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Expression of Macrophage Scavenger Receptor CD36 in Humans – its Implication in Type 2 Diabetes Mellitus

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ABSTRACT

CD36 (Cluster of Differentiation 36) is an integral membrane protein found on the surface of many cell types of class B scavenger receptor family. Macrophage accumulation is a feature of type 2 diabetes mellitus (T2DM) and is associated with diabetic complications. There are different views regarding the possible role and levels of expression of CD36 in T2DM. We investigated expression level of CD36 on macrophages from T2DM patients and controls. Macrophages were isolated from blood samples of T2DM and controls (n=50) using HiSep-1077 and proliferated in Macrophages-Serum-Free-Media with Macrophages-Colony-Stimulating-Factor, Insulin-Transferin-Selenium-A and antibiotics. After incubation with FITC-coated monoclonal antibody (SC-7309), cells were subjected to FACS analysis. Age of T2DM cases showed significant association (P = 0.0424) while BMI, Fasting glucose (F), Post Prandial (PP) glucose, Glycosylated Haemoglobulin (HbA1c), Total Cholesterol, High density lipoproteins (HDL) and Low density lipoproteins (LDL) were highly significant when compared with that of controls (P<0.000) by student's t test (PSAW ver.17.0). CD36 expression is increased ~4 times in T2DM and it can be used as a biomarker monocyte activation and ascertain the proinflammatory state.

Key Words: CD36-Expression, Macrophoge Culture, FACS-Analysis, T2DM, North Indian Population.

INTRODUCTION

Insulin resistance (IR) is the major cause of T2DM resulting in ineffective insulin receptors thereby leading to impaired regulation of blood sugar level. T2DM is the most common form of diabetes in the Indian population and India has been predicted to become the world diabetes capital with an estimate of 350 million by the year 2030 [1]. People (>40 yrs) with dyslipidemia, impaired glucose tolerance and hypertension, the so-called metabolic syndrome (MetS) carry an increased risk of developing T2DM and cardiovascular diseases. Abnormalities in lipid metabolism results in elevations of plasma free fatty acids (FFAs) during T2DM which is reported to contribute to IR, inflammation and endothelial dysfunction. An integral membrane protein CD36 (Cluster of Differentiation 36) belonging to class B scavenger receptor family has been Implicated in hemostasis, thrombosis, malaria, inflammation, lipid metabolism and atherogenesis [2-10]. It can bind to many ligands such as oxidized low density lipoprotein, native lipoproteins, oxidized phospholipids and long-chain fatty acids, erythrocytes parasitized with Plasmodium falciparum, collagen and thrombospondin. This active uptake of these FFAs (specially oxLDL into

cells) by multiligand cell surface scavenger receptor, CD 36 revealed its functional role in mediating atherosclerosis, inflammation and oxidative stress in macrophages and other cell types [11].

Current evidence supports a critical role of macrophages in the evolution of diabetic complications. There are different views regarding the possible role and levels of expression of CD36 in IR and T2DM [12-15]. In this study we investigated the expression level of CD 36 on macrophages from T2DM patients and normal healthy controls and tried to correlate with the biochemical parameters.

MATERIALS AND METHODS

Patient selection and sample collection

Whole-blood samples were collected in 0.5M EDTA from 50 North Indian T2DM cases at the Diabetic Clinic of Department of Medicine, Chatrapati Shahuji Maharaj Medical University (CSMMU), Lucknow, India and 50 healthy nondiabetic individuals. The diagnosis of T2DM was based on clinical characteristics, fasting/post-prandial glucose and HbA1c levels. The healthy nondiabetic controls were those without a family history of diabetes and normal blood glucose levels even after 2-hours of food intake. Diabetic and control subjects recruited in this study were matched with respect to age, sex and ethnic background. The study was conducted after obtaining informed consent from each study subject and approval of Ethics Committee of CSMMU, Lucknow, India.

