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Influence of cytokine status on insulin resistance and circulating endothelial progenitor cells in type 2 diabetes mellitus

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ABSTRACT

Background: Type 2 diabetes mellitus (T2DM), a chronic metabolic disorder caused by insulin resistance (IR) and elevated blood glucose level, may lead to endothelial dysfunction. This can result in the development of various vascular complications, even in clinically controlled glycemic state.

Aim: It has been experimentally proven that cytokine influences both IR and endothelial progenitor cell (EPC) dysfunction in T2DM patients. The present study evaluated the effect of clinical and metabolic risk factors and cytokine levels on IR and EPC, which are used as critical early biomarkers for estimating the risks associated with T2DM.

Methods: The study involved 58 T2DM patients. They were further subdivided into three groups based on IR score: 32 (55.17%) with normal, 11 (18.97%) with mild-moderate and 15 (25.86%) with severe IR. The relationship of clinical, metabolic and immune mediators with IR and EPCs was verified.

Results: HbA1c% was significantly elevated in severe ($P = 0.022$) and mild-moderate IR groups ($P = 0.012$) than the normal group. The IR normal group had significantly elevated TNF levels compared to mild-moderate and severe groups. The regression analysis indicated that patients with increased body mass index (BMI) were 19.5% more likely to be significantly associated with severe IR. Association studies demonstrated that IL6 and IL10 values correlated with EPCs.

Conclusion: IL6 and IL10 were associated with circulating EPCs than IR and other clinical characteristics including glycemic control (glycated hemoglobin). TNF-α was associated with IR, but had no relationship with EPCs. The effect of cytokine status on IR and circulating EPCs in T2DM may indicate the risk of vascular complications.

1. Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by the development of micro and macrovascular complications. Inadequate control of blood sugar and inflammatory parameters has been demonstrated to positively influence the development of these complications. Many studies postulate local and systemic inflammation as one of the underlying pathophysiological mechanisms in cardiovascular diseases [1,2]. Pro-inflammatory (IL6, TNF-α) and anti-inflammatory cytokines (IL10) are found to be significantly associated with metabolic abnormalities [3]. Plasma biomarkers produced by adipose tissue including TNF are elevated in patients with insulin resistance (IR) [4]. Therefore, these cytokine may serve as biomarkers, if evaluated periodically, to estimate the cardiovascular risks associated in DM.

The circulating endothelial progenitor cells (EPC) play an essential role in regulating the vascular tone and structure of the blood vessels [4]. EPCs are regarded as a significant biomarker as they depict the patient's cardiovascular stress level. Classical cardiovascular risk factors such as elevated BMI, smoking, hypertension, dyslipidemia, and diabetes induce adverse inflammatory microenvironment, which modulates EPC survival and function [5]. Circulating EPCs mediate endogenous endothelial repair. Reduced levels of circulating EPCs has been reported in patients with increased risk of cardiovascular diseases [6].

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Knowledge on circulating EPCs, and its relationship with traditional risk factors and cytokine levels may help in understanding the pathophysiology of vascular complications in T2DM. Studies have correlated conventional risk factors of vascular diseases with changes in EPC levels [7–9]. Non-classical risk factors such as CRP and vitamin D are also known to be involved in vascular damage, and have an impact on circulatory EPC levels [5,6]. CRP attenuates endothelial nitric oxide synthase mRNA expression in EPCs, thereby inhibiting their differentiation, survival, and function [10]. Literature evidence suggests positive association between vitamin D levels and EPCs [11]. A study conducted in human umbilical vein cord endothelial cells (HUVEC) has reported that calcitriol treatment have an inhibitory effect on the pro-inflammatory parameters such as adhesion molecules, receptor of advanced glycation end product (RAGE), and IL-6. The same study demonstrated that anti-inflammatory properties of calcitriol are mediated through modulation of endothelial pro-inflammatory transcription factor nuclear factor κ B (NFkB) and phosphorylated-p38 mitogen-activated protein kinase (MAPK) activities [12]. Additionally, cytokines and therapy could modulate inflammatory processes and mobilization of peripheral circulating EPCs [9]. Circulating cytokines are involved in recruiting EPCs to the site of vascular injury from the bone marrow [13].

Improved clinical applicability of EPCs and insulin resistance by identifying risk factors influencing them can lead to therapeutic interventions in T2DM patients to reduce cardiovascular risk. The purpose of this study was to evaluate the effect of clinical and metabolic risk factors and cytokine status on IR and circulating endothelial cells in type 2 diabetes mellitus.

2. Methodology

2.1. Study design

2.1.1. Patient recruitment

The study was conducted as a part of the main study organized to estimate the impact of vitamin D3 supplementation on cardiovascular risk in patients with T2DM. This sub-study was approved by the institutional ethics committee and informed consent was obtained from all the participants. The patients with T2DM complying with the American Diabetes Association (ADA) 2015 criteria were recruited in 2015 for the main study. The inclusion criteria considered were: patients within the age range 25–65 years, T2DM with HbA1c < 9%, vitamin D levels < 30 ng/ml, and those with dyslipidemia and on adequate doses of statins based on the ADA guidelines. Patients with comorbidities that can influence the cytokine status were excluded from the study.

