Original Article

Relationship between Serum Cholesterol Efflux Capacity and Glucose Intolerance in Japanese-Americans

Mitsunobu Kubota[1], Shuhei Nakanishi[1], Masatoshi Hirano[1], Shusaku Maeda[1], Masayasu Yoneda[1], Tomokazu Awaya[1], Kiminori Yamane[2] and Nobuoki Kohno[1]

[1]Department of Molecular and Internal Medicine, Graduate School of Biomedical & Health Sciences, Hiroshima University, Hiroshima, Japan
[2]Nippon Telegraph and Telephone (NTT) West Corporation Chugoku Health Administration Center, Hiroshima, Japan

***Aim*: Serum cholesterol efflux has been suggested to be a key anti-atherogenic function of reverse cholesterol transport. Meanwhile, the quantitative and qualitative alteration of the levels of lipoproteins in the serum has been reported in patients with diabetes, although it remains unclear whether the serum cholesterol efflux capacity is impaired in cases of newly diagnosed glucose intolerance. We thus assessed the relationship between the serum cholesterol efflux capacity and glucose intolerance as detected using oral glucose tolerance tests (OGTTs).**

***Methods*: We measured the capacity of whole serum to mediate cholesterol efflux from human THP-1 macrophages in a cohort of 439 Japanese-Americans who underwent 75-g OGTTs. A multiple regression analysis was performed to examine the relationship between the serum cholesterol efflux capacity and glucose intolerance.**

***Results*: The serum cholesterol efflux capacity was found to be negatively correlated with the area under the curve for the serum glucose concentration during the 75-g OGTTs in all subjects. In addition, the serum cholesterol efflux capacity was found to be modestly but significantly lower in the glucose intolerance group (31.4±6.2%) than in the normal glucose tolerance group (33.2±6.1%). There was also a negative association between the serum cholesterol efflux capacity and glucose intolerance after adjusting for age and sex. Moreover, this association remained significant even after further adjustments for serum total cholesterol, high-density lipoprotein cholesterol, apolipoprotein AI and C-reactive protein.**

***Conclusions*: The serum cholesterol efflux capacity is impaired in Japanese-Americans newly diagnosed with glucose intolerance. This impairment may contribute in some manner to increasing the risk of atherosclerotic disease in subjects with glucose intolerance.**

J Atheroscler Thromb, 2014; 21:1087-1097.

Key words: Reverse cholesterol transport, Cholesterol efflux, Glucose intolerance, Atherosclerosis

Introduction

In patients with diabetes, atherosclerotic disease is an important determinant of the life expectancy, developing two to three times more often than that observed in patients without diabetes[1)] and accounting for 30-40% of mortality in this population[2)]. The risk of atherosclerotic disease may increase even at the stage of impaired glucose tolerance (IGT), which is generally considered to be a pre-diabetic state[3-4)]. One of the causes of the high frequency of atherosclerosis in patients with glucose intolerance is the accumulation in serum of atherogenic lipoproteins, such as remnant lipoproteins, small dense low-density lipoproteins (LDL) and oxidized LDL[5-6)]. Namely, patients with glucose intolerance display a typical pattern of atherogenic dyslipidemia. In contrast, the

Address for correspondence: Shuhei Nakanishi, Department of Molecular and Internal Medicine, Graduate School of Biomedical & Health Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima, 734-8551, Japan
E-mail: n-shuhei@umin.net
Received: February 6, 2014
Accepted for publication: April 11, 2014

plasma concentration of anti-atherogenic high-density lipoprotein cholesterol (HDL-C) tends to decrease in the presence of glucose intolerance[5]. Glucose intolerance, moreover, promotes the glycation of HDL itself[5]. Taken together, in patients with glucose intolerance, both quantitative and qualitative alteration of the levels of lipoproteins is hypothesized to decrease the anti-atherogenic effects in the serum.

The most widely accepted anti-atherogenic property in organisms is the centripetal return of excess cholesterol from macrophage foam cells in peripheral arteries to the liver for excretion into the bile and ultimately the feces via the reverse cholesterol transport (RCT) pathway[7]. Cholesterol efflux is the initial step of the RCT and represents the ability of serum-containing HDL particles to promote the RCT by accepting cholesterol from lipid-laden macrophages[8]. Consequently, measuring the serum cholesterol efflux capacity is expected to have clinical utility in the functional assessment of RCT. In fact, recent studies have demonstrated a robust association between the serum cholesterol efflux capacity and cardiovascular outcomes, independent of the serum HDL-C concentration[9], and that statin treatment results in an improved cholesterol efflux capacity[10]. Moreover, the use of whole serum as a cholesterol acceptor for efflux assay has been hypothesized to be a physiological method for ex vivo study, as the capacity of whole serum to induce cholesterol efflux from cells provides comprehensive and accurate data regarding lipoproteins and serum components, such as cholesteryl ester transfer protein (CETP), lecithin-cholesterol acyltransferase (LCAT) and phospholipid transfer protein (PLTP), involved in modifying HDL and promoting cellular cholesterol efflux[11-13].

According to several reports that have examined cholesterol efflux to the serum or plasma in subjects with type 2 diabetes mellitus (DM)[14-16], serum cholesterol efflux is thought to be influenced by various effects of glucose intolerance. However, to the best of our knowledge, no reports have yet assessed the serum cholesterol efflux capacity in patients with newly diagnosed glucose intolerance, as detected using 75-g oral glucose tolerance tests (OGTTs).