Biochemical assay

Serum was isolated for lipid profile which included Total Cholesterol (TC) (mg/dl); Low Density Lipoprotein (LDL)-cholesterol (mg/dl); High Density Lipoprotein (HDL) - cholesterol (mg/dl); Very Low Density Lipoprotein (VLDL)-cholesterol (mg/dl) and Triglyceride (TG) (mg/dl) after overnight fasting. The estimations were done with Ecoline kits (Merck, USA) using UV-Vis double beam spectrophotometer (Shimadzu, Japan).

Isolation of human monocytes – derived macrophages

Mononuclear cells were isolated by centrifugation of EDTA-blood at 1200 rpm using HiSep 1077 (HiMedia) at room temperature. EDTA-blood (2.0 ml) was diluted with 0.9% NaCl in 1:4 ratio and lightly poured over 3.0 ml HiSep in a centrifugation tube. The middle layer containing mononuclear cells was transferred to a new vial after centrifugation and cells were washed twice by EDTA containing phosphate buffered saline (PBS).

Primary macrophage cell culture

Mononuclear cell suspension (0.5 ml) as transferred to a culture plate containing macrophage serum free (MSF) media with macrophage colony stimulating factor (M-CSF, 100 ng/ml), 1.0% media supplement, Insulin Transferin Selenium – A (ITS-A) and penicillin streptomycin antibiotics (100U/ml : 1mg/ml). The plate was transferred to a CO_2 incubator (37^0C; 5.0% CO_2) for cell proliferation. The media was changed at 24 hours interval. Cells were stained with trypan blue to check the cell viability. The cells were proliferated upto 96 hrs and assessed for quantity and quality. The maximum proliferation rate was observed at 48 hours of incubation when the cell density reached 10^6 cells per ml (Figure 1).

Immunofluoresent flow cytometry

Immunofluoresent flow cytometry analysis was performed using peripheral blood monocytes and a mouse monoclonal IgM (200 µg/ml) raised against human tonsil cells (SC-7309, Santa Cruz). The cells were washed with PBS lightly because of their semi adherent nature and were incubated with 0.4 µg/ml FITC-coated CD36 antibody for 90 minutes in dark. The FITC-coated CD36 antibody treated cells were subjected to FACS scan. Cells were properly gated and side (SSC)/ front scattered (FSC) based graphs were obtained showing the X-mean value of the graph peak (mean channel fluorescence) which indicated the activity of CD36 receptor expression.

Statistical Analysis

Student's and paired t-test were used to compare age, body mass index (BMI), Waist Hip Ratio (WHR) and other biochemical parameters (mentioned earlier) between T2DM subjects and controls using PSAW software (ver. 17.0).

RESULTS

The study was conducted on 50 each of T2DM cases normal healthy controls. The mean age ± SD of cases and controls was 45.74 ± 6.54 and 48.34 ± 6.09 respectively. Anthropometric and clinical data of cases showed significant association when compared to controls (Figure 2). Age of T2DM cases showed significant association (*P* = 0.0424) while BMI, F, PP, HbA1c, TChol., HDL and LDL were highly significant when compared with that of controls (*P*<0.000). However, TGL and VLDL were only significantly associated with T2DM (*P* = 0.011; 0.0267 respectively) (Figure 2).

The representative pattern of flow cytometric analysis for surface expression of CD36 on macrophages is shown in Figure 3. Figure 3A shows the relative number of macrophages of control subjects which were not bound to anti CD36 – FITC coated antibody (mean channel fluorescence = 1.74) while Figure 3B shows the relative number of macrophages of control subjects which were bound to anti CD36 – FITC coated antibody (mean channel fluorescence= 2.48). In contrast, the cases (mean channel fluorescence = 3.68) showed an abundant expression of CD36 antigen on the surface of macrophages derived from T2DM patients (Figure 3D) when compared to those not bound to the antibody (mean channel fluorescence = 1.01, Figure 3C). The clear indication of FITC signal used against the CD36 antigen on macrophages have been shown in Figure 4 with a peak shift of 0.683 times in controls (Figure 4A) and 2.681 times in cases (Figure 4B). The increase in peak of diabetics is ~4.0 times that of controls which indicated that CD36 in T2DM cases were 4.0 times more active than in controls. This observation clearly indicated that CD36 expression had increased in the presence of high glucose concentration.

