compared to control subjects (p value <0.0001). There was no statistical difference between study groups 1 and 2.

Phagocytic index of the neutrophils with the influence of opsonins present in the serum

PI (WBO)

In group 1 there was a highly significant decrease in the PI (WBO) when compared to control subjects (p value<0.0001). Group 2 showed a highly significant (p value<0.0001) decrease in PI (WBO) compared to the control group 3.

On comparison of group 2 with group 1, 11 patients of group 2 showed similar results to that of group 1. In order to evaluate if the PI(WBO) values could predict IHD in group 2 patients, they were categorized as group 2a based on a cut off value 1.03 ±0.246 which is the PI(WBO) of group 1. 11 patients in group 2 had similar PI(WBO) values to those in group 1 and were categorized as group 2b. The remaining 40 patients showed a highly significant (p value <0.0001) decrease in PI(WBO) compared to the group 1 and were categorized as group 2a. These results suggested that the 11 patients of group 2b may have defective C3 and IgG opsonisation.

PI (C3)

In group 1 there was a highly significant decrease in the PI (C3) when compared to control subjects (p value<0.0001). Group 2 showed a highly significant (p value<0.0001) decrease in PI(WBO) compared to the control group 3.

Group 2a when compared to group 1 had a significant change (p value<0.0001) in the PI(C3) as observed in PI(WBO) and results of PI(C3) of group 2b were similar to group 1. There is a highly significant difference in values of PI(C3) between 2a and 2 b.

PI (IgG)

In group 1 there was a highly significant decrease in the PI (IgG) when compared to control subjects (p value<0.0001). Group 2 showed a highly significant (p value<0.0001) decrease in PI(IgG) compared to the control group 3.

Group 2a when compared to group 1 had a highly significant change (p value<0.0001) in the PI (IgG) as observed in PI (WBO) and results of PI (IgG) of group 2b were similar to group 1. There is a highly significant difference in values of PI (IgG) between 2a and 2b (p value<0.0001).

T and B cell proliferation in response to mitogens

T and B cell proliferation rate significantly decreased (p value<0.0001) in both Groups 1 and 2 when compared to the control group. There was no change in T and B cell proliferation rate between Groups 1 and 2.

DISCUSSION

The present investigation suggests decreased neutrophil phagocytic capacity in type 2 DM and DM IHD patients when compared to control subjects. PI (NO) is similar in DM and DM IHD patients and in both the groups it is statistically decreased when compared to healthy controls hence it may not be possible to use PI (NO) as a predictor for IHD events in DM patients. PI (WBO), PI (IgG) is reduced in both groups 1 and group 2. Forty of the 51 DM patients (group 2) without any ECG changes presented higher PI (WBO, C3, IgG) the remaining 11 patients displayed values similar to DM IHD patients pointing out that these patients may be at a risk of developing IHD. PI (WBO, C3, IgG) can be used as a marker to predict IHD events in DM patients whose lipid values cannot predict IHD events. However, larger sample size needs to be examined for further confirmation.

Inactivation of C3 reduces the opsonic capacity and bringing down the PI[26] and is in agreement our results. In DM patients with IHD higher levels of C3 are seen due to the inflammatory responses during the ischaemic event [27]. Though C3 levels are higher in DM patients they do not enhance the PI probably due to the decreased activity of the neutrophils itself. Other heat labile opsonins are likely to be gylcosylated or inhibited in DM patients leading to decrease in overall opsonization capacity[28]. Immune dysfunction is reported in DM IHD patients with overactivation of pro-inflammatory cytokines and higher levels of immunoglobulins and circulating immune complexes[29]. IgG levels are increased in DM and DM IHD patients[30]. In DM IHD patients the IgG are mainly against Cardiolipins. Glycosylation of IgG leads to decreased antibody function[31]. Due to these reasons though the IgG levels increase, opsonization capacity and PI do not seem to alter in DM and DM IHD compared to control group. Neutrophils play an essential role in the host inflammatory response. Mowat and Baum[32] have showed that the chemotactic activity of neutrophils from diabetic patients is significantly lower than in cells from healthy controls leading to a decrease in PI. Decreased bactericidal activity[33] and impairment of phagocytosis[34] by neutrophils of diabetic patients have been described. Patients with DM also display significant increment in the basal levels of calcium in PMNLs which is associated with marked impairment in their phagocytosis[14]. Proper phagocytic function is required to prevent formation of atherosclerotic lesions, which are characterized by the presence of a large number of macrophages. One of the reasons for dysfunction of these macrophages is that they become less efficient phagocytes[35]. This study can provide an insight into the possible predictive role of PI values for cardiac events in IHD patients in whom lipid profile is unaltered.

T and B cell proliferation rate in groups 1 and 2 when compared to healthy controls was decreased suggesting that the innate immunity in these patients is grossly reduced. There is decreased cell

proliferation in both DM and DM IHD patients hence this cannot be used as a marker to predict risk of developing IHD. Proliferation of lymphocytes which is impaired in response to mitogen could be due to potential increase in basal levels of intracellular calcium in the lymphocytes of diabetic patients. Impairment in B cell proliferation and in antibody production has been reported in other clinical conditions such as chronic renal failure[36]. Impaired response both in T and B lymphocytes in type 2 DM patients has been observed[37]. The redox imbalance in DM patients[38] extends to circulating lymphocytes where several antioxidant enzyme indices and oxidative modifications of proteins, higher concentrations of the freely diffusible H_2O_2 point towards oxidative imbalance affecting the lymphocytes[39].

In conclusion, neutrophil function and opsonising capacity can be evaluated as markers for risk assessment for developing IHD in those DM patients in whom lipid profile does not give any suggestion to the ongoing development towards IHD or events such as inflammation, decreased immune response. Our study suggests the potential importance of modulating neutrophilic and immune response as part of a novel, improved strategy for preventing and treating atherosclerosis.

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