Pro- and anti-inflammatory cytokines in uncomplicated type 2 diabetes patients

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Abstract: ***Background.*** Role of systemic pro- and anti-inflammatory cytokines are associated with type 2 diabetes mellitus (T2DM) in adults is unclear. In this cross section study, we compared cytokine concentrations in patients with T2DM and healthy individuals to test the hypothesis that differences of cytokine concentrations between them are attributable to diabetes and metabolic control. ***Methods.*** The pro-inflammatory cytokine as interferon gamma (IFN-γ) and anti-inflammatory cytokines as adiponectin and interleukin (IL)-10 were measured in 50 participants with T2DM and 70 healthy controls using ELISA kits. ***Results.*** IFN-γ was significantly increased while adiponectin and IL-10 were significantly decreased in T2DM than healthy controls (P <0.0001). In T2DM patients, negative correlations were found between fasting blood glucose with adiponectin (r= -0.293, P <0.039) and IFN-γ with IL-10 (r= -0.320, P <0.023). BMI was positively correlated with IFN-γ (r= 0.723, P <0.0001) and glycozylated hemoglobin (r=0.399, P <0.016). ***Conclusion.*** T2DM showed elevated serum levels of inflammatory cytokine IFN-γ and decrease serum levels of anti-inflammatory cytokines (adiponectin and IL-10) and negative association between anti-inflammatory cytokine and blood glucose levels indicated importance of inflammation in pathogenesis of T2DM. The hypothesis that T2DM is inflammatory disease opens new clinical perspectives for diagnosis and treatment but still requires further investigation.
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1. Introduction

Type 2 diabetes mellitus (T2DM) is a growing health problem all over the world widely discussed in literature. There is good evidence that both insulin resistance and progressive pancreatic beta cell failure are key pathomechanisms in natural history of T2DM. However, it is unclear how these abnormalities arise. It has been postulated that T2DM is a manifestation of an ongoing chronic low-grade inflammation **(1)**. This low-grade inflammation state reflects the activation of innate immunity with the implication of metabolic, environmental, and genetic factors **(2)**.

Previous studies have indicated that the enhanced inflammation in T2DM is associated with elevated levels of the prototypic inflammatory marker C-reactive protein (CRP) as well as the proinflammatory cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-6 (**3**). Both T helper (Th)1 cells and natural killer (NK) cells express interferon gamma (IFN-γ) on activation. Despite the established role of IFN-γ in mediating the immune response in type 1 diabetes and diet-induced obesity **(4)**, the role of IFN-γ in inducing inflammation in T2DM have not been studied.

Adiponectin is a predominantly anti-inflammatory adipokine that inhibits pro-inflammatory cytokines IL-6 and TNF-α and nuclear factor-kB (**5**) and induces anti-inflammatory cytokines [IL-10 and IL-1 receptor antagonist (IL-1Ra)] (**6**). Under certain conditions, adiponectin however has pro-inflammatory effects as well (**7**). In the circulation, adiponectin exists as low-, medium-, and high-molecular-weight complexes (LMW, MMW, and HMW, respectively) that may vary in efficacy regarding their effects on target tissues. Studies suggested that the HMW isoform is the most biologically active isoform of systemic adiponectin in regulating insulin resistance (**8**). IL-10 is a major regulatory cytokine of inflammatory responses secreted mainly by numerous cell types such as activated monocytes, macrophages, lymphocytes, keratinocytes, and mature adipocyte fraction of human white adipose tissue. Increasing evidence has demonstrated that IL-10 acts as a general inhibitor of proliferative and cytokine responses of both Th 1 and Th2 cells *in vitro* and *in vivo* **(9; 10).** It attenuates inflammatory and immune responses through various mechanisms as suppression of proinflammatory cytokines and chemokines (e.g. IL-1, IL-6, IL-8, TNF-α, RANTES) production, while promoting IL-1Ra release (**11**), but the biological activity of this cytokine seems to be more complex and there is evidence of a pro-inflammatory effect **(12)**.

Aim:

In this cross section study, we tested and compared circulating serum concentrations of pro-inflammatory cytokines IFN-γ, and anti-inflammatory cytokines adiponectin and IL-10 in male patients with uncomplicated type 2 diabetes and healthy participants and analyzed their associations with age, body mass index (BMI) and glycemic control.

