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Gene expression levels of S100 protein family in blood cells are associated with insulin resistance and inflammation (Peripheral blood S100 mRNAs and metabolic syndrome)

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ABSTRACT

Objective: Visceral fat obesity is located upstream of metabolic syndrome and atherosclerotic diseases. Accumulating evidences indicate that several immunocytes including macrophages infiltrate into adipose tissue and induce chronic low-grade inflammation. We recently analyzed the association between visceral fat adiposity and the gene expression profile in peripheral blood cells in human subjects and demonstrated the close relationship of visceral fat adiposity and disturbance of circadian rhythm in peripheral blood cells. In a series of studies, we herein investigated the association of visceral fat adiposity and mRNA levels relating to inflammatory genes in peripheral blood cells.

Approach and Results: Microarray analysis was performed in peripheral blood cells from 28 obese subjects. Reverse transcription-polymerase chain reaction (RT-PCR) was conducted by using blood cells from 57 obese subjects. Obesity was defined as body mass index (BMI) greater than 25 kg/m^2 according to the Japanese criteria. Gene expression profile analysis was carried out with Agilent whole human genome 4 × 44 K oligo-DNA microarray. Gene ontology (GO) analysis showed that 14 genes were significantly associated with visceral fat adiposity among 239 genes relating to inflammation. Among 14 genes, RT-PCR demonstrated that S100A8, S100A9, and S100A12 positively correlated with visceral fat adiposity in 57 subjects. Stepwise multiple regression analysis showed that S100A8 and S100A12 mRNA levels were closely associated with HOMA-IR and S100A9 mRNA was significantly related to adiponectin and CRP.

Conclusions: Peripheral blood mRNA levels of S100 family were closely associated with insulin resistance and inflammation.

1. Introduction

Obesity, especially visceral fat-accumulated obesity, is closely associated with the development of atherosclerotic diseases and is strongly linked to metabolic syndrome [1]. Molecular mechanism for metabolic syndrome has been investigated but it has not been fully understood at present. Increasing evidences indicate that several immunocytes including macrophages infiltrate into adipose tissue and induce chronic low-grade inflammation, which develops into insulin resistance and metabolic syndrome [2]. Moreover, these immune cells interact with adipocytes through free fatty acids and adipocytokines, generating a vicious metabolic cycle that accelerates the development of metabolic syndrome and atherosclerosis [3,4].

These pathologies in obese fat tissue suggest that gene expression profile in peripheral blood cells may reflect the visceral fat condition. Recently, we examined and analyzed the association between visceral fat adiposity and the gene expression profile in peripheral blood cells to search novel surrogate markers relating to visceral fat adiposity and to establish novel diagnostic tools for metabolic syndrome [5]. Interestingly, genes relating to circadian rhythm were significantly correlated with visceral fat adiposity, suggesting that visceral fat adiposity links to disturbance of

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circadian rhythm. In a series of studies for the impact of visceral fat adiposity on the gene expression profile in peripheral blood cells, we here analyzed the association of visceral fat adiposity and expression levels of inflammatory genes in peripheral blood cells.

2. Methods

2.1. Study population and clinical examinations

All subjects were inpatients of the Division of Endocrinology & Metabolism, Osaka University Hospital, Osaka, Japan. Written informed consent was obtained from each subject after explaining the purpose and potential complications of the study. The study protocol was approved by the human ethics committee of Osaka University and the study was registered with the University hospital Medical Information Network (Number: UMIN 000001663). Subjects and clinical examinations were described previously. Patients with type 1 diabetes mellitus, autoimmune diseases, malignant diseases, and infectious diseases were excluded from the study. Patients treated with statin and/or thiazolidinediones were also excluded. The estimated visceral fat area (eVFA) was measured by abdominal bioelectrical impedance analysis (BIA), as reported previously [6,7]. The homeostasis model-assessment of insulin resistance (HOMA-IR) was calculated by the equation: HOMA-IR = fasting insulin (μU/mL) × fasting glucose (mg/dL)/405.