DISCUSSION

The expression of CD36 on macrophages was investigated in north Indian T2DM population. The current *in vitro* study demonstrated that the human monocyte derived macrophages were proliferated in 48 hours and the expression of CD36 receptors on macrophages was four times in T2DM cases as compared to normal healthy controls. Our results indirectly indicated that the accumulation of lipoproteins in the blood vessels increased up to 4.0 times than that of controls. Increased efficiency of CD36 receptors may lead to an increased accumulation of cholesterol esters such as Low density lipoproteins (LDL) in macrophages. It has been reported that oxidized form of LDL (OxLDL) binds to the macrophage CD36 receptor and triggers the activation of PPARγ and kinases such as Lyn, MEKK2, JNK1 and JNK2 which leads to an increased expression of CD36 [16]. Lipids, including cholesterol are transported in serum by low-density lipoprotein (LDL). The scavenger receptor CD36 expressed on the surface of macrophages bind to and internalize these lipids causing the conversion of macrophages to pro-atherogenic foam cells leading to atherosclerosis. Present therapies are limited in slowing the progression of macrophage-mediated injury. Novel strategies that are more specific at targeting macrophages may provide better protection against the development of T2DM complications [17]. Antidiabetic thiazolidinediones (TZD) also activate the nuclear receptor PPAR-γ, leading to increased expression of CD36. Similarly, NFκB is targeted by OxLDL stimulation inducing the synthesis of diverse cytokines. T2DM increases inflammation and reactive oxygen species (ROS) which finally leads to atherosclerosis by both creating modified LDL ligands and causing endothelial dysfunction. The CD36 ligands such as OxLDL are capable of mediating a CD36-dependent inflammatory paracrine loop between adipocytes and their associated macrophages. This facilitates chronic inflammation in adipose tissue and contributes to IR common in obesity and dyslipidaemia.

CD36 scavenger receptors were found to have increased expression only with decreased whole body glucose disposal rather than increased serum FFA levels [11, 18, 19]. The myeloperoxidase–hydrogen peroxide–nitrite system of phagocytic cells gets activated to act upon the internalized LDL to make it OxLDL and other lipid derived CD36 specific ligands leading to macrophage mediated foam cell formation [20]. The internalized OxLDL goes through lipoxygenase or other pathways to generate lipid byproducts like 9- and 13-hydroxyoctadecadienoic acid [HODE], prostaglandin J2 [PGJ2] which act as ligands for the transcription factor PPARγ. After molecular interaction with these ligands PPARγ has been reported to charge the complex for nuclear translocation and activates transcription of target genes. Activation of target genes creates a positive feedback loop which increases the expression of both PPARγ and CD36 receptors. The hyperglycemic conditions resulting in increased expression of CD36 has been found to be a valuable tool for cardiovascular risk assessment among cases with Type 2 diabetes. Earlier reports have shown that expression of the class A scavenger receptor is increased in monocytes from hyperlipidemic cases [21].

