Evaluation of adiponectin and TNF-α expression in diabetic patients and its relationship with cardiovascular diseases

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ABSTRACT

Diabetes is caused by peripheral insulin resistance and lack of insulin secretion due to the apoptosis of pancreatic beta cells. Tumor necrosis factor alpha (TNF-α), a pro-inflammatory cytokine secreted from the tissue on the insulin signaling pathway, can play a role in causing fat resistance to insulin in type 2 diabetes patients. Adiponectin is a specific protein of adipose tissue. It belongs to the collectin family, which is present in human plasma at a high level and can protect against vascular lesions. Considering the importance of epigenetic changes in the development of multifactorial diseases, this study was conducted to investigate the methylation of TNF-α gene promoter in patients with type diabetes with cardiovascular disease and compare it with diabetic people without cardiovascular disease. Also, the serum concentration of adiponectin was investigated in diabetic patients with and without cardiovascular disease. For this purpose, 95 patients with type 2 diabetes referred to Isfahan Endocrine and Metabolism Research Center were divided into two groups: cardiovascular disease and without cardiovascular disease, based on the angiography results. Serum adiponectin level was measured by the RIA method. In addition to adiponectin, indicators such as FBS, cholesterol, triglycerides, and HDL were also measured in these patients. Then, the promoter region of the TNF-α gene was investigated by bisulfite treatment method, nested PCR, and finally, sequence determination. The results showed that the serum level of adiponectin was higher in diabetic patients without cardiovascular disease than in diabetic patients with cardiovascular disease, but this difference was not statistically significant. Also, no change was observed between men and women in TNF-α gene promoter methylation in diabetic and non-diabetic groups. In general, the decrease in adiponectin concentration in diabetic people can be a factor in causing macroangiopathy, so it can be predicted that the production of recombinant adiponectin can be helpful in the treatment and protection of cardiovascular disease in these patients. Also, it seems that the epigenetic changes of cytokines that play a role in causing insulin resistance in type 2 diabetic patients are not noticeable in the peripheral blood sample. In this regard, other tissues should probably be investigated.

Introduction

Today, diabetes is becoming a global problem in developed and developing countries (1). Diabetes is a chronic inflammatory condition caused by genetic and environmental factors. Although the pathophysiology of diabetes is not fully understood, genetics form an integral part of the disease (2). Obesity, a primary risk factor in diabetes, is a pro-inflammatory condition in adipocytes. In genetically predisposed individuals, when body weight increases with age, a parallel state of chronic inflammation characterized by an increase in pro-inflammatory cytokines can cause changes (3). In the adipose tissue of obese people, the migration of immune cells, especially macrophages, increases due to fat accumulation. Tumor necrosis factor alpha (TNF-α), an important pro-inflammatory cytokine, is secreted from these immune cells by activating MAPK and NFxB signaling pathways. It causes the secretion of other cytokines, such as 1-IL and 6-IL. TNF and 6-IL stimulate JNK and NF-KB/IKK inflammatory pathways through classical insulin receptors (IR). TNF and other adipokines, by phosphorylating 1-IRS prevent its function for PI3K/Akt and thus interrupt the activation pathway of the insulin signaling pathway (4, 5).

Adipose tissue is the source of production of various factors derived from fat cells. Adipocytokine refers to biologically active molecules derived from fatty tissue cells. A number of these adipocytokines mediate the systemic effects of obesity on health (6). Recently, adiponectin has been recognized as an adipocytokine with acute metabolic effects. Adiponectin is a protein with 244 amino acids and is secreted in a large amount from human adipose tissue cells (7). This cytokine is a collagen-like protein structurally similar to collagen VIII and X and complement factor C19 and circulates in human plasma in large quantities (8).

Contrary to resistin and other adipocytokines, which levels increase during obesity, the expression of adiponectin and its plasma levels decrease during obesity and insulin resistance (9). Studies have shown that performing gastric bypass in obese patients increases the adiponectin

level (7-9). Also, low levels of adiponectin predict the risk of type 2 diabetes. A specific physiological role for adiponectin has yet to be discovered. However, experimental data suggest that adiponectin may have anti-atherogenic and anti-inflammatory properties, which may explain the protective function of this hormone in the initiation and progression of atherosclerosis. Inflammation is an essential factor in the initiation and formation of atherosclerosis (10). The first change in this process is the creation of atherosclerosis plaques and endothelial damage, which is done by various inflammatory stimuli, including TNF-α (11).

