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The Expression of Interleukins 10 and Leptin Receptor in Peripheral Mononuclear Cells from Patients with Metabolic Syndrome

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ABSTRACT

Metabolic syndrome (MetS) is a common disease and a serious public health problem in developed countries. Chronic inflammation plays a key role in MetS. Interleukins 10 and LpR are considered to involve in inflammatory state and may be associated with pathogenesis of MetS. Therefore the aim of this study was to determine the rate of gene expression of IL-10& LepR genes in peripheral blood mononuclear cells (PBMCs) in MetS compared to control subjects.

Using real-time RT-PCR, the relative amount of mRNA of the IL-10 & LepR in PBMC from 20 patients with MetS was determined and compared with those of twenty control healthy subjects. Classical plasma and anthropometric parameters that are characteristics of MetS were also assessed.

While total cholesterol, triglyceride, and fasting blood sugar were significantly higher in MetS patients compared to healthy subjects, no significant differences was observed in HDL-C and LDL-C.

However there was no significant difference in BMI between two studied groups, statistical analysis indicated that BMI more than 30 identified as a risk factor for MetS (OR=2.931). Excessive waist circumference was the most frequent of MetS components. PBMC IL-10 expression was significantly decreased in MetS versus control subjects (fold of change was 0.55±0.09), while LpR expression increased in MetS participants 2.69±0.51. IL-10 expression had negative strong correlation with systolic pressure (r=0.46 P=0.03) while positive correlation with waist circumferences was observed (r=0.4 P=0.01). Also LepR showed correlation with TC, LDLc and waist circumferences in MetS group (r=0.4 P<0.05).

To our knowledge, this is the first research that investigated IL-10 & LepR expression in PBMCs of MetS subjects. We found an increase in LepR and a decrease in IL-10 expression on PBMC of MetS compared to control subjects. Study on a larger population and detecting synthesis of these proteins can reveal more details.

KEYWORD: Metabolic syndrome, real-time PCR, IL-10, Leptin receptor, PBMC, gene expression

1. INTRODUCTION

Metabolic syndrome (MetS) (also referred to Syndrome X, Insulin Resistance Syndrome) is one of the major public health challenges worldwide that is characterized by clustering of waist circumference, blood triglycerides(TG), HDL cholesterol, fasting glucose and blood pressure with different cut-off [1-3]. MetS and its components are risk factors which lead to developing cardiovascular disease and type 2 diabetes [3-5]. MetS can cause dyslipidemias, prothrombotic and proinflammatory states, ovarian polycystosis and fatty liver disease and finally increased CVD-related and all-cause mortality [1,6,7]. MetS affects approximately 25% of the adult population in western countries and also increase quickly in young populations [8-10]. The etiology is complex, genetic and environmental factors both have important roles [4]. Although it is unknown which components start and drive MetS to worsening condition, it is believed that visceral obesity can lead to development of insulin resistance, impaired glucose tolerance, hyperglycemia, and type 2 diabetes [11-13]. Adipose tissue as secretory organ release variety of molecules referred to adipocytokines [13]. Leptin is proinflammatory adipokine involved in different inflammatory diseases [14, 15]. It is produced mainly by fat tissue and is always correlated with BMI and is also unusually high in obese individuals [15]. Of course these leptin cannot suppress eating and promote weight loss that termed leptin resistance [16]. Leptin also involves in immune and inflammatory responses [17], which is mediate by leptin receptor (OB-R) that expressed by many types of immune cells, pancreatic β cells, adipose tissue, and muscle [18, 19]. Inflammation is now thought to play a key role in the pathophysiology of MetS [20]. Recent

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evidence has shown association between MetS and chronic low-grade inflammation. Moreover inflammation is closely related to obesity [2]. Association between leptin receptor, immune system, and MetS is still uncertain. This led to the hypothesis that MetS is mediated directly or indirectly by leptin receptor and its effects on immune system. Low-grade systemic inflammation is result of unbalanced production of pro-inflammatory and anti-inflammatory cytokines [21]. IL-10 plays a key role in balance of pro-inflammatory versus anti-inflammatory status [22]. The worth of IL-10 as an anti-inflammatory cytokine is shown by a recent study that reports a low-producing IL-10 was associated with a higher cardiovascular morbidity and mortality [21]. Also Esposito et al. showed that low levels of IL-10 in MetS [23].Another report proves that low IL-10 production capacity is associated with high plasma glucose, high HbA1c, type 2 diabetes, and dyslipidemia and also with metabolic syndrome and type 2 diabetes [24]. In addition, in a report it was detected association between circulating concentrations of leptin and IL-10 [25]. We hypothesize that leptin receptor and IL-10 may affect on each other. To our knowledge there is no report in expression study of IL-10, LpR and their relation in PBMC of MetS patients. Therefore the purpose of the present study was to investigate IL-10 and LepR expression in patients with MetS compared with non-MetS and to assess a possible correlation between the expression of these genes and parameters of disease.

