Proinflammatory cytokine polarization in type 2 diabetes

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Abstract

Subclinical inflammatory reaction is associated with non-insulin dependent diabetes. Therefore, the aim of the present study is to describe the effect of the three cytokines: interferon γ (IFN-γ), interleukin (IL)-4 and IL-5 on the development of type 2 diabetes (T2D). Forty-five volunteers (after their permission) were participated in this work; according to their clinical examination and laboratory investigations (fasting blood sugar, 2 hours postprandial, HbA1c and lipid profile), they were divided into thirteen control (non-diabetic) (five females and eight males) and thirty-two diabetic patients (twenty-one females and eleven males). Thereafter, their sera were evaluated for C-reactive protein (CRP), IFN-γ, IL-4 and IL-5. The results revealed an increasing trend of CRP and a significant increase of IFN-γ in diabetic patients with no sex difference. A positive correlation between IFN-γ and both IL-4 and IL-5 in control, and a positive correlation between IL-4 and IL-5 in diabetic patients had been visualized. These results denoted that there may be an association of the pro-inflammatory cytokines in the etiology of diabetes mellitus type 2.

Key words: cytokines, proinflammatory, type 2 diabetes, interleukin 4, interferon γ, interleukin 5.

Introduction

Several evidences revealed that type 2 diabetes (T2D) is a state of low-grade inflammation and reducing inflammation is an important outcome for T2D treatment since it caused balance between the pro-inflammatory and anti-inflammatory cytokines. Removal of tumor necrosis factor α (TNF-α) which is an inflammatory mediator protects against insulin resistance and salicylates intake improves insulin sensitivity [1-5]. As mentioned earlier, inflammation plays a major role in the development of T2D [6, 7]. However, there are other factors like genetic determinants, nutritional factors, and life style [8], that may contribute in the pathogenesis of non-insulin dependent diabetes.

Specific T cell subsets imbalance are responsible for many inflammatory and autoimmune disorders [9, 10]. Th1/Th2 cytokines imbalance has been reported in chronic disease progression [11-14] and in metabolic syndrome [15]. The Th1 pro-inflammatory cytokines such as interferon γ (IFN-γ) and interleukin (IL)-2 activate macrophages and are involved in inflammatory immune responses. IFN-γ had been identified by Rocha *et al.* [16] to have a role in diet-induced adipose inflammation, obesity, and glucose intolerance in vivo. McGillicuddy *et al.* [17] suggested that IFN-γ caused a time-dependent reduction of insulin-stimulated glucose uptake which is indicative of insulin resistance.

Activated Th2 cells secrete cytokines such as IL-4, IL-5, IL-10 and IL-13, which have anti-inflammatory properties and are involved in antibody production, eosinophil activation, and suppression of macrophage functions [12, 13, 18]. IL-4 which is secreted also by basophils and mast cells has pleiotropic functions including Th2 differentiation, B-cell proliferation and immunoglobulin class switching [19]. IL-5 is another important Th2 cell-derived cytokine. It regulates the expression of diverse genes; these genes are partly responsible in proliferation, cell survival, maturation and effector functioning of B cells and eosinophils [20].

Therefore, the aim of the present study is to evaluate the levels of cytokines termed IFN-γ, IL-4 and IL-5 in Egyptian males and females suffering from T2D in an attempt to study the imbalance between the pro-inflammatory and anti-inflammatory immune response in diabetic patients.

Material and methods

Patients

Thirty-two patients (21 females and 11 males) with T2D and 13 non-diabetic controls (five females and eight males) were participated in the present study. All patients with T2D were receiving appropriate diet and oral hypo-

glycemic agents as glucose-lowering medication at the time of experiment. The age of the diabetic subjects ranged from 30 to 65 years while the age of the healthy control subjects lied between 32 and 60 years. Family history, duration of diabetes, current medication and past history of infectious diseases or heart attack were thoroughly collected from every subject. With the exception of oral hypoglycemic agents, no other medications were taken. Clinical examination was recorded including, height, weight, body mass index (BMI – weight/height^2), blood pressure, heart and chest examination. Patients with acute or chronic infection, hepatic or renal disease, malignancy, rheumatologic disorder and any clinical cardiovascular disease were excluded. The BMI of all subjects were between 19 and 24.9 kg/m^2.