The patient's demographic data and clinical characteristics such as height, weight, body mass index, and blood pressure were documented at screening visit. Biochemical and serological tests performed included: fasting blood glucose and postprandial blood glucose, HbA1c, triglyceride, total cholesterol, high density lipoprotein-cholesterol (HDL-C), low density lipoprotein-cholesterol (LDL-C), very low-density lipoprotein-cholesterol (VLDL-C), total cholesterol to high density lipoprotein cholesterol (TC/HDL-C) ratio, blood urea levels, serum creatinine, high-sensitivity c-reactive protein (hs-CRP), and vitamin D at fasting insulin levels. Assessment of cytokines was also done using the collected blood samples.

The Homeostatic Model assessment (HOMA) was used to calculate IR score for each patient and was graded into three categories as follows: < 3: normal, 3 to 5: mild-moderate, and > 5: severe IR.[14]

The cardiovascular risk index was assessed using atherogenic index of plasma (AIP), which is logarithmically transformed value of triglyceride to high density lipoprotein ratio. The concentrations were taken in mg/dl.[15,16,17,18]

2.1.2. Quantification of cytokines by ELISA

Serum levels of the cytokines IL-6, IL-10 and TNF-α were measured by enzyme linked immunosorbent assay (ELISA) following the manufacturer's instructions (BD Biosciences, USA). The assay detection limits of the cytokine detection system were 2.2 pg/ml for IL-6, 2 pg/ml for IL-10, and 2 pg/ml for TNF-α. IL6 and IL10 levels were categorized as below detection limit (BDL) and above detection limit (ADL) values: ≤2.2 pg/ml and > 2.2 pg/ml for IL-6, and ≤2 pg/ml and > 2 pg/ml for IL-10. The TNF values in all the subjects were above assay detection limit values. Hence it could not be classified into BDL and ADL values, and continuous values of TNF were considered for analysis.

2.1.3. Quantification of peripheral blood EPCs by flow cytometry

The EPCs were analyzed by flow cytometry (FC500; Beckman Coulter, MarselleCedex 9, France) using mouse anti-human CD34 phycoerythrin (PE) (555822; BD Biosciences, San Jose, CA, USA), mouse anti-human CD45 PE (555484; BD Biosciences, San Jose, CA, USA) and mouse anti-human CD133VioBright Fluorescein isothiocyanate (130-105-225/226; MiltenyiBiotec GmbH, BergischGladbach, Germany). 100 μl of whole blood was incubated with 10 μl each of CD34, CD45, and CD133 antibodies after gentle mixing in dark for 15 min at room temperature. Subsequently, 500 μl of optilyse (Beckman Coulter, France) was added for red blood cell lysis, vortexed and incubated in dark for 15 min at room temperature. The sample was evaluated after adding 1 ml of sheath fluid (Beckman Coulter, France), followed by 100 μl of flow count beads (Beckman coulter, France) and vortexing. After appropriate gating, the peripheral blood cells positive for the stained antibody reagents were determined by two-dimensional side scatter fluorescence for $CD45^+$. $CD34^+$ cells were gated followed by the examination of dual positive CD34+ and CD133+ population of circulating cells. Circulating EPCs considered were $CD45^+34^+133^+$ cell population. A total of 2×10^4 events were acquired per tube and scored. Internal calibration was performed at each analysis of EPCs for exactness and stability of cell count by using CAL 992 (Beckman Coulter, Marselle Cedex 9, France).

2.2. Statistics

Data were reported as mean ± standard deviation or median (range) for continuous and counts for categorical variables. The IR groups were compared for differences in demographic, clinical, biochemical and immune parameters by ANOVA and Kruskal-Wallis test for continuous variables. Post-hoc analysis by Turkey's honest significant difference (HSD) and Games-Howell was done for ANOVA, and Dunn-Bonferroni method for Kruskal-Wallis test. Chi-square or Fisher's exact test were performed for categorical variables. The relationship between endothelial progenitor cells and independent variables was examined by Mann-Whitney *U* test or Kruskal-Wallis test for categorical variables and Spearman's correlation test for continuous variables. Statistical significance was assessed at $P < 0.05$. The association of the independent variables with IR was verified by univariate and multivariate ordinal regression. The variables were entered by simultaneous entry method. Association of independent variables with endothelial progenitor cells was tested by univariate and multiple linear regressions. For univariate analysis, $P \leq 0.2$ was considered as cut-off for inclusion of variables in multivariate regression. Statistical significance was considered at < 0.05 level for multivariate regression analysis.

3. Results

A total of 65 consecutive patients who met the inclusion criteria were recruited for the study. Seven patients were excluded due to incomplete data, consent withdrawal, loss to follow-up or extreme values of fasting insulin levels (> 50 mlU/L). The study finally considered a sample size of 58 patients. Their mean ± sd age was 53.09 ± 7.89 with F:M ratio of 0.93:1 (28 women and 30 men). The patients had 101.34 ± 69.58 (mean ± sd) months of duration of diabetes. The

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