2.2. Microarray analysis

Blood samples were collected into PaxGene Blood RNA tubes (PreAnalytiX/QIAGEN Inc., Valencia, CA) at 7:30 am. Total RNA was extracted by using PaxGene Blood RNA Kit (PreAnalytiX/QIAGEN) according to the protocol supplied by the manufacturer. After RNAs were qualified by the Agilent 2100 Bioanalyzer, total RNA was converted to cDNA, amplified, and labeled with Cy3-labeled CTP using the Quick Amp Labeling kit (Agilent Technologies, Santa Clara, CA). The amplified RNA and dye incorporation were quantified using a ND-1000 Spectrophotometer (Nano Drop Technologies, Wilmington, DE) and hybridized to Agilent whole human genome 4 × 44 K oligo-DNA microarray (Agilent Technologies, Santa Clara, CA). After hybridization, the arrays were washed consecutively by using Gene Expression Wash Pack (Agilent Technologies). Fluorescence images of the hybridized arrays were generated using the Agilent DNA Microarray Scanner, and the intensities were extracted with Agilent Feature Extraction software ver.10.7.3.1. The raw microarray data are deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO Series GSE28038).

2.3. Real-time RT-PCR

First-strand cDNA was synthesized from 180 ng of total RNA using Thermoscript RT (Invitrogen, Carlsbad, CA) and oligo dT primer. Real-time quantitative PCR amplification was performed with the LightCycler 1.5 (Roche Diagnostics, Tokyo, Japan) using LightCycler-FastStart DNA Master SYBR Green I (Roche Diagnostics, Tokyo, Japan). The final result for each sample was normalized to the respective GAPDH (glyceraldedyde-3-phosphate dehydrogenase) value. The primer sets used were: S100A8, 5′-CATGCCGTCTACAGGGATGA-3′ and 5′-GACGTCTGCACCCTTTTTCC-3′; S100A9, 5′-GGGAATTCAAAGAGCTGGTG-3′ and 5′-CACTGTGATCTTGGCCACTG-3′; S100A12, 5′-GCTCCACATTCCTGTGCATTGAGG-3′ and 5′-CCCTCATTGAGGACATTGCTGGG-3′; GAPDH, 5′-AAGGGCATCCTGGGCTACA-3′ and 5′-GAGGAGTGGGTGTCGCTGTTG-3′.

2.4. Microarray data analyses

The raw microarray intensities were processed by the percentile shift method (75th percentile) using the GeneSpring GX11 (Agilent Technologies) so as to normalize the range of expression intensities for inter-microarray. Only those genes whose expression data were available in more than 50% of hybridizations were included for further analyses. The normalized data were exported from the GeneSpring GX software. The correlation between peripheral blood gene expression levels and Log-eVFA levels was examined by Pearson's correlation under the R environment (http://cran.at.r-project.org). Gene Ontology (GO) information was retrieved from the annotations in GeneSpring GX11.

2.5. Clinical data analysis

Geometric mean values were used for C-reactive protein (CRP) due to the skewed distribution of the data. Non-normally distributed variables were log-transformed before analysis. The Spearman rank correlation coefficients for the study population as a whole were analyzed for Log-eVFA levels and other clinical variables. P values less than 0.05 denoted the presence of significant difference. Pearson's correlation coefficient was used to examine the relationship between S100A8, S100A9 and S100A12 and metabolic parameters. Stepwise multiple regression analysis was conducted to identify those parameters that significantly contributed to S100A8, S100A9 and S100A12, and parameter with F value > 4.0 were subsequently entered into the regression analysis as independent variables. All calculations were performed using the JMP software (JMP 9.0; SAS Institute Inc., Cary, NC). Data are expressed as mean ± SD.

3. Results

3.1. Characteristics of the subjects

The clinical characteristics of the participating subjects are listed in our previous report [5]. Briefly, the mean BMI, eVFA, and HOMA-IR of 57 patients were 30.6 kg/m^2, 168.8 cm^2, and 3.0, respectively. The proportion of patients with diabetes mellitus, dyslipidemia, and hypertension was 73%, 75%, and 58%, respectively. These clinical characteristics indicate that present study population is typical obesity with multiple complications.