2. Patients and Methods:

Subjects

Fifty adult male Saudi patients with type 2 diabetes mellitus were recruited in this cross sectional study from diabetes outpatient clinic at King Abdelaziz University Hospital, Jeddah, Saudi Arabia; between January 2011 and January 2012. The diagnosis of T2DM was made according to the new criteria of the American Diabetes Association based on fasting plasma glucose ≥126 mg/dL or glycozylated hemoglobin (HA1c) ≥ 6.5% **(13)**. T2DM patients were treated with oral hypoglycemic drugs including metformin and sulfonylureas, lipid lowering drugs, aspirin. Seventy male age-matched healthy Saudi volunteers were recruited as control subjects from hospital stuff and patients relatives. Excluded from the study were patients with: 1) type I diabetes mellitus, 2) patients with complicated T2DM, 3) clinical or laboratory evidence of other hormonal abnormalities or serious systemic diseases such as acute/chronic inflammations or malignancies, 4) history of hospitalization or ketoacidosis in the preceding 6 months; 5) insulin - treated patients because exogenous insulin might lead to a falsely high plasma insulin concentration that was used in the calculation of the insulin resistance index; 6) patients who were taking medication that is supposed to influence carbohydrate or lipid metabolisms or oxidative stress or related endocrine functions (e.g., β-blocker, steroids, diuretics, vitamins, antioxidants); 7) patients with connective tissue disease such as rheumatoid arthritis. Control subjects had no family history of diabetes mellitus. Before enrolment in the study, all subjects signed an informed consent after explanation of the nature and aim of the study. The study protocol was reviewed and approved by the hospital Human Ethics Review Committee and complied with the Declaration of Helsinki.

Demographic and clinical data of all participants were reported as follow: age, height and weight, duration of illness and associated comorbidity. Body mass index was calculated as weight (kg) divided by height squared (m^2). Weight was measured to the nearest 0.10 kg on a calibrated balance beam scale. Height was measured to the nearest 0.50 cm by a tape measure.

Biochemical measurements

Subjects were asked to overnight fast for 12 hours prior to blood draws (5 ml) which were performed the following morning between 8 and 10 a.m. into plain tubes via venipuncture. Whole blood were used to measured fly plated hemoglobin HbA1c and it is measured by using Roch copus integra 400 plus. Hemoglobin was measured with automated cell counts on the Beckman coulter LH 750 machine within 6 hours of phlebotomy. Fasting serum glucose was measured by the glucose oxidase method. Blood samples were centrifuged at 1500 X g for 10 min and serum was obtained and stored in 300 ml aliquots at -70°C until analysis. The enzyme-linked immunosorbent assay (ELISA) kits were used for determination of serum adiponectin (catalog# EA2500-1; Assaypro LLC, MO, USA) with sensitivity 0.5 ng/ml and coefficient of variations (CVs) of inter-assay and intra-assay 4.2% and 7.3%, respectively; interferon gamma (catalog# EI1023-1; Assaypro LLC, MO, USA) with sensitivity 16 pg/ml and CVs of inter-assay and intra-assay 4.4% and 7.3%, respectively and interleukin-10 (catalog# EI3010-1; Assaypro LLC, MO, USA) with sensitivity < 100 pg/ml and coefficient of variations (CVs) of inter-assay and intra-assay 4.8% and 7.2%, respectively.

Statistical analysis

Statistical Package for the Social Science (SPSS, Chicago, IL, USA) version 16 was used for data analysis. Data are expressed as means +/- standard deviation (SD) and minimum -maximum. Quantitative data that normally distributed were analyzed using one-way analysis of variance [ANOVA] (post-hoc test) for parameteric variables. Correlations between variables were tested with the Person test for parametric variables. For all tests, values of P <0.05 (two-tailed) were considered statistically significant.

3. Results:

Table (1) showed that BMI, fasting blood glucose and glycozylated hemoglobin were significant increased while, hemoglobin was significantly decreased in T2DM than healthy controls (P <0.0001 for all).

Interferon-γ was significantly increased while adiponectin and IL-10 were significantly decreased in T2DM than healthy controls (P <0.0001 for all) (Table2).

In T2DM patients, negative correlations were found between fasting blood glucose was with adiponectin (r= -0.293, P <0.039) and interferon gamma with IL-10 (r= -0.320, P <0.023). BMI was positively correlated with IFN-γ (r= 0.723, P <0.0001) and glycozylated hemoglobin (r=0.399, P <0.016) (Table 3).