2. MATERIALS AND METHODS

Participants

Twenty patients with metabolic syndrome (MetS) and 20 age matched healthy subjects participated in this study. MetS diagnosis was performed according to the modified criteria for MetS from the National Cholesterol Education Program-Adult Treatment Panel III (NCEP-ATP III) [26]. Based on this criteria our patients had three or more of the following conditions : a) central obesity (waist circumference > 90 cm); b) high blood pressure ≥ 130/85 mmHg or documented use of antihypertensive therapy; c) high fasting glucose (≥ 100 mg/dL); d) hypertriglyceridemia (≥ 150 mg/dL), and e) decreased high density lipoprotein cholesterol (HDLc) (< 50 mg/dL).

Participants with the following conditions were excluded from the study: estro-progesterone or testosterone treatment including oral contraceptives; smoking; pregnancy; amenorrhea; polycystic ovarian syndrome. They were not affected by chronic illness, nor taking drugs or supplements known to modify the immune system. No subjects showed evidence of chronic diseases (hepatic, renal, thyroid, cardiac), smoking, alcohol consumption, or family history of cardiovascular disease of early onset, no they were taking drugs. Informed consent was obtained from all participants prior to beginning the study and the study was approved by the Hamadan University of Medical Sciences ethics committee, the study also conformed to the principles outlined in the Declaration of Helsinki. Each participant underwent a baseline visit after an overnight fast. Height, weight, waist circumference and blood pressure were measured.

Sample collection

Following an overnight fasting (≥10 h), venous blood samples were collected between 7 and 8 a.m. Samples for CBC and gene expression analysis were collected in tubes containing 1 g EDTA/L. Serum was separated from whole blood by centrifugation at 1110 g for 15 min at 4°C within the hour after extraction.

Analytical methods

Blood pressure was measured in the sitting position after 5 minutes rest. FBS, TC, TG, LDLc & HDLc were measured using an autoanalyzer (Hitachi 911 sunrise Corporate company Kobe, Japan) by a colorimetric method kit (ParsAzmun - Iran). CBC analysis was carried out by hematology analyzer Sysmex Kx-21N (Sysmex Corporation, Japan).

Peripheral blood mononuclear cell (PBMC) isolation

Buffy coats were diluted 1:1 in BSS (Balanced salt solution); 4ml of diluted peripheral blood was layered on the 3ml of Ficoll–Hypaque solution (Amerhsam Biosciences, Piscataway, NJ). Density gradient centrifugation was carried out at 400 × *g* for 35 minutes. PBMC were harvested from the interface layer, washed twice with BSS. Harvested PBMCs were used to extract RNA directly.

RNA extraction and cDNA synthesis

Total RNA was extracted from PBMCs using RN easy Mini Kit (Qiagen, Hilden, Germany). Quantity and purity of the extract were measured by Nanodrop (Epoch,BioTek) spectrophotometer and the ratio of A260/A280 nm of all the samples were about 2. RNA integrity was assessed using 1% agarose gel, 1× TBE. RNA was judged suitable for gene expression only if samples exhibited intact bands corresponding to 18S and 28S ribosomal RNAs.

Equal amounts of total RNA, approximately 0.5 micrograms, was reverse transcribed into single-stranded cDNA using QuantiTect Reverse Transcription kit (Qiagen, Hilden, Germany) following the kit manufacturer's instructions. Briefly, one step gDNA wipeout in 42°C, then cDNA synthesis was performed at 42°C for 20 min, followed by RT inactivation at 95°C for 3 min.

Quantitative real-time PCR

PCR analyses were performed using C1000 Thermocycler and CFX96 real time system (BioRad) and *QuantiFast SYBR Green PCR Kit* (Qiagen, Hilden, Germany) in a final volume of 25 µl with 10 pmol of each primer. Each reaction was performed on 1 µl of 1:9 (v/v) dilution of the first cDNA strand.

