Adipose Tissue Gene Expression of Adiponectin, Tumor Necrosis Factor- α and Leptin in Metabolic Syndrome Patients with Coronary Artery Disease

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Abstract

Objective Metabolic syndrome (MS) is associated with an increased risk of coronary artery disease (CAD) and type 2 diabetes mellitus (DM). In MS, adipose tissue has been shown to function as a paracrine and an endocrine organ secreting various adipocytokines. In the current study, adiponectin, tumor necrosis factor- α (TNF- α) and leptin gene expressions in the epicardial adipose tissue (EAT), paracardial adipose tissue (PAT) and subcutaneous adipose tissue (SAT) were investigated in MS patients with CAD and in non-MS patients without CAD.

Methods and Results Thirty-seven patients with MS undergoing coronary artery bypass grafting due to CAD (MS group) and twenty-three non-MS patients without CAD undergoing heart valve surgery (control group) were recruited prospectively to the study. Relative gene expressions of adiponectin, TNF- α and leptin in EAT, PAT and SAT were compared between two groups of patients. Adiponectin gene expression in EAT and PAT were significantly lower in MS group compared to the control group ($p < 0.0001$, $p = 0.04$, respectively) while SAT adiponectin gene expression did not differ significantly ($p = 0.64$). TNF- α and leptin gene expressions were found to be statistically significantly higher in EAT, PAT and SAT of the MS group ($p < 0.0001$, for all).

Conclusion Our results demonstrate that TNF- α and leptin gene expressions increase prominently in the EAT, PAT and SAT while adiponectin gene expression decreases significantly in EAT and PAT in MS patients with CAD. These findings suggest that disturbances in expression of adiponectin, TNF- α and leptin in EAT, PAT and SAT might play an important role in MS patients with CAD.

Key words: metabolic syndrome, atherosclerosis, chemokines, inflammation, genes

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Introduction

The metabolic syndrome (MS) is one of the leading pub-

lic health issues around the world (1). The prevalence of MS is increasing in parallel with obesity and diabetes worldwide (2). Among the various criteria for the identification of MS, its major components are atherogenic dyslipidemia, in-

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sulin resistance, hypertension and abdominal obesity (3, 4). MS is associated with an increased risk of coronary heart disease (CAD) and type 2 diabetes mellitus (DM) (5, 6). It is also known that abdominal obesity and insulin resistance play a central role in MS.

In the last decade, numerous published clinical and experimental studies have focused on visceral adipose tissue and abdominal obesity. Today it is known that visceral adipose tissues function as a paracrine and an endocrine organ and secrete a number of adipocytokines which have proinflammatory, atherogenic or protective effects including leptin, adiponectin, tumor necrosis factor- α (TNF- α), resistin, interleukin-6, and fatty acid binding protein 4 (7-9). Adiponectin is an adipocyte-derived hormone with antiatherogenic, antidiabetic and anti-inflammatory properties. It attenuates insulin resistance by increasing insulin sensitivity of the liver. In muscle, adiponectin enhances glucose utilization and fatty acid oxidation. In addition, adiponectin increases endothelial nitric oxide (NO) secretion and inhibits monocyte adhesion and smooth muscle cell proliferation in the vascular wall (10, 11). TNF- α is an inflammatory cytokine, secreted from the macrophages within the adipose tissue and adipocytes. TNF- α contributes to insulin resistance via multiple direct endocrine and indirect modulatory effects of the genes involved in glucose and lipid metabolism (12, 13). TNF- α increases lipolysis, diminishes insulin-stimulated glucose transport and inhibits endothelial NO production (14, 15). Leptin is an anorexogenic hormone which is predominantly produced in adipose tissue (16). In addition to its effect on neuroendocrine, immune and reproductive systems, leptin regulates food intake, body weight and energy homeostasis (17). Recently, increased adiposity was shown to be associated with hyperleptinemia which subsequently causes endothelial dysfunction, hypertension and cardiovascular diseases (18).

In the current study, adiponectin, TNF- α and leptin gene expressions in epicardial adipose tissue (EAT), paracardial adipose tissue (PAT) and subcutaneous adipose tissues (SAT) were compared in MS patients with CAD and in non-MS patients without CAD.