Biochemical analysis

After clinical examination, 12 hours fasting blood samples were taken, introduced into two tubes, one tube with EDTA for analysis of glycated hemoglobin (HbA1c) and from the other tube serum was separated for determination of CRP, fasting blood sugar (FBS) and lipid profile (cholesterol, triglyceride, HDL, LDL). Another specimen was taken from the volunteers after 2 hours for the evaluation of 2 hours postprandial blood sugar (2h PP). FBS, 2h PP and HbA1c were measured to evaluate the diabetic condition of the individuals; CRP was evaluated to check the pro-inflammatory condition of patients and the lipid profile was analyzed to assess changes accompanying T2D.

Cytokines measurements

After the evaluation of these confirmatory results, five milliliters of whole-blood samples were collected from both patients and controls, using a standard venipuncture technique. Serum samples were obtained after centrifugation at 800 × g for 10 min and stored at –80°C until assayed.

The concentration of the pro-inflammatory cytokine, IFN-γ, was evaluated by radio-immunoassay (RIA). IL-4 and IL-5 which are anti-inflammatory cytokines, were both determined in the sera by ELISA kits (enzyme-linked immunosorbent assay) according to the manufacturer's instructions.

Measurement of serum IFN-γ

The concentration of IFN-γ was evaluated by radio-immunoassay using Bio-source Europe S.A. (Belgium) kit. IFN-γ IRMA is an immunoradiometric assay based on coated-tube separation and on the oligoclonal system R, in which several monoclonal antibodies directed against distinct epitope of IFN-γ have been used. The capture antibodies are attached to the lower and inner surface of the plastic tube. Standards or samples, added to the tubes will at first show low affinity for antibodies. The signal anti-

body labeled with I-125, will trigger the immunological reaction. After washing, the remaining radioactivity bound to the tube reflects the antigen concentration.

Measurement of serum IL-4

IL-4 was determined by ELISA using RayBiotech (Inc. 3607 Parkway Lane, Suite 100, Narcross GA 30092) kit. This assay employs an antibody specific for human IL-4 coated on a 96-well plate. Standards and samples are pipetted into the wells and IL-4 present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-human IL-4 antibody is added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of IL-4 bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

Measurement of serum IL-5

IL-5 was estimated using Boster biological technology LTD (USA) kit. Boster's human IL-5 ELISA Kit is based on standard sandwich enzyme-linked immune-sorbent assay technology. Human IL-5 specific-specific monoclonal antibodies are precoated onto 96-well plates. The human specific detection monoclonal antibodies are biotinylated. The test samples and biotinylated detection antibodies are added to the wells subsequently and then followed by washing with PBS or TBS buffer. Avidin-Biotin-Peroxidase Complex is added and unbound conjugates are washed away with PBS or TBS buffer. HRP substrate TMB is used to visualize HRP enzymatic reaction. TMB is catalyzed by HRP to produce a blue color product that changes into yellow after adding acidic stop solution. The density of yellow is proportional to the human IL-5 amount of sample captured in plate.

Statistical analysis

Results were presented as means ±standard error (SE). Paired Student test (*t*-test) was used to analyze sample averages. The correlation between the variables were analyzed by Pearson's correlation test [21]. The normality of distribution were evaluated by Kolmogorov-Smirnov test. No significant changes were observed between males and females; therefore, we added all the individuals, males and females, in one group.

Results

Type 2 diabetes raises the levels of all biochemical tests

All the tested parameters (FBS, 2h PP, cholesterol, triglyceride, HDL, LDL, HBA1c and CRP) showed increase in

Table 1. Statistical analysis of FBS, 2h PP, HBA1c, CRP, cholesterol, triglycerides, HDL and LDL values in controls and diabetic patients

Parameters	Control	T2D	Normal range
FBS (mg/dl)	87 ±2.17	114 ±3.01	80-120
2h PP (mg/dl)	89 ±10.11	169 ±3.12	80-120
HbA1c (%)	5.5 ±0.87	8.55 ±1.01	Up to 6
CRP (mg/l)	6.08 ±0.68	10.33 ±1.34	0-10
Cholesterol (mg/dl)	189 ±4.52	257 ±5.72	Up to 200
Triglycerides(mg/dl)	83 ±7.33	137 ±2.93	Up to 150
HDL (mg/dl)	42 ±1.07	44 ±1.75	35-55
LDL (mg/dl)	107 ±2.33	144 ±3.17	Up to 170