The reaction was incubated at 95°C for 5 min, followed by 40 cycles of 15s at 95°C, 30s at annealing temperature, 30s at 72°C and then fluorescence was measured. Primers, designed by software AlleleID7.6, were:

IL-10-F (5'- GAGGACTTTAAGGGTTAC -3'),
IL-10-R (5'- CTATGTAGTTGATGAAGATG -3'),
LpR-F (5'- AGCCAATCTTCCTATTATC -3'),
LpR -R (5'- CCTCATACATCTTCCATT -3'),
βactin-F(5'- AAGATCAAGATCATTGCT -3'),
βactin-R (5'- TAACGCAACTAAGTCATA -3').

The gene numbers of different variants of LepR were NM_001198687, NM_001198688, NM_001198689, NM_001003679, NM_001003680, NM_002303 and the number belongs to IL-10 was NM_000572.

Annealing temperature and PCR products were 48°C (292), 46.5°C (261) and 47.5°C (177) for IL-10, LepR and βactin, respectively. Specificity of PCR amplifications was verified by a melting curve program (70-95°C with a heating rate of 0.5°C/s and a continuous fluorescence measurement) and analyzed by electrophoresis on a 1% agarose gel, 1× TBE. Primer efficiencies were: IL-10, 101%; LpR, 106%; βactin, 98%. Expression values were obtained as relative expression of the target gene versus the constitutively expressed βactin gene (relative expression = 2-(Ct, Target gene-Ct, Reference gene)).

Statistical methods

General values were expressed as means ± SD and gene expression level was reported as means ± SEM of three independent experiments. Values of $p < 0.05$ were considered significant. Results were analyzed using t-test for comparison between normal and MetS subjects and Mann–Whitney U-test for non-normal distribution. Spearman correlation coefficient was used for the determination of relations among variables. All statistical analyses were conducted using SPSS software (Version10).

3. RESULT

Characteristics of study population

Patient‘s baseline characteristics are shown in Table 1. There were no differences in weight and height between two groups. All patients in MetS group and 40% of the subjects of non-MetS had waist circumferences more than 90cm. The studied subjects were divided to 4 groups according to their BMI. These groups contained: normal weight (BMI=18.5-25), overweight (BMI=25-30), obese type1 (BMI=30-35) and obese type2 (BMI>35) as it is shown in Table 2. According to this classification, despite of existence more obese subjects (type1 & 2) in MetS group, no significant difference is discerned between MetS and non-MetS groups (P>0.05 by Pearson Chi-Square test) but when subjects classified into two groups (cut off is BMI=30) and comparing MetS in these groups, Fisher exact test showed significant difference (p=0.039) and we indicated the subjects that had BMI more than 30 are more susceptible to MetS than subjects with BMI less than 30 (OR = 2.931, 95% CI = 0.818–10.508).

In an attempt to investigate any existing correlation between stricken to MetS and family history, the subjects were asked for any MetS parameters in their family, both groups had approximately unique distribution of family history of disease and no remarkable difference was reported between these two groups (55 and 60 % in non-MetS and MetS respectively).

CBC measurement in all studied subjects did not show any significant differences in the mean RBC, WBC, neutrophil, lymphocyte, monocyte, eosinophil count, hematocrit percent, and hemoglobin concentration between two groups (data not shown).

Metabolic syndrome components

All subjects with MetS had waist circumferences more than 90cm as a diagnostic factor of MetS, frequency of other components in MetS group were: 75% high FBS, 65% hypertension, 60% hypertriglyceridemia, 45% low HDL. Correlation between MetS and MetS components is shown in the Table3. Results showed a relationship between MetS and total cholesterol (Table3).

In MetS group waist circumference correlates with TC(r =0.503, P=0.024) and LDL-c(r =0.614, P=0.004) and in non-MetS with FBS. Although FBS did not correlate to any variable in MetS group, in non-MetS it correlated to BMI(r=0.526, P=0.021) and waist circumference (r=0.519, P=0.019).

Gene expression in PBMC

For each person and gene, ΔCT was computed and compared between the two groups. While there was a significant difference in the IL-10 expression between the two groups (11.39±1.18 in MetS group versus 9.97±1.22 in non-MetS subjects p=0.001), also LepR expression was statistically different (13.85 ± 0.81 versus 14.76 ± 0.91 in MetS and non-MetS groups respectively).