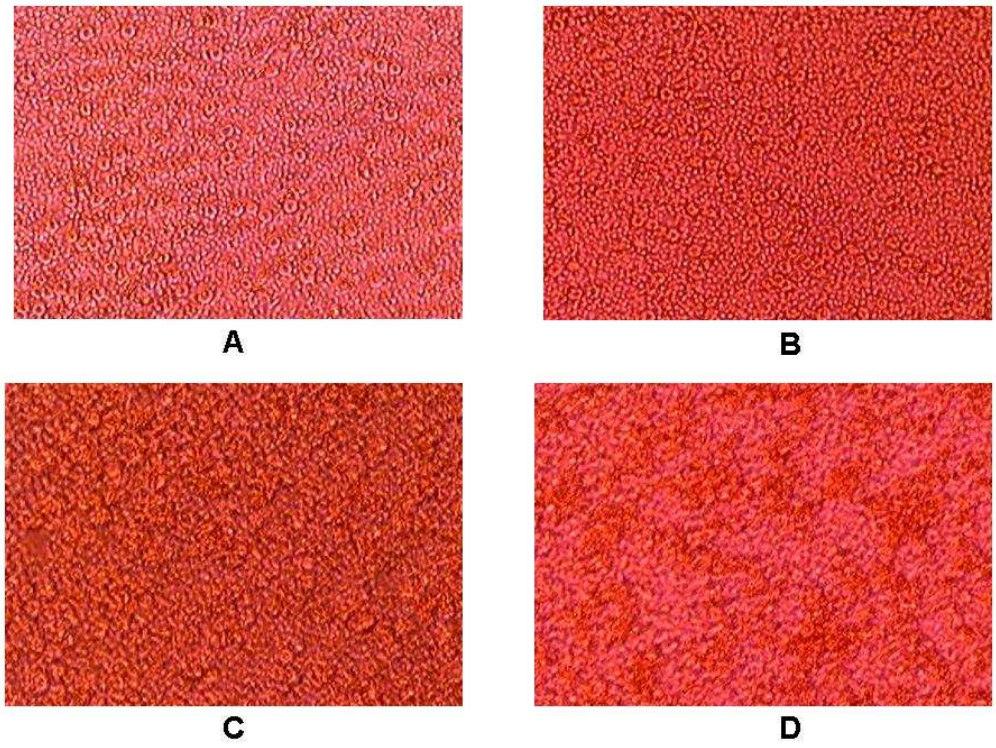

Figure 1: Primary macrophage cell culture of macrophage cells after A: 24 hrs; B: 48 hrs; C: 72 hrs (necrotic stage) and D: 96 hrs (debris after cell death).

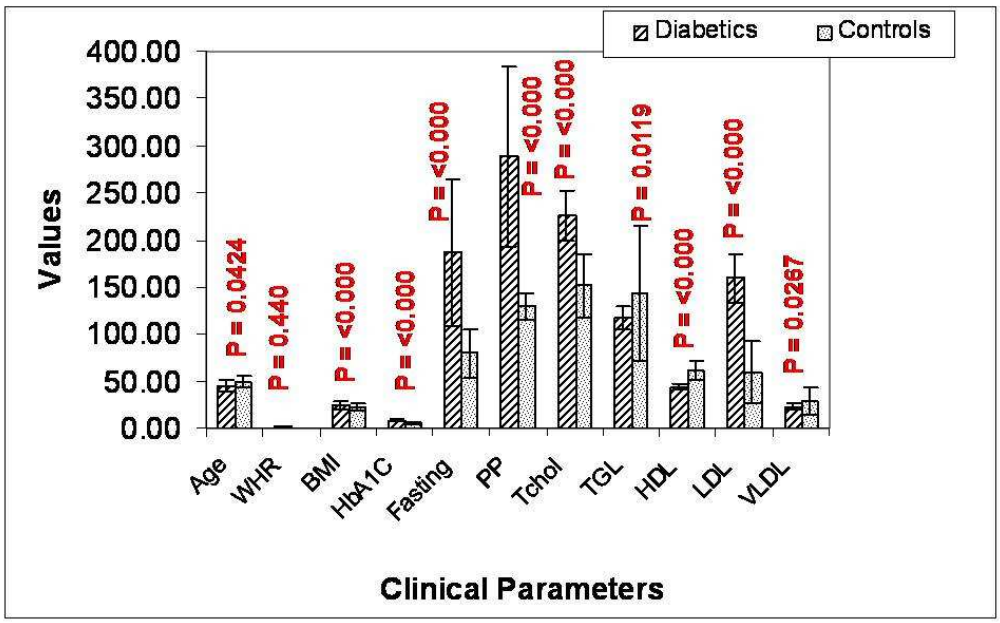

Figure 2: Anthropometric and clinical data of cases and controls with their *P*-values.