Materials and Methods

Study population

Between November 2007 and January 2009, 37 patients with MS undergoing coronary artery bypass grafting due to CAD (MS group) and 23 non-MS patients without CAD undergoing heart valve surgery (control group) in our institution were enrolled prospectively in this study. Written informed consent was obtained from all of the patients prior to recruitment. The study was approved by the local ethical committee and performed in accordance with the ethical standards formulated in the Helsinki Declaration. Patients were included to the MS group according the National Cholesterol Education Program, Adult Treatment Panel III crite-

ria which defined the clustering of three or more of the following abnormalities: waist circumference >102 cm in men and >88 cm in women, serum triglycerides (TG) ≥ 150 mg/dL; high-density lipoprotein cholesterol (HDL-C) <40 mg/dL in men and <50 mg/dL in women or specific treatment for this lipid abnormality (fibrates and nicotinic acid), blood pressure $\geq 130/85$ mmHg; or fasting serum glucose (FSG) ≥ 110 mg/dL or drug treatment for hypertension or type 2 diabetes, respectively (19).

Patients with pregnancy, chronic renal and hepatic insufficiency, connective tissue disorders, cancer, endocrine and severe psychiatric disease were excluded from the study. Oral anti-diabetics, metformin in particular, and lipid lowering drugs which may interfere with adipocytokine gene expression were stopped 3 days before blood and tissue sample collection. None of the patients were treated with thiazolidinediones. Plasma and tissue samples were obtained after 8-10 hours (overnight) fasting. All patients had normal dietary regimes at least three days before taking blood samples. Fasting venous blood samples were separated as serum, plasma and cellular portions. Serum and plasma were divided as aliquot of 0.5 mL and stored at -20°C for biochemical analysis. Serum adiponectin levels were assayed by sandwich enzyme-linked immunosorbent assay (ELISA) kits (Orgenium, Finland). EAT samples were collected from the anterior surface of the right ventricle while PAT samples were taken from the pericardio-diafragmatic junction. SAT were obtained from retrosternal region. Tissue samples were then immediately frozen in liquid nitrogen and stored at -80°C prior to total RNA, cDNA and protein preparation. FSG was measured using enzymatic reference method with glucose oxidase, total cholesterol (TC) was measured using the enzymatic, colorimetric method with cholesterol esterase, HDL-C and low-density lipoprotein cholesterol (LDL-C) were directly determined with homogeneous enzymatic colorimetric assay, TG was measured by the enzymatic colorimetric method (GPO/PAP) with cholesterol phosphate oxidase and 4-aminophenazone method on the opeRA analyzer (Bayer AG, Leverkusen, Germany). All analyses were performed on Cobas 6,000 analyzer (Roche Diagnostics GmbH, Mannheim, Germany). Insulin resistance was measured by the homeostasis model assessment of insulin resistance (HOMA-IR) calculated as following formula: insulin ($\mu\text{U/mL}$) $\times$ glucose (mmol/L)/22.5 (20). Body fat amount was assessed by the bioelectrical impedance technique (MC 180, TANITA Corporation, Tokyo, Japan).

RNA isolation and cDNA synthesis

RNA was isolated from EAT, PAT and SAT by the use of Allprep kit (Qiagen, GmbH, Hilden, Germany) according to manufacturer's instructions and as previously published (21). RNA of 1 μg was reverse transcribed in 20 μL total volume using random hexamers and Superscript II reverse transcriptase (Invitrogen Corporation, California, USA). Lightcycler 480 SYBR Green Master (Roche, Mannheim, Germany) was used for the quantitative real-time polymerase chain reaction

Table 1. Clinical and Metabolic Data of Study Groups

	MS group (n=37)	Control group (n=23)	p value ^a	p value ^b
Age (years)	60.6 ± 9.0	55.13 ± 7.0	0.0154	-
Male gender (n, %)	21 (56.8)	11 (47.8)	0.60	-
Hypertension (%)	28 (80.0)	13 (56.5)	0.078	0.135
Diabetes (%)	31(88.6)	4(17.4)	<0.00001	<0.00001
Hyperlipidemia (%)	31 (88.6)	11 (47.8)	0.001	0.005
Fasting serum glucose (mg/dL)	157.4 ± 51.6	106.9 ± 11.6	<0.00001	0.001
Waist circumference (cm)	106.5 ± 9.7	89.5 ± 14.1	<0.0001	0.001
HOMA-IR	3.80 ± 2.3	1.36 ± 0.92	<0.0001	<0.0001
LVEF (%)	52.34± 9.7	53.26 ± 17.7	0.1	0.8
Fasting serum insulin (μU/mL)	9.9 ± 4.7	7.8 ± 5.9	0.129	0.268
BMI (kg/m ²)	31.4 ± 3.9	29.1 ± 5.4	0.057	0.098
Body fat (kg)	30.11± 8.5	25.0 ± 10.7	0.044	0.055
hs-CRP (mg/dL)	15.6 ± 35.5	4.5 ± 3.50	0.068	0.315
Serum adiponectin level (μg/mL)	5.29 ± 1.56	7.13 ± 1.03	<0.00001	<0.00001