Gene expression (fold changes) in MetS group compared to non-MetS subjects was calculated by $2^{-\Delta\Delta CT}$ formula. In case of IL-10 MetS patients expressed 0.55±0.09 (mean ± SEM) fold compared to non-MetS subjects. The LepR expression change in MetS patients was significantly different from non-MetS (2.69±0.51 as mean ± SEM).

Correlation study of IL-10 ΔCT showed reverse association with systolic pressure in MetS group (P=0.037, r = - 0.469) and with waist circumferences in all subjects participated in this study (P=0.01, r = 0.401). On the other hand LepR Delta CTs related to TC, LDLc and waist circumferences in MetS group (with P<0.05 and r = 0.445, r = 0.452 and r = 0.494) and had connected to systolic pressure in all participants(P=0.027, r = - 0.349).

Table1. Demographic data of the studied population

Characteristic	**mean ± S.D**		**P-value**
	MetS	Non-MetS	
Age (year)	53±10	47±6	NS
BMI (kg/m2)	29.7±4.3	27.3±3.5	NS
WC (cm)	101±7	91±6	0.000
Sys.P (mmHg)	139±26	114±13	0.000
Dias.P (mmHg)	90±13	78±16	0.003
FBS (mg/dl)	123±38	96±6	0.000
tChol (mg/dl)	218±50	177±45	0.014
TG (mg/dl)	187±120	87±32	0.000
HDLc (mg/dl)	57±21	57±16	NS
LDLc (mg/dl)	127±39	111±38	NS

MetS=metabolic syndrome, BMI= body mass index, WC= waist circumference
Sys.P=systolic pressure, Dias.P= diastolic pressure, FBS= fasting blood sugar,
TG= triglyceride, HDLc= high density lipoprotein cholesterol,
LDLc= low density lipoprotein cholesterol, NS= non significant,

Table2. Frequency of different degree of obesity in MetS and non-MetS groups

Group	**normal weight**	**Overweight**	**obese type1**	**obese type2**
MetS (%)	10	50	20	20
Non-MetS (%)	40	50	5	5

Table3. Correlation between MetS and some parameters

Correlation	Parameters						
	WC	FBS	TG	tChol	HDLc*	SysP	DiasP
r	0.692	0.691	0.576	0.394	0.022	0.593	0.469
P value	0.000	0.000	0.000	0.012	0.894	0.000	0.002

FBS= fasting blood sugar, TG= triglyceride, HDLc= high density lipoprotein cholesterol, WC = waist circumferences, tChol = total cholesterol, SysP = Systolic pressure, DiasP = Diastolic pressure

*: no significant relation

4. DISCUSSION

As a report indicates fifty million Americans now have the diagnosis of metabolic syndrome [5], a group of life-shortening morbidities like type 2 diabetes and hypertriglyceridemia [27]. Many studies have demonstrated that inflammation is an important etiology of MetS [28] through its various effectors (eg, leptin, adiponectin, TNF-α, IL-6, IL-10, etc) [29]. Report reviews have displayed the importance of obesity and insulin resistance in low-grade inflammation [30]. Recent studies concentrate on the role of inflammation on obesity and insulin resistance in MetS. Thus the genes involved in inflammation pathways have been evaluated for risk susceptibility and understanding of disease mechanism. Therefore in the present study, we quantified the relative gene expression of IL-10 and LepR in PBMCs in MetS subject and investigated correlation between these gene expression and other biochemical parameters.

Leptin is a multifunctional cytoadipokine which involved in immune regulation through leptin receptor [19]. Leptin receptor belongs to the cytokine receptor class I superfamily [31]. Several isoforms (a-f) and a soluble form

(sOB-R) exist as a result of alternative mRNA splicing [32]. Our result shows 2 fold increases in gene expression of LepR in MetS compared to Non-MetS patients. Data relating to correlation of LepR with MetS and its risk factors are very limited. We found only one report that surveyed sOB-R and showed its concentrations were inversely correlated with BMI, waist circumference, systolic and diastolic blood pressure, triglycerides, and fasting glucose and positively with HDL cholesterol [16], therefore they considered sOB-R as a beneficial factor. Although we investigated all variants of LpR, we suggest negative effect of this protein, as LepR correlated with TC, LDLc and waist circumferences in MetS group in our study.

The OB-Rb is the longest isoform that considered to be the functional receptor and contain intracellular motifs for the activation of janus kinase 2 (JAK2) and activator of transcription 3 (STAT3) followed by STAT3 dimerization and nuclear translocation. Also leptin can activate PI3K as a direct result of binding to LpR [31-33].