The data are presented as mean ±SE. FBS – fasting blood sugar, 2h PP – 2 hours postprandial, HbA1c – glycated hemoglobin, CRP – C-reactive protein, HDL – high density lipoprotein cholesterol, LDL – low density lipoprotein cholesterol

Table 2. Statistical analysis of IFN-γ, IL-4 and IL-5 values in controls and diabetic patients

Parameters	Control ($n = 13$)	T2D ($n = 32$)	Percent of change	*p* value
IFN-γ (IU/ml)	1.86 ±0.53	9.64 ±3.68*	418	< 0.05
IL-4 (pg/ml)	0.04 ±0.03	0.13 ±0.05	225	> 0.05
IL-5 (pg/ml)	12.82 ±5.5	15.64 ±4.99	22	> 0.05

The data are presented as mean ± SE and percentage of change. Significant at $p < 0.05$*. IFN-γ – interferon γ, IL-4 – interleukin 4, IL-5 – interleukin 5

Table 3. Correlation coefficient of IFN-γ, IL-4 and IL-5 in controls

Correlated parameters	*r* value	*p* value	95% CI (lower limit – upper limit)
IFN-γ × IL-4	0.586*	< 0.05	(0.04 – 1.3)
IFN-γ × IL-5	0.841**	< 0.01	(0.6 – 1.85)
IL-4 × IL-5	0.25	> 0.05	(–0.37 – 0.88)

Values of correlation coefficient are significant at $p < 0.05$* and $p < 0.01$**. IFN-γ – interferon γ, IL-4 – interleukin 4, IL-5 – interleukin 5

Table 4. Correlation coefficient of IFN-γ, IL-4 and IL-5 in diabetic patients

Correlated parameters	*r* value	*p* value
IFN-γ × IL-4	0.107	> 0.05
IFN-γ × IL-5	0.283	> 0.05
IL-4 × IL-5	0.499*	< 0.05

Values of correlation coefficient are significant at $p < 0.05$*. IFN-γ – interferon γ, IL-4 – interleukin 4, IL-5 – interleukin-5

the diabetic persons compared to controls; they also showed an increase as regards to the normal range (Table 1).

Rise in IFN-γ level in diabetic patients

The IFN-γ is the only cytokine which showed significant ($p < 0.05$) increase in diabetic patients where the percentage of change mounted to 418%. There was an insignificant increase in both IL-4 and IL-5 in the diabetic group compared to the control group, with a percentage of change 225% and 22% respectively (Table 2).

Correlation between IFN-γ and both IL-4 and IL-5 in the control group

There was a positive significant correlation in the control group between IFN-γ and both IL-4 and IL-5 at $p < 0.05$ and $p < 0.01$ respectively and a positive insignificant correlation between IL-4 and IL-5 (Table 3).

Correlation between IL-4 and IL-5 in the diabetic patients

In diabetic patients, there was a positive significant ($p < 0.05$) correlation between IL-4 and IL-5 and a positive insignificant correlation between IFN-γ and both IL-4 and IL-5 (Table 4).

No correlations were observed between the tested cytokines and the duration of diabetes or BMI.

Discussion

In the present study HbA1c and CRP were chosen as an indicator of hyperglycemia in the past 3 months [22] and as a sensitive physiological marker of subclinical systemic inflammation [23] respectively. There is a correlation between cytokines production and CRP. Schultz and Arnold [24] and Sabanayagam *et al.* [25] stated that cytokines such as IL-6, IL-1 and TNF-α stimulate hepatic synthesis of CRP. Similarly Pepys and Hirschfield [26] concluded that the acute phase CRP was produced as part of pathophysiological conditions including infection and inflammation. The link that exist between diabetes and systemic inflammation is reflected in the circulatory levels of CRP in the present study and confirmed the finding of many studies [27-30].

T2D besides its classification as a metabolic disease, it is considered also as a subclinical inflammatory one with changes in immune cell function. In diabetes, the metabolic and immune pathways are interdependent; metabolic disorder in T2D and the associated inflammatory process seems to play an important role in the development of diabetes and its late complications [31].