Categorical variables are expressed as numbers (%) and continuous variables as mean±SD. BMI: Body mass index, HOMA-IR: Homeostasis model assessment of insulin resistance, hs-CRP: High-sensitivity C-reactive protein, LVEF: Left ventricular ejection fraction. Variables that are significantly different between cases and controls are shown in bold.

p-value^a: Results from parametric or non-parametric test of continuous variables, or Fisher's exact test.

p-value^b: Results from logistic regression analysis, adjusted for age and sex.

(qRT-PCR) carried out on the Lightcycler 480 Instrument (Roche Applied Sciences, Germany). Cyclophilin gene was used as an internal standard. The qRT-PCR protocol was as follows: initial denaturation at 95°C for 7 minutes, amplification segment is 5 sec at 95°C, 10 sec at 53°C-65°C (primer specific), 10 sec at 72°C for 45 cycles and melting curve segment is 15 sec 60°C for one cycle. The gene expression data were collected as Ct values (Ct is the cycle number at which logarithmic qRT-PCR plots cross a calculated threshold line). These values were used to determine the ΔCt (ΔCt is calculated as Ct of the target gene minus Ct of the internal control gene). Relative expressions were calculated according to the Δ-ΔCt method based on the mathematical model as described by Livak and Schmittgen (22). Ct values over 40 were excluded from further mathematical analysis.

Statistical analysis

Kolmogorov-Smirnov test was used to test for normality of continuous variables. Comparison of mean values between MS and control samples were performed using Mann-Whitney-U for non-parametric, Student's T-test for parametric variables. mRNA expression levels were log transformed before regression analysis. We performed logistic regression analysis in order to adjust for age difference between MS and control groups. Pearson's correlation test was used to evaluate the correlation between continuous variables. All analyses were performed using SPSS (version 15.0 for Windows, SPSS Inc.).

Results

Table 1 shows the clinical and metabolic data of the study samples. Age (p=0.02), FSG (p<0.00001), waist circumference (p<0.0001), HOMA-IR (p<0.0001) and body fat (p=0.04) were significantly higher in MS group compared to control group. As expected, the prevalence of diabetes (88.6% vs 17.4%), hypertension (80% vs 56.5%) and hyperlipidemia (88.6% vs 17.4%) was higher in MS group. The difference between groups remained statistically significant after adjustment by age, except body fat (p=0.06). No significant difference was observed between the two study groups in body mass index (BMI), fasting serum insulin, high-sensitivity C-reactive protein (hs-CRP) and left ventricular ejection fraction (LVEF). Figure 1, 2 and 3 represent the mRNA expression levels of adiponectin, TNF-α and leptin. Adiponectin mRNA expression of EAT and PAT were lower in MS group compared to the control group (p<0.001, p<0.05, respectively) while SAT adiponectin gene expression did not differ significantly. Serum adiponectin levels were higher in controls compared to MS group (p=0.00001). Serum adiponectin levels were correlated to EAT (r=0.33, p=0.018) and PAT (r=0.47, p=0.0004) adiponectin levels but not with SAT (p=0.4) levels. TNF-α and leptin mRNA expressions were found to be higher in EAT (p<0.01 and p<0.0001), PAT (p<0.001 and p<0.01) and SAT (p<0.001 and p<0.01) of the MS group. No major change in p values was observed after adjustment for age. In EAT, adiponectin expression was strongly negatively correlated with leptin and TNF-α (r=-0.5, p<0.001 for both), whereas leptin and TNF-

Figure 1. Adiponectin mRNA expression levels in EAT, PAT and SAT of MS and control groups. Logistic regression analysis shows that adiponectin mRNA expression levels are lower in EAT ($p < 0.001$) and PAT ($p < 0.05$) of MS group, while there was no significant difference in SAT ($p = 0.93$).