Some researcher report that association of leptin and insulin resistance may be independent of fat mass, and suggest leptin resistance is considered as a component of the MetS [34]. In agreement with this suggestion, another paper show that inflammatory stimuli can induce elevated systemic leptin concentrations [35] and also leptin affects cytokine secretion patterns like increase of IL-6 secretion, while IL-6 is suggested to activate the NF-κB, STAT3 and proinflammatory cascade [36]. Moreover leptin might regulate the inflammatory cell infiltration in visceral adipose tissue [15].

There are mentioned that leptin changes the cell cytokine secretion and facilitates inflammation. Also some reports show that leptin inhibits expression of IL-10 [37] or leads to down regulation of IL-10 in monocytic cell line U937 [25]. Against these data, other reports suggested highly significant positive association between leptin and IL-10[25], therefore we tried to study IL-10 gene expression plus LepR and found no relation between them.

IL-10 is an anti-inflammatory cytokine and has multiple effects like inhibition of NFκB leading to suppressed cytokine production [39]. It seems that IL-10 is a regulator against the cascade of pro-inflammatory cytokines, including IL-6[21]. IL-10 expressed by a variety of cell types includes macrophages, dendritic cells, B cells, T cells and NK cells [40]. Also T cells are thought to be the main source of IL-10 in vivo [41]. It is known that there are clear associations between IL-10 and disease susceptibility [41]. Our result show 0.55 folds of IL-10 expression in MetS compare to Non-MetS and correlation of IL-10 ΔCT with waist circumferences and reverse association with systolic pressure, these findings are similar to other report that show more circulating level of IL-10 in obese subject [42]. Of course these reports showed weight loss causes increasing in this level [42]. The fact that number of macrophages in the visceral fat of subjects increased by obesity, justify this finding [43]. This report also shows that IL-10 level in MetS subject is less than controls. Other research shows that IL-10 production induced by LPS, is inhibited in MetS [24]. Nishida et al shows no significant differences in IL-10 level between MetS and controls, whereas they report lower level of adiponectin in MetS subjects. Also they indicated a correlation between IL-10 and adiponectin in MetS subjects [44]. Thus some authors deduce that low IL-10 production capacity is associated with the metabolic syndrome [24].

Signaling of IL-10 receptor briefly includes of recruitment of Jak1 to receptor then phosphorylation of the receptor cytoplasmic tail, thus recruitment of STAT3 and its phosphorylation, next homodimerization and entry into the nucleus for transcription of IL-10-regulated genes [22]. However we could not find a correlation between LpR and IL-10 ΔCTs, consideration of some reports indicates leptin and IL-10 related to each other, therefore we suggest high leptin level and leptin resistance, accompany with increase LpR, may affect IL-10 expression that result unbalance between pro- and anti-inflammatory state and produce low grade inflammation that cause incorrect cycle and worsening condition [41].

As mentioned above LpR cause activation of JAK2 and STAT3 similar to IL-10 receptor, on the other hand STAT3 complex can trigger a negative feedback loop of IL-10 regulation [40].

Therefore possibly STAT3 is the key protein in induction and suppression of IL-10 expression. Some researchers like Benkhart demonstrated a direct interaction of Stat3 with the human IL-10 promoter at –120 , and suggested an autoregulation mechanism of IL-10[45]. Another report indicated that Stat3 correspond to regulation of the IL10 gene via several different signaling pathways and have role in a feedback mechanism [41]. Of course major papers prove that IL-10 production is under tight genetic control, with heritability estimates as high as 75% [46].

One limitation of our report is the small sample size that could increase the risk of statistically false negative results (type II errors). Another limitation of this study was the fact that we intended the control group not to have any parameter of MetS but finding age-matched women without any MetS component was very difficult. Also it would be better to measure plasma level of leptin, sOB-R and IL-10 for more information and acquiring a better understanding of the involved mechanisms. Additional work and functional studies are needed to ascertain the biological importance and study the suggested mechanism reported in the current work.

To our knowledge, this is the first research that investigated IL-10 & LepR expression in PBMCs of MetS subjects. In summary, we reported the increase LepR expression and decrease expression of IL-10 on PBMC of MetS

compared to control subjects. Study on a larger population, detecting synthesis of these proteins and cell culture study might reveal more details.

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