The relation between inflammation and insulin resistance, the key primary defect underlying the development of T2D, originates through different mechanisms. Impair-

ment of muscle cell insulin action can be caused by over-production of nitric oxide resulting from the inflammatory cytokine stimulation, leading to induction of inducible nitric oxide synthase (iNOS) [32, 33]. The inflammatory signaling pathways can also become activated by metabolic stresses originating intracellular and extracellular from signaling molecules [34, 35]. Additionally, the rise in mitochondrial production of reactive oxygen species (ROS) enhanced activation of inflammatory pathways [36, 37].

In the present study the high significant increase of the proinflammatory IFN-γ might cause insulin resistance as suggested by McGillicuddy *et al.* [17]. They examined the effect of IFN-γ on insulin sensitivity, lipid storage, and differentiation in human adipocytes. They found that IFN-γ induced a time dependent reduction of insulin-stimulated glucose uptake and Akt (protein kinase B) phosphorylation, both indicative of insulin resistance.

IFN-γ as a cytokine can be produced by NK, macrophages and helper T cells. Macrophages, as examined by Mosser and Edwards [38], have remarkable plasticity that allows them to change their phenotype and their physiology according to environmental signals, including alterations in the expression of surface proteins, the production of cytokines and pro-inflammatory mediators. It has been established that macrophages contributes to the proinflammatory milieu of the diabetic islet [39]. Several authors suggested that monocytes from T2D patients constitutively and inducibly secrete elevated levels of IL-6, IL-8, TNF-α and IL-1 beta [38, 40-43] and decreased levels of the anti-inflammatory cytokine IL-10 [44]. However, it was demonstrated that monocytes and macrophages might have different origin with different lineage, though there might be some plasticity [45].

The present study agreed with the work of Jagannathan-Bogdan *et al.* [46], which showed that blood from T2D patients had increased circulating Th17 cells (proinflammatory subsets of T cells) and that T cells from T2D patients had increased production of IFN-γ, but produced healthy levels of IL-4. In contrast, they found that T2D patients had decreased percentage of CD4+ Tregs (anti-inflammatory subsets of T cells). In addition, it was stated that T2D patients also had elevated levels of serum IL-12 [47, 48], a cytokine that promotes Th1 differentiation and elevated IFN-γ production [49-51].

The present work disagreed with that of Ahmad *et al.* [52] who showed in their study that plasma IL-5 levels were significantly lower in diabetic individuals as compared with non-diabetic counterparts. It seems that there is another factor which interferes with this cytokine, as the same authors observed in their study that IL-5 levels were significantly higher in obese diabetic population than overweight/ lean diabetic individuals. Madhumitha *et al.* also showed the suppression of Th2 serum cytokines in subjects with diabetic coronary artery disease [15]. The difference

in results from ours may be caused due to different laboratory methods of cytokines analysis.

There was a positive correlation between IFN-γ and both IL-4 and IL-5 in control subjects (Table 3) contrary to that observed in diabetic group. This condition in the control may reflect the importance of the balance between pro-inflammatory and anti-inflammatory subsets of T cells which is critical to maintain homeostasis and avoid inflammatory diseases. Since T2D had been identified in recent findings as an example of a chronic inflammatory disease, it is expected that DM promote pro-inflammatory cytokines production. The loss of correlation between IFN-γ, IL-4 and IL-5 in T2D patients most probably means that there is loss of homeostasis in T cell subsets balance and the T cells in T2D patients are skewed toward pro-inflammatory subsets that promote chronic inflammation in T2D.

The non-significant changes of IL-4 in diabetes may point to the nature of DM as a subclinical inflammatory disease, allowing no obvious release of this anti-inflammatory cytokine and supporting the finding of Jagannathan-Bogdan *et al.* [46] who stated that there was healthy levels of IL-4 in T2D patients. The significant positive correlation between IL-4 and IL-5 in diabetic patients (Table 4) pointed to the close association between the two cytokines.

The increase of IFN-γ, in addition to the positive correlation between IL-4 and IL-5 in diabetic patients may point to the association between inflammation and T2D In particular, the increase of proinflammatory cytokine IFN-γ in diabetic individuals denoted this close association.

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