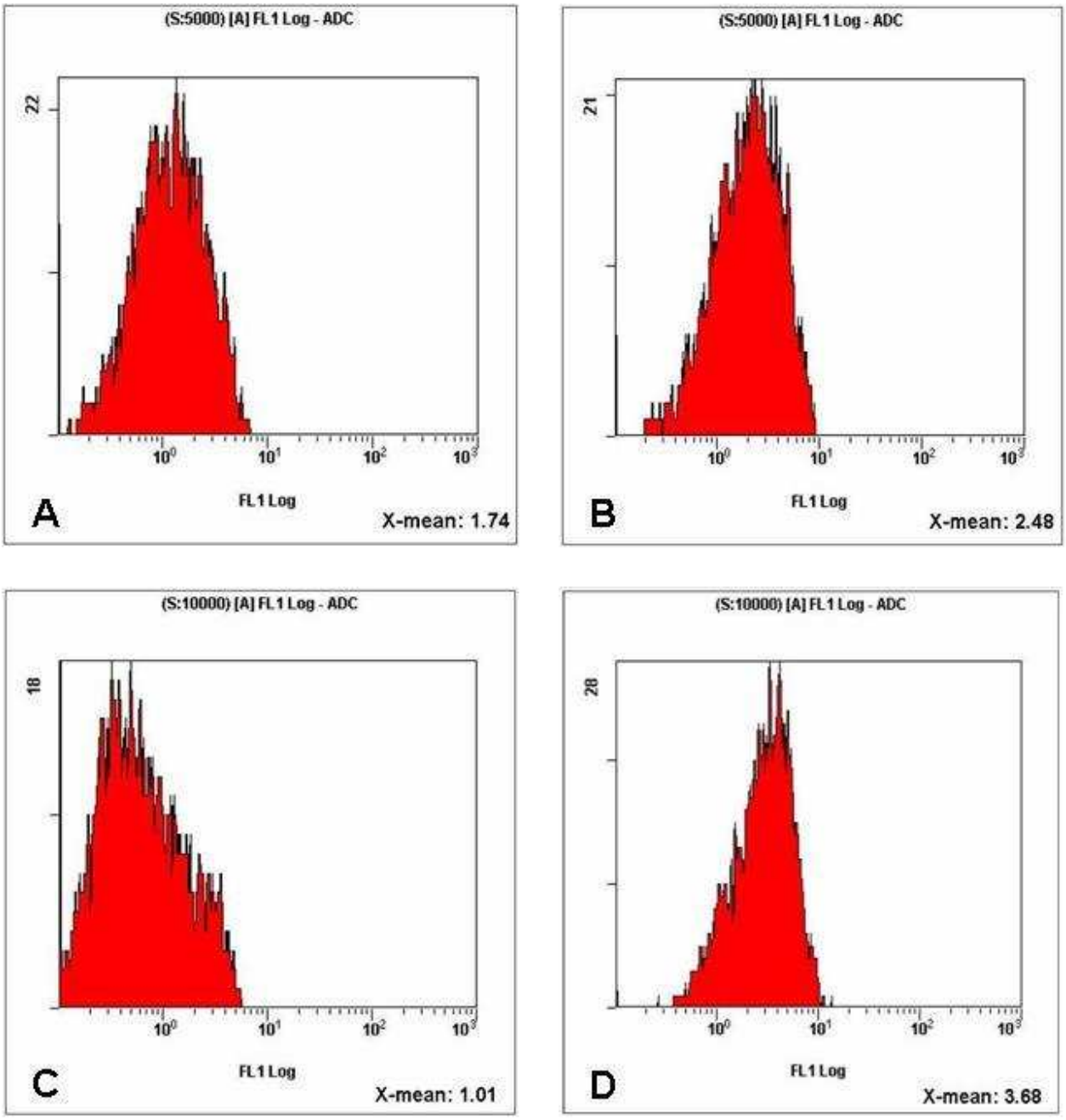

Figure 3: Flow cytometric analysis of CD36 expression on macrophages using anti CD36 – FITC coated antibody. A: control subjects (not bound); B: control subjects (bound); C: cases (not bound); D: cases (bound).