3.2. Analysis of gene expression profiles

Microarray analysis was performed by using peripheral blood RNA samples from 28 subjects (BMI 31.9 ± 6.0 kg/m^2, VFA 199.4 ± 89.4 cm^2). The target probes were selected under the condition that significant signals were detected in more than 14 cases and finally 27,969 genes were extracted for gene expression analysis. To examine the correlation of visceral fat adiposity and peripheral blood mRNA expression levels relating to inflammation, genes classified as inflammation by gene ontology were extracted from among genes associated with visceral fat adiposity. Table 1 lists the significant genes related to inflammation (GO:0006954). Significant association was observed in 14 genes among 239 genes (5.9%) relating to inflammation. Nuclear factor related to kappaB binding protein (NFRKB), which belongs to family of eukaryotic transcription factors that control the expression of a large number of genes regulating inflammation and immunity, was most strongly correlated with eVFA (P = 0.0018). Interestingly, 3 genes, S100A12, S100A8, and S100A9, which belong to S100 protein family, emerged as significantly correlated genes with eVFA.

Table 1
Genes relating to inflammation (GO:0006954).

Gene symbol	Gene name	Correlation	*P* value
NFRKB	Nuclear factor related to kappaB binding protein	−0.563	0.0018
S100A12	S100 calcium binding protein A12	0.538	0.0031
ADORA3	Adenosine A3 receptor	−0.536	0.0033
IL15	Interleukin 15	0.524	0.0042
S100A8	S100 calcium binding protein A8	0.468	0.0120
CCL21	Chemokine (C–C motif) ligand 21	0.463	0.0131
S100A9	S100 calcium binding protein A9	0.439	0.0194
BMP6	Bone morphogenetic protein 6	−0.430	0.0224
PROK2	Prokineticin 2	0.428	0.0230
CCR3	Chemokine (C–C motif) receptor 3	−0.412	0.0293
CCR7	Chemokine (C–C motif) receptor 7	−0.411	0.0296
PXK	PX domain containing serine/threonine kinase	0.406	0.0320
LY96	Lymphocyte antigen 96	0.384	0.0434
TLR5	Toll-like receptor 5	0.374	0.0497

RT-PCR was next performed in 57 subjects to revalue the association of eVFA and S100A8, S100A9, and S100A12 in peripheral blood cells. As shown in Fig. 1, S100A8, S100A9 and S100A12 mRNA levels significantly and positively correlated with eVFA (S100A8, $P = 0.018$, $R = 0.31$; S100A9, $P = 0.047$, $R = 0.26$; S100A12; $P = 0.0013$, $R = 0.41$).

3.3. Association between S100 protein family and clinical parameters

Tables 2–4 lists the correlation coefficients for the relationship between S100A8, S100A9, and S100A12 and various clinical parameters, respectively. In age, sex-adjusted simple correlation analysis, S100A8 mRNA level correlated positively with log-eVFA, HOMA-IR, WBC, neurotrophilis, and CRP, while its mRNA level correlated negatively with log-adiponectin (Table 2). S100A9 mRNA level correlated positively with WBC, neurotrophilis, and CRP, whereas its mRNA level correlated negatively with HDL-C and log-adiponectin (Table 3). S100A12 mRNA level correlated positively with BMI, WC, log-VFA, HOMA-IR, and CRP, while its mRNA level correlated negatively with log-adiponectin (Table 4). Finally, stepwise multiple regression analysis was performed. HOMA-IR was identified as significant determinants of S100A8 ($F = 5.10$) and S100A12 ($F = 6.84$) (Table 2 and 4). Log-adiponectin ($F = 6.23$) and CRP ($F = 11.0$) were significant determinants of S100A9 (Table 3).

4. Discussion

In the present study, we show that peripheral blood S100-relating genes were strongly associated with CRP, adiponectin, and HOMA-IR in obese subjects.