Table (1): Clinical characteristics of participants with type 2 diabetes and control

Variables	Control	Type 2 diabetes mellitus	Significance
age (years)			
Mean+/- SD	50.43±4.81	49.88-52.44	0.400
minimum- maximum	40.00-59.00	43.00--59.00	
Body mass index (kg/m^2)			
	22.76±1.63	28.91±3.08	0.0001
	16.98-26.99	20.45-36.58	
Duration (years)	-	5.96±4.30	
		1.00-17.00	
Fasting glucose (mg/dl)	85.70±8.67	153.60±34.05	0.0001
	69.00-102.00	106.00-250.00	
Glycozylate hemoglobin (%)			
	4.78±0.43	8.81±1.96	0.0001
	4.00-5.50	7.00-15.80	
Hemoglobin (gram/dl)			
	14.28±0.89	12.27-12.89	0.0001
	13.00-16.40	9.00-14.00	

Table (2): Serum levels of pro and anti-inflammatory cytokines in participants with type 2 diabetes and control individuals

Variables	Control	Type 2 diabetes mellitus	Significance
Interferon- γ (pg/ml)			
Mean+/- SD	6.21±5.25	18.72±12.65	0.0001
minimum- maximum	0.00-24.00	1.00-45.00	
Adiponectin (ug/ml)			
	16.19±3.03	5.33±2.58	0.0001
	12.00-22.00	0.00-9.20	
Interleukin-10 (pg/ml)			
	6.38±4.82	0.93±0.73	0.0001
	0.00-16.00	0.00-3.50	

Table (3): Correlation between circulating cytokine concentrations and metabolic variables for type 2 diabetic patients.

Variables	Duration	Fasting blood glucose	Glycozylate hemoglobin	Interferon gamma	Adiponectin
Fasting blood glucose	-0.119				
	0.411				
Glycozylate hemoglobin	-0.015	0.148			
	0.920	0.305			
Interferon gamma	-0.082	0.205	-0.105		
	0.570	0.152	0.467		
Adiponectin	-0.037	-0.293	-0.077	-0.143	
	0.796	0.039	0.595	0.323	

Interleukin-10	0.163	0.077	0.215	-0.320*	-0.215
	0.258	0.595	0.133	0.023	0.133
Body mass index	0.253	0.021	0.339	0.723	-0.085
	0.076	0.885	0.016	0.0001	0.556

4. Discussion:

Diabetes mellitus remains a chronic metabolic disorder that is often associated with an unacceptably high disease burden especially in developing countries (**14**). Inflammation, deranged glucose and lipid metabolism, and over activated adipocytes have been implicated in the pathogenesis of T2DM. Hypercytokinaemia and activated innate immunity may be the common antecedent of T2DM (**15**). Hyperglycemia and insulinopenic state are considered to have negative influence on immune-competent cells, a hypothesis supported by the evidence of improvement in the immune-cellular response of diabetic patients after metabolic control (**16**).

In this cross section study, we demonstrated activated Th1 cytokine (IFN-γ) was significantly increased in T2DM patients than healthy controls. Previously, it was observed that newly diagnosed or established T2DM patients had significantly higher values of acute phase proteins and proinflammatory cytokines compared with non-diabetic controls **(17, 15, 18)**. In one of these studies, elevated levels of IL-6, which is known to be a main stimulator of the production of most acute-phase proteins, were shown to increase the risk of diabetes (**19**). However, in addition to IL-6, other cytokines, such as IL-1β or TNF-α, are central mediators of inflammatory reactions. It is well known that cytokines operate as a network in stimulating the production of acute-phase proteins. For example, the effects of IL-6 on CRP synthesis largely depend on an interaction with IL-1β (**19**). The acute-phase response in various artificial inflammatory models requires both IL-6 and IL-1β, as demonstrated in respective knockout mouse models (**20; 21**). These data strongly suggest that inflammatory reactions do not depend on single mediators, but rather that the pattern of various cytokines is crucially important for the perpetuation of an acute-phase response. In this study, BMI was positively correlated with IFN-γ and HBA1c. Similarly, Pham *et al.* study, all individuals with higher BMI simultaneously had higher circulating pro- as well as anti-inflammatory cytokine concentrations. These data point to a positive influence of obesity on the secretion of systemic cytokines and it is an additional risk factor for impairment of disease progression in T2DM.