In the next step, leukocytes adhere to the endothelium and migrate to the wall of arterioles, where they become macrophages. Macrophages and smooth muscle cells ingest the modified LDL and transform it into foam cells. Scavenger receptors play an essential role in lipid accumulation and foam cell formation (12). The physiological concentration of adiponectin inhibits the production of TNF-α in macrophages attached to blood vessels. It prevents the expression of molecules such as VCAM-1 E Selectin and 1-ICAM on the endothelium (13). The mechanisms responsible for the control and synthesis of adiponectin have not yet been identified, and insulin is the only hormone that regulates adiponectin gene expression. TNF-α is one of the molecules responsible for insulin resistance. TNF-α significantly reduces the expression and secretion of adiponectin from fat cells (12). Also, new findings show that the reduction of adiponectin gene expression and its serum level is related to the pathogenesis of obesity and type-2 diabetes (insulin resistance) (14). Considering the anti-atherosclerosis property of this hormone and its plasma reduction in cardiovascular patients, and considering that diabetic patients are at risk of cardiovascular diseases, it is necessary to measure this hormone in these patients (15).

Materials and Methods

This case-control study was conducted on 95 patients with type 2 diabetes who were referred to the hospital for angiography and diagnosis of cardiovascular disease. After angiography, these patients were divided into two groups: diabetic patients with cardiovascular disease and diabetic patients without cardiovascular disease.

Approximately 7ml of blood was taken from the patients in the fasting state. 2ml of it was poured on EDTA anticoagulant with potassium salt to measure HbA1C and the rest of the sample was poured into acid wash tubes. After separating the serum, 0.5 ml of it was separated to measure adiponectin and stored at -70°C, and the rest was used to perform biochemical tests of FBS, cholesterol, HDL, TG and LDL by the analyzer. Smoking, a history of high blood pressure and having chronic diseases such as

cancer caused patients to be excluded from the study.

HbA1C was measured using an ion exchange system and with the help of a DS5 chromatography machine made in Germany. To measure adiponectin, a commercial kit (Adiponectin (human) RIA (DRG, Germany) was used. All steps of the RIA test were performed according to the instructions of the kit, and then the tubes were read in a Berthold model gamma counter for 1 minute and the concentration of adiponectin in the sample was calculated in ng/ml.

DNA extraction and bisulfite treatment

Blood samples were collected in tubes containing 0.5M EDTA. Genomic DNA was extracted using a DNA extraction kit. The quality of the samples was checked using the NanoDrop 2000 device (Thermo Fisher Scientific, Waltham, MA, USA). Bisulfite treatment was done using Qiagen, cat Nos:59824 EpiTect Fast DNA Bisulfite Kit, Germany. During this process, unmethylated cytosine is converted to uracil, but methylated cytosines remain unchanged.

Primer design and PCR reaction

The promoter region of the TNF-α gene (NG_007462.1) was amplified using the Nested PCR method and methylated primers specific to the promoter. There are 10 CG regions in the promoter and UT region of the TNF-α gene around the coding region (CDS), which were investigated in this study (Figure 1).

PCR primers were designed using Meth Primer software. Primer sequences for Need PC are shown in Table 1. The PCR reaction temperature program for both steps consisted of initial annealing at 95°C for 5 minutes, 35 cycles including 95 °C for 30 seconds, 51 °C for 30 seconds and 72 °C for 30 seconds and finally the final elongation at 72 °C for 5 minutes. Finally, the quality of the PCR product was checked using 8% polyacrylamide gel. The PCR products of each sample were sequenced to check the amount of methylasone. Sequencing was done with ABI 3500 machine.

Statistical Method

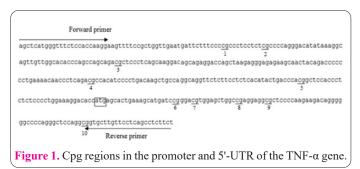T-test was used for statistical analysis. In this study, P less than 0.05 were considered significant.