Our group for the first time demonstrated that S100A8 is highly expressed in obese adipose tissues and adipose S100A8 is significantly reduced by peroxisome proliferator-activated receptor-γ (PPARγ) agonist, indicating that S100A8 is one of adipocytokines [8]. Furthermore, we recently showed that circulating level of calprotectin (S100A8/A9 complex) is positively correlated with visceral fat area [9] and is associated with low ultrasonographic low carotid plaque density [10]. Several groups have also showed the clinical significance of calprotectin and suggested that calprotectin is a novel biomarker of cardiovascular events [11–13]. Calprotectin is also higher in obese subjects than in non-obese subjects and is decreased by weight reduction [14,15]. Calprotectin promotes ROS generation via NADPH oxidase activation [16], binds to toll-like receptor 4 (TLR4) [17], and associates with the receptor for advanced glycation end-products (RAGE) [18], suggesting that calprotectin accelerates vital signaling pathways involved in the pathogenesis of atherosclerosis. Plasma S100A12 concentration is increased in type 2 diabetes and cardiovascular diseases [19–21]. Interestingly, the overexpression of human S100A12 induced arterial calcification in mice [22], suggesting that S100A12 is associated with the development of atherosclerosis. Collectively, S100A8, S100A9, and S100A12 may take part in the development of atherosclerosis and type 2 diabetes, because of their pro-inflammatory properties.

However, little is known in the association between peripheral blood mRNA expression levels of S100 family and inflammatory diseases in human. In patients with Kawasaki disease, leukocyte mRNA levels of S100A8, S100A9, and S100A12 were increased at acute phase compared to stable phase [23]. S100A12 mRNA level in peripheral blood mononuclear cells (PBMCs) was higher in non-diabetic subjects with pre-mature coronary artery disease than in the subjects with multiple coronary risk factors [24]. In present study, mRNA expression levels of peripheral blood S100A8, S100A9, and S100A12 were significantly correlated with visceral fat area, although its mechanism remains uncertain.

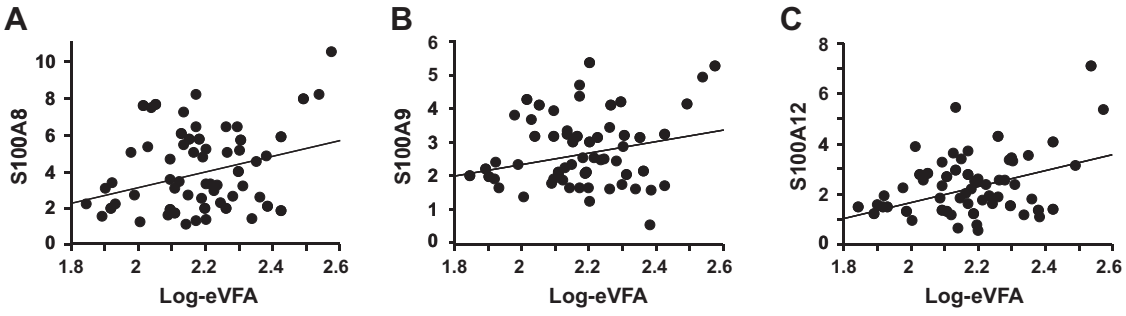

Fig. 1. Correlation of visceral fat area and peripheral blood mRNA levels of S100 family. Total RNAs from peripheral blood cells of 57 subjects were subjected to RT-PCR. S100A8, $P = 0.018$, $R = 0.31$; S100A9, $P = 0.047$, $R = 0.26$; S100A12; $P = 0.0013$, $R = 0.41$.

Table 2
Correlation between peripheral blood S100A8 mRNA and clinical parameters.