Chronic hyperglycemia, more specifically due to the increase of glycated proteins, seems to activate the immune and macrophage-monocyte systems and to stimulate the production of cytokines and acute phase proteins (IL-6 is the main stimulus to CRP production in the liver) (**23**). In addition, hyperglycemia induces oxidative stress that is responsible for nuclear factor-kB activation that increases serum circulating proinflammatory cytokines levels **(24)**. Longer disease duration results in increased advanced glycosylation end products (AGE) and AGE-modified proteins, which could bind to macrophages stimulating synthesis and release of proinflammatory cytokines in T2DM **(25)**.

In this cross section study, we demonstrated significantly decreased in serum levels of anti-inflammatory cytokines adeponectin and IL-10 in T2DM patients than healthy controls. In T2DM, we reported a significant negative correlation between adiponectin and fasting blood glucose level and between IL-10 and IFN-γ. In this respect, several studies (**26, 27)** reported decreased in adiponectin levels in T2DM patients. Also, **it was** observed that adiponectin levels were significantly lower in diabetics and negatively correlated with BMI adjusting for age, diabetic status, and gender (28). However, they did not report any significant correlation between adiponectin and inflammatory markers. **It was** reported that adiponectin correlates negatively with fasting serum triglycerides and plasma glucose concentrations (29) and there was an inverse correlation between adiponectin with fasting plasma glucose levels (30). Adiponectin—displays anti-inflammatory activity by inhibiting the production of TNF-α and IL-6 by macrophages, and by binding lipopolysaccharide **(31)**. Adiponectin also decreases hepatic gluconeogenesis and increases lipid oxidation in skeletal muscle (**32**). Adiponectin modulates glucose metabolism and insulin resistance by 5'-adenosine monophosphate-activated kinase signaling pathway, and decreases also free fatty acid concentrations by stimulating fatty acid oxidation in muscle **(33)**. Furthermore, others **(34; 35)** reported that adiponectin concentrations correlate negatively with CRP, IL-6 and TNF-α. Adiponectin may be largely independent of systemic levels of many cytokines and chemokines (**36**) but has been shown to have strong anti-inflammatory effects on the molecular and cellular level **(37; 34)** so that it may also be considered as immune marker. (**38)** discovered that low IL-10 production capacity is associated with metabolic syndrome and T2DM. In addition, they found that serum concentrations of total cholesterol, low-density lipoprotein cholesterol, triglycerides, glucose, and HbA1c gradually decreased as IL-10 production capacity increased, whereas high-density lipoprotein cholesterol concentration gradually increased. This

observation implies that low IL-10 production capacity may be involved in metabolism and may be associated with T2DM. IL-10 is considered to be a pleiotropic Th2 cytokine, playing an important role in down regulating the secretion of pro-inflammatory cytokines, such as IL-1, TNF-α, IL-6, and IL-12 from activated monocytes/macrophages, and in inhibiting T-cell activation by decreasing major histocompatibility complex class II and B7 expression on antigen presenting cells. In addition, IL-10 stimulates antibody production by B lymphocytes and promotes their proliferation and differentiation **(39)**, properties that are contributing to the development of autoimmune phenomena **(40)**.

Epidemiological, clinical and experimental data suggest that T2DM has inflammatory basis. Thus, decrease of activity of inflammation should result in lower incidence of T2DM and its complications. When we look at the results of intervention studies we find that several of them confirm the role of inflammation in T2DM. It has been shown that Mediterranean-style diet (**41**), increase of physical activity (**42**), loss of weight (**43**), treatment with statins (**44**), high dose of aspirin (**45**), thiazolidinedione (**46**) and converting enzyme inhibitors as well as angiotensin-receptor blockers (**47, 48**) reduce incidence of diabetes and improve metabolic control of diabetes partially via anti-inflammatory mechanism. The hypothesis that T2DM is inflammatory disease opens new clinical perspectives for diagnosis and treatment but still requires further investigation.

Limitation:

The study design was cross-sectional. A longitudinal follow-up study would be more appropriate and enable the investigation of different stages in progression from healthy control to prediabetic states and overt diabetes. Finally, possible associations between glucose and lipid toxicity, beta cell function and circulating cytokine concentrations in different diabetes groups could not be investigated because suitable data were not collected.

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