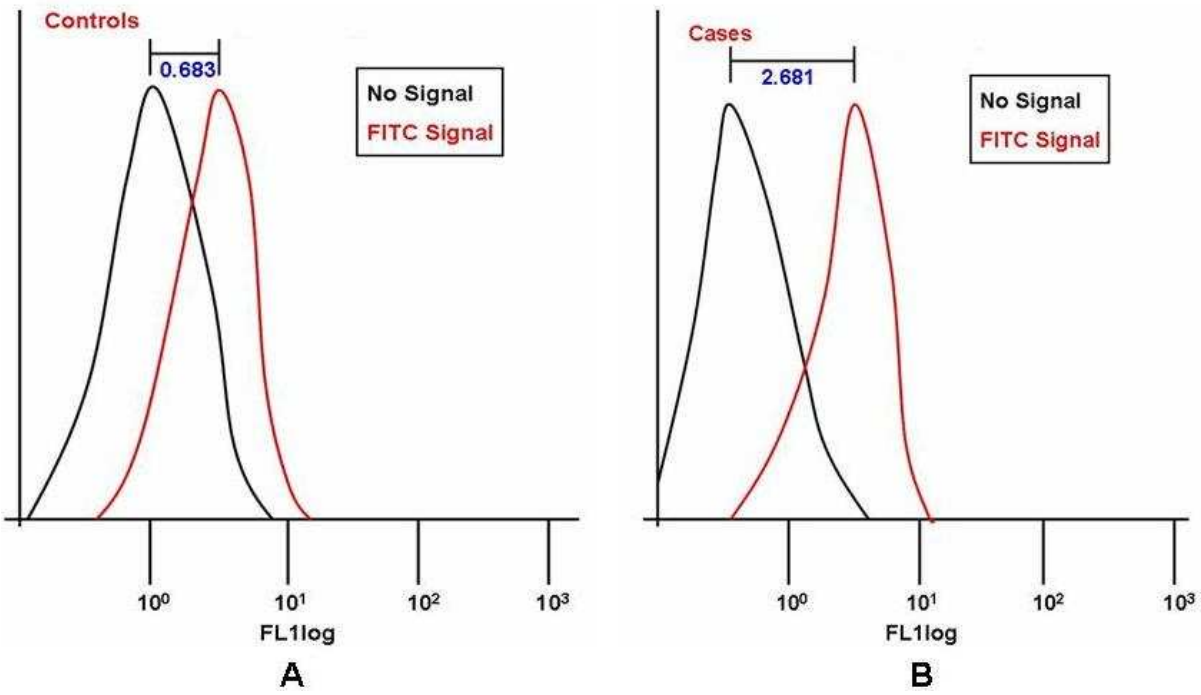

Figure 4: Comparison of CD36 FITC signal in (A) controls and (B) cases bound to the anti CD36 – FITC coated antibody.

CONCLUSION

The present finding on CD36 expression in T2DM confirms and extends the results of earlier studies performed in rodents in relation with its expression in human skeletal muscle [22-25]. The size of atherosclerotic lesions is reduced by inactivation of CD36 and increased by reintroduction of CD36 in apolipoprotein E/CD36-deficient mice. Our data also corresponds to the previously reported studies done *in vitro* with LDL regulation and lipid accumulation in CD36 [23]. This finding suggests that the CD36 expression in increased upto four folds and it can be used as a biomarker monocyte activation and ascertain the proinflammatory state.

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