Clinical parameters	Univariate (non-adjusted)		Univariate (age, sex-adjusted)		Multivariate	
	R	*P* value	*R*	*P* value	*P* value	*F* value
Age	−0.57	<0.0001	–	–		
Sex	0.19	0.16	–	–		
BMI	0.31	0.02	0.20	0.10		
Waist circumference (WC)	0.25	0.06	0.20	0.10		
Log-eVFA	0.30	0.02	0.27	0.02	0.54	0.37
Systolic blood pressure	−0.08	0.54	−0.09	0.44		
Diastolic blood pressure	0.20	0.14	−0.13	0.32		
Fasting glucose	0.05	0.69	0.03	0.82		
Hemoglobin A1c (JDS)	0.22	0.10	0.18	0.11		
HOMA-IR	0.33	0.06	0.29	0.04	0.03	5.10
AST	0.18	0.17	0.15	0.19		
ALT	0.29	0.03	0.16	0.18		
γ-GTP	−0.10	0.46	−0.06	0.59		
Total cholesterol	0.03	0.81	−0.08	0.52		
LDL-C	0.005	0.97	−0.09	0.42		
Triglyceride	0.32	0.02	0.19	0.12		
HDL-C	−0.30	0.02	−0.21	0.08		
Creatinine	−0.26	0.06	−0.18	0.22		
Log-adiponectin	−0.49	0.0001	−0.35	0.004	0.55	0.37
WBC	0.50	<0.0001	0.32	0.01	0.26	1.32
Neutrophilis	0.45	0.0003	0.32	0.008	–	–
Lymphocytes	0.43	0.0005	0.20	0.13		
Monocytes	0.36	0.0004	0.21	0.08		
Eosinophilis	0.28	0.03	0.18	0.14		
Basophilis	−0.08	0.55	−0.18	0.11		
CRP	0.39	0.003	0.35	0.001	0.21	1.65
Complication of DM	0.005	0.97	0.09	0.43		
Complication of HT	0.02	0.90	0.15	0.19		
Complication of DLP	0.14	0.30	0.02	0.88		
Mean IMT	−0.18	0.22	0.09	0.50		

Data are mean ± SD. BMI; body mass index, eVFA; estimated visceral fat area, HOMA-IR; homeostasis model assessment of insulin resistance, LDL-C; low density lipoprotein–cholesterol, HDL-C; high density lipoprotein–cholesterol, IMT; intima-media thickness.

Table 3
Correlation between peripheral blood S100A9 mRNA and clinical parameters.

Clinical parameters	Univariate (non-adjusted)		Univariate (age, sex-adjusted)		Multivariate	
	R	*P* value	*R*	*P* value	*P* value	*F* value
Age	−0.34	0.009	–	–		
Sex	0.04	0.75	–	–		
BMI	0.30	0.02	0.20	0.14		
Waist circumference (WC)	0.24	0.07	0.20	0.18		
Log-eVFA	0.26	0.05	0.23	0.06		
Systolic blood pressure	−0.24	0.08	−0.24	0.07		
Diastolic blood pressure	−0.05	0.73	−0.27	0.07		
Fasting glucose	0.06	0.64	0.05	0.72		
Hemoglobin A1c (JDS)	0.24	0.08	0.21	0.10		
HOMA-IR	0.33	0.06	0.30	0.07		
AST	0.14	0.31	0.11	0.41		
ALT	0.18	0.17	0.11	0.42		
γ-GTP	−0.18	0.20	−0.13	0.34		
Total cholesterol	−0.06	0.67	−0.14	0.29		
LDL-C	−0.04	0.78	−0.12	0.36		
Triglyceride	−0.18	0.20	0.14	0.30		
HDL-C	−0.32	0.02	−0.29	0.02	0.13	2.42
Creatinine	−0.31	0.02	−0.20	0.23		
Log-adiponectin	−0.31	0.02	−0.37	0.007	0.02	6.23
WBC	0.40	0.002	0.37	0.01	0.42	0.65
Neutrophilis	0.42	0.001	0.42	0.002	–	–
Lymphocytes	0.25	0.06	0.08	0.59		
Monocytes	0.29	0.03	0.21	0.11		
Eosinophilis	0.14	0.30	0.10	0.44		
Basophilis	−0.07	0.58	−0.17	0.20		
CRP	0.42	0.001	0.37	0.003	0.002	11.0
Complication of DM	0.06	0.67	0.12	0.35		
Complication of HT	0.06	0.71	0.07	0.61		
Complication of DLP	0.11	0.40	0.03	0.81		
Mean IMT	−0.19	0.19	−0.01	0.92		

Data are mean ± SD. BMI; body mass index, eVFA; estimated visceral fat area, HOMA-IR; homeostasis model assessment of insulin resistance, LDL-C; low density lipoprotein–cholesterol, HDL-C; high density lipoprotein–cholesterol, IMT; intima-media thickness.