Figure 1. Cpg regions in the promoter and 5'-UTR of the TNF-α gene.

Table 1. The primer sequence of the TNF-α gene for nested PCR.

		Primer sequence (5'-3')	PCR product length
First stage primers	F1	GAGATAGAAGGTGTAGGGTTTATTAT	488 bp
	R1	TCCAAAATACAACAAACAAAAAAAC	
Second stage primers	F1	AGTTTATGGGTTTTTTTATTAAGG	394 bp
	R1	AAAAAAAACTAAAAAACAAACACC	

Results

Biochemical factors and serum levels of adiponectin were measured in 95 patients with type 2 diabetes. The average age, gender, body mass index, biochemical factors and serum levels of adiponectin are shown in Table 2. Serum adiponectin levels were lower in diabetic patients with cardiovascular disease compared to patients without cardiovascular disease, but this comparison was not statistically significant. Also, the serum level of adiponectin was compared between men and women, with women in two groups and men in two groups.

The number of methylation of CpG regions in the TNF-α gene by gender in the studied groups was also investigated. Our results did not show a significant difference between men and women in relation to TNF-α gene promoter methylation (Table 3).

Discussion

Adiponectin is known as a cysteine-rich protein specific to adipose tissue. Adiponectin is a protein with 244 amino acids and the product of the apml gene, which is specifically and highly expressed in human fat cells (11). It seemed necessary to conduct the present study in order to predict cardiovascular disease in people with type 2 diabetes, therefore, to achieve this goal, the serum level of adiponectin hormone in patients with type 2 diabetes was measured and its difference in It was investigated between diabetic patients with cardiovascular disease and without cardiovascular disease. Previous studies have shown that plasma levels of adiponectin are decreased in obese individuals and those with cardiovascular disease (16). Hotta *et al.* (17) showed that the plasma level of adiponectin in diabetic patients is lower than in non-diabetic patients; in addition, they showed a decrease in the plasma level of adiponectin in patients with cardiovascular disease compared to people without cardiovascular disease. Also, Kunada *et al.* (18) showed that the reduction of adiponectin levels in men alone and independently of other factors doubles the risk of cardiovascular disease.

Our results showed that the serum concentration of adiponectin in diabetic patients without cardiovascular disease was higher than that of diabetic patients with cardiovascular disease, which was consistent with previous studies and confirmed the previous results, although this result was not statistically significant. did not have Obtaining this result could be because diabetics without CAD may have had mild CAD that could not be detected by angiography. A study on 967 Japanese subjects with normal weight showed that plasma adiponectin has a negative relationship with body mass index of total cholesterol, LDL and triglycerides, but a positive relationship with HDL (18). It seems that increasing the sample size can make the result meaningful. Decreased levels of adiponectin in patients with cardiovascular disease raise an important question in any discussion examining the relationship between adiponectin and atherosclerosis (19). The pos-

Table 2. Information and amount of biochemical factors and adiponectin level in studied patients.

	Diabetic Male		Diabetic Female		Total	
	without cardiovascular disease	with cardiovascular disease	without cardiovascular disease	with cardiovascular disease	without cardiovascular disease	with cardiovascular disease
Number	28	32	18	17	46	49
Age	48±2	50±4	46±3	48±1	47±2	49±2
BMI	26±0.6	26±0.5	26±0.6	26±0.7	26±0.4	26±0.4
Fasting blood sugar (mg/dl)	155±7	181±10	146±2	173±0.6	178±7	181±9*
Cholesterol (mg/dl)	185±8	205±9	180±13	218±9	183±7	210±48*
Triglyceride (mg/dl)	176±19	200±22	160±4	226±38	170±20	209±19
HDL (mg/dl)	42±1	37±1	48±2	42±2	44±1	38±1*
Hemoglobin A1c (%)	7.8±0.3	8.8±0.3	7.8±0.43	7.58±0.3	7.8±0.3	8.5±0.2*
Adiponectin (ng/ml)	9.94±1.9	9.6±1.4	15.5±5.9	13±2.7	12.2±2.6	10.7±1.3**

*: A significant difference was observed between the diabetic group with cardiovascular disease and the diabetic group without cardiovascular disease at the 0.05 level. **: A significant difference was observed between the diabetic group with cardiovascular disease and the diabetic group without cardiovascular disease at the 0.01 level.