Figure 2. TNF- α mRNA expression levels in EAT, PAT and SAT of MS and control groups. Logistic regression analysis shows that TNF- α mRNA expression levels are higher in EAT ($p < 0.01$), PAT ($p < 0.001$) and SAT ($p < 0.001$) of MS group.

Figure 3. Leptin mRNA expression levels in EAT, PAT and SAT of MS and control groups. Logistic regression analysis shows that leptin mRNA expression levels are higher in EAT ($p < 0.0001$), PAT ($p < 0.01$) and SAT ($p < 0.01$) of MS group.

α expressions were positively correlated ($r=0.5$, $p<0.001$). There was no significant correlation between gene expression levels in PAT and SAT. In addition, no correlation was found between the gene expressions and descriptive data (metabolic & clinical), including HOMA-IR, hs-CRP, BMI, LVEF and waist circumference.

Discussion

In this study, we showed that TNF- α and leptin gene expressions were higher in EAT, PAT and SAT, however, adiponectin gene expression was lower in EAT and PAT in MS patients with CAD compared with the control group. Adiponectin expression level of SAT was similar in both groups.

In recent years, attention has focused on the visceral adipose tissue due to the presence of many adipocytokines synthesized and released from adipocytes (7-9, 23-25). Increased visceral adipose tissue in MS disturbs the adipocytokine secretion and leads to a low-grade chronic inflammatory state by the infiltration of macrophages in adipose tissue (24). This inflammatory state is found to be associated with insulin resistance and atherosclerosis (23, 24). In MS patients, the levels of serum adiponectin are decreased, while TNF- α concentration is elevated (26). However, the relation between leptin and MS is controversial. The concentration of serum leptin is generally higher in obese people, reflecting a leptin-resistant state which is found to be associated with insulin resistance, obesity and endothelial dysfunction (27-29). In the present study, leptin expression from three different adipose tissues was found to be high and also associated with MS but not with the control group.

These adipocytokines are known to interact with each other. It has already been shown that adiponectin counteracts the pro-inflammatory effects of TNF- α on the arterial wall (30). Also leptin and TNF- α regulate each other's production (31, 32). In the present study, we showed a significant correlation between these adipocytokines in EAT.

It is known that EAT shares a common embryological origin with visceral adipose tissue. Adipocytokines released from EAT have been suggested to have a central role in the development of coronary artery disease and have been shown to promote atheromatous plaque formation in the intima layer by passing into the myocardium via the vasovarium (33, 34). A limited number of gene expression studies have showed decreased levels of adiponectin and increased levels of inflammatory adipocytokines in EAT such as TNF- α and leptin in patients with CAD and obesity (8, 9, 25). Iacobellis et al reported that adiponectin expression was lower in EAT of non-obese patients with CAD compared to controls without CAD (8). Mazurek et al showed that TNF- α , interleukin (IL)-6 and IL-1 β expressions were increased in EAT compared to SAT of obese patients with CAD (9). The present results are in concordance with the above-mentioned data in terms of adiponectin expression. Baker et al demonstrated that in CAD patients, the adiponectin ex-

pression level was significantly lower in EAT compared to gluteal and abdominal adipose tissues while leptin expression was significantly lower in EAT and omental adipose tissue compared with SAT and gluteal adipose tissue (25). The same study showed that leptin mRNA expression was significantly lower in both EAT and omental adipose tissue compared to SAT and gluteal adipose tissue while TNF- α expression did not differ across adipose tissues. In the present MS group, we did not observe a significant difference between EAT and SAT considering TNF- α and leptin expressions. The present study enabled us to directly evaluate the expression of all three adipocytokines in EAT, PAT and SAT in MS patients with CAD compared with the control group. We also observed a similar adipocytokine expression pattern in EAT and PAT which suggests that PAT may be as important as EAT. The possible association between the altered gene expressions and MS presented in this study would enable us to exclude obesity as a confounding factor due to the similar BMI values in both groups. Contrary to expectations, the absence of a significant correlation between adipose tissue adiponectin, TNF- α and leptin gene expression and clinical or metabolic risk factors may be explained by the modest study sample size.

In conclusion, our results demonstrate that TNF- α and leptin gene expressions increase prominently in the EAT, PAT and SAT while adiponectin gene expression decreases significantly in EAT and PAT in MS patients with CAD. These findings suggest that disturbances in the expression of adiponectin, TNF- α and leptin in EAT, PAT and SAT might play an important role in MS patients with CAD.

The authors state that they have no Conflict of Interest (COI).

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