Table 4
Correlation between peripheral blood S100A12 mRNA and clinical parameters.

Clinical parameters	Univariate (non-adjusted)		Univariate (age, sex-adjusted)		Multivariate	
	R	*P* value	*R*	*P* value	*P* value	*F* value
Age	−0.44	0.0006	–	–		
Sex	0.13	0.33	–	–		
BMI	0.38	0.004	0.31	0.02	–	–
Waist circumference (WC)	0.38	0.004	0.31	0.005	–	–
Log-eVFA	0.40	0.002	0.37	0.002	0.69	0.16
Systolic blood pressure	−0.09	0.51	−0.09	0.45		
Diastolic blood pressure	0.02	0.16	−0.05	0.72		
Fasting glucose	0.03	0.85	0.005	0.97		
Hemoglobin A1c (JDS)	0.20	0.14	0.17	0.17		
HOMA-IR	0.56	0.0008	0.52	0.0004	0.01	6.84
AST	0.19	0.16	0.16	0.19		
ALT	0.27	0.04	0.17	0.18		
γ-GTP	−0.12	0.39	−0.09	0.57		
Total cholesterol	−0.07	0.58	−0.16	0.19		
LDL-C	−0.07	0.62	−0.15	0.23		
Triglyceride	0.12	0.36	0.009	0.94		
HDL-C	−0.21	0.13	−0.13	0.31		
Creatinine	−0.20	0.15	−0.11	0.37		
Log-adiponectin	−0.40	0.002	−0.29	0.03	0.46	0.56
WBC	0.34	0.009	0.19	0.18		
Neutrophilis	0.30	0.02	0.19	0.15		
Lymphocytes	0.28	0.04	0.07	0.64		
Monocytes	0.22	0.11	0.09	0.50		
Eosinophilis	0.09	0.51	−0.01	0.94		
Basophilis	−0.07	0.59	−0.16	0.20		
CRP	0.44	0.0006	0.42	0.0005	0.06	3.88
Complication of DM	0.05	0.74	0.12	0.26		
Complication of HT	0.02	0.87	0.11	0.36		
Complication of DLP	0.04	0.79	0.07	0.63		
Mean IMT	−0.18	0.21	0.003	0.97		

Data are mean ± SD. BMI; body mass index, eVFA; estimated visceral fat area, HOMA-IR; homeostasis model assessment of insulin resistance, LDL-C; low density lipoprotein–cholesterol, HDL-C; high density lipoprotein–cholesterol, IMT; intima-media thickness.

Visceral fat accumulation causes dysregulation of adipocytokines and results in chronic low-grade inflammation in a whole body. There is a possibility that visceral fat-mediated inflammatory signal affects on peripheral blood cells and induces mRNA expressions of S100 family.

As shown in Table 2–4, HOMA-IR was directly associated with S100A8 ($F = 5.10$) and S100A12 ($F = 6.84$). Adiponectin ($F = 6.23$) and CRP ($F = 11.0$) were significant determinants of S100A9. These results suggest that S100A8, S100A9 and S100A12 in peripheral blood cells may be closely related to insulin resistance and inflammation in visceral fat accumulation.

The present study has several limitations. All subjects were inpatients and thus diabetes mellitus, dyslipidemia, and hypertension were common in the study population. These conditions could influence on the expression levels of various genes in peripheral blood cells directly or indirectly. In addition, the study participants were obese Japanese subjects (BMI $\geqslant$ 25 kg/m^2) and thus future studies are needed to perform among not only obese subjects but also non-obese healthy (low VFA) subjects.

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Disclosures

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