Table 3. Comparison of methylation number of CpG regions in the TNF-α gene by gender in the studied groups.

Variable	Group	Gender	Average methylation number
CpG TNF-α	Diabetic without cardiovascular disease	Male	0.50 ± 0.2
	Diabetic without cardiovascular disease	Female	0.45 ± 0.1
	Diabetic with cardiovascular disease	Male	0.41 ± 0.1
	Diabetic with cardiovascular disease	Female	0.54 ± 0.1

sible mechanisms could be the reduction of adiponectin production in adipocytes, the increase of its catabolism in the blood, or both. It is a clear issue that patients with cardiovascular disease also have metabolic syndrome, which is closely related to diabetes and insulin resistance. The anti-inflammatory properties of adiponectin indicated that this protein has a good and very efficient protective effect to prevent the progression of atherosclerosis (11).

Today, it is known that in addition to genetic changes, epigenetic changes play an important role in the development of multifactorial diseases (20). Diabetes is also a multifactorial disease, which during several years of study and discovery of many genes that play a role in the creation of its genetic part, it has been determined that environmental factors play an important role in its creation, according to this study, to examine the TNF-α gene, which is involved in the molecular pathway of diabetes, bisulfite treatment was used in this method, which is the main method to check the methylation status of genes. In this case, unmethylated cytosines are converted to uracil, and during the PCR reaction, uracils are converted to thymine, but methylated cytosines remain unchanged, which can be detected after sequencing. The TNF-α gene plays an initial role in the molecular pathway of diabetes pathogenesis and initiates subsequent molecular events (21). TNF-α is the first cytokine secreted by accumulated macrophages in adipose tissue, which initiates the release and activation of other cytokines and apoptosis of B islets of Langerhans or interrupting the insulin signaling pathway, causing insulin resistance in white adipose tissue, liver and muscle as one of the main causes of diabetes. and subsequently activation of intracellular enzyme pathways that cause inflammatory conditions and production of reactive oxygen species (ROS) in diabetes (22).

Regarding the TNF-α gene, several studies have been conducted on people with diabetes or the methylation status of its promoter in people with high fat. In a study conducted in 2016 to investigate the serum level of some cytokines, including TNF-α, in type 2 diabetes, it was shown that the risk of type 2 diabetes is greatly increased with an increase in the level of inflammatory cytokines, including IL-18, IL-6 IL-1 is related to TNF-α and CRP (19). Also, some studies showed that in women with higher central obesity, the TNF-α gene promoter has less methylation, but its plasma level is higher (5). Our study did not show a relationship between TNF-α gene promoter methylation and type 2 diabetes, as we know, control Expression of genes due to epigenetic changes, in addition to DNA methylation, also takes place through other pathways such as histone changes and micro RNA. The absence of methylation in the TNF-α promoter in our study may be due to the role of other regulatory pathways. The fact that there was no significant difference in BMI in our studied groups can be another reason for not observing a difference in the methylation level in the studied groups. Because studies have shown that obesity can be an effective factor in the epigenetic changes of the TNF-α gene (5).

Recently, a study on hydroxymethylation and total DNA methylation in blood cells of well-controlled type 2 diabetes patients and poorly controlled patients compared to healthy subjects showed that the TNFα gene promoter in the drug-treated patients was not properly controlled, it is less methylated (23-28). We did not check the level of diabetes control in the patients and probably the lack of change in the methylation level could be related to the control of diabetes in the patients studied. Epigenetic studies on blood tissue, which is an accessible sample compared to other tissues that are related to the development of diabetes, can help in the early diagnosis of the disease. Although we did not observe a change in TNF-α gene promoter methylation in the peripheral blood sample between the studied groups, it may be possible to study the epigenetics of other genes involved in the pathogenesis of the disease in the peripheral blood from the blood sample as a marker in diagnosis.

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