We therefore hypothesized that serum cholesterol efflux as a key anti-atherogenic function of the RCT may be impaired in subjects with newly diagnosed glucose intolerance, leading to a residual risk of atherosclerosis. In order to investigate this hypothesis, we assessed the relationship between the serum cholesterol efflux capacity and glucose intolerance in Japanese-Americans who underwent 75-g OGTTs. Japanese-Americans living in the United States are genetically equivalent to Japanese residing in Japan, although they have experienced rapid and intense westernization of their lifestyle. We previously reported that the prevalence of diabetes, metabolic syndrome and atherosclerotic disease is significantly greater in Japanese-Americans than in Japanese living in Japan[17-19]. The effects of glucose and lipid abnormalities on the serum cholesterol efflux capacity are therefore expected to be more readily apparent in this population than in native Japanese.

Materials and Methods

Study Subjects

The present study is part of a long-term epidemiological survey (Hawaii-Los Angeles-Hiroshima study) initiated in 1970 that continues to investigate risk factors for diabetes and cardiovascular disease among subjects limited to a population genetically equivalent to the native Japanese population. The epidemiological survey has been previously described in detail elsewhere[17-20]. The subjects of the present study were Japanese-Americans enrolled in the Los Angeles portion of the long-term epidemiological study carried out in 2010. The study population consisted of 439 Japanese-Americans (186 men and 253 women) who were not diagnosed with DM and not using medications for dyslipidemia. We also excluded individuals with a fasting triglyceride (TG) level of more than 400 mg/dl in consideration of the use of the Friedewald equation[21]. The glucose tolerance status was ascertained in each subject and classified as follows: normal glucose tolerance (NGT), IGT or DM according to the results of the 75-g OGTT. The diagnosis of DM was made in accordance with the American Diabetes Association 1997 glucose-tolerance standards[22], as follows: a fasting serum glucose (FSG) level of ≥ 126 mg/dl or two-hour serum glucose (2-h SG) level of ≥ 200 mg/dl after oral glucose loading. The smoking status (never, past or current) was assessed using standard interview procedures. All subjects received an explanation of the procedures and provided their written informed consent. This study was approved by the Ethics Committee of Hiroshima University, and the Council of Hiroshima Kenjin-Kai Association in Los Angeles.

Biochemical Analyses

All subjects underwent physical measurements and provided blood and urine samples after an overnight fast. The collected blood samples were centrifuged, and the obtained serum samples were immediately frozen and transported to Japan. The serum glu-

cose levels were measured according to the glucose oxidase method. Immunoreactive insulin (IRI) was measured using a double-antibody radioimmunoassay (Yamasa, Tokyo, Japan), and the degree of insulin resistance was evaluated with the homeostasis model assessment for insulin resistance (HOMA-IR) value[23]. The hemoglobin A1c (HbA1c) values were assessed according to the high-performance liquid chromatography method using the HLC-723G7 Automated Glycohemoglobin Analyzer (Tosoh, Tokyo, Japan). The obtained HbA1c values were converted to National Glycohemoglobin Standardization Program (NGSP)-equivalent values using the official equation[24]. The C-reactive protein (CRP) levels were measured using a highly sensitive, latex-enhanced immunonephelometric assay (Denka Seiken, Tokyo, Japan). The serum total cholesterol (T-Cho) and TG levels were assessed according to an enzymatic method (Kyowa Medex, Tokyo, Japan). The HDL-C levels were measured directly using a homogenous assay (Kyowa Medex, Tokyo, Japan), and the LDL-C levels were calculated using the Friedewald equation[21]. The apolipoprotein AI (apo-AI) and apolipoprotein B (apo-B) levels were measured using an immuno-nephelometry assay (Sekisui Medical, Tokyo, Japan).

Cholesterol Efflux Assay

The cholesterol efflux assay has been previously described[25]. Briefly, THP-1 cells (Riken Cell Bank, Tsukuba, Japan) were maintained in RPMI 1640 (Nikken Bio, Kyoto, Japan) containing 10% fetal bovine serum. THP-1 cells were differentiated into macrophages with 320 nmol/l phorbol 12-myristate13-acetate for 72 hours. For the measurement of cholesterol efflux, the cells were cultured in a 24-well plate at a density of 1.0×10^6 cells/well. The macrophages were washed twice with phosphate buffered saline (PBS) and labeled via incubation in the presence of [^{3}H]-cholesterol (PerkinElmer, Boston, USA; final concentration 0.33 μCi/ml) in medium containing 0.2% bovine serum albumin (BSA) for 24 hours. The cells were washed twice with PBS containing 0.2% BSA and incubated for 24 hours at 37℃ in medium containing 0.2% BSA in the presence of 0.5% serum (v/v) obtained from the study subjects as a cholesterol acceptor[25-27]. Following incubation, the cultures were centrifuged to remove cell debris, and the medium was removed to determine the level of radioactivity. At the end of the chase period, the macrophages were dissolved in a 3:2 (v/v) mixture of hexane/isopropanol, and the level of radioactivity per aliquot was measured. The percentage of cholesterol efflux was calculated by dividing the media-derived radioactivity by the sum of the radioactivity in the media and cells[28]. The ability of individual serum to promote cholesterol efflux was calculated by subtracting values obtained without serum in order to exclude the effects of passive diffusion. To minimize intra-assay variability, each serum sample was run in triplicate, and the average and standard deviation values were calculated for each percent of efflux obtained. To correct for inter-assay variability, a pool of human serum was tested in each assay as a reference standard, and its efflux capacity was used to normalize the subject sample values obtained from different experiments. The calculated mean intra- and inter-assay coefficients of variation were 6.64% and 7.76%, respectively.

Measurement of Atherosclerotic Lesions

The carotid artery intima-media wall thickness (IMT) was measured using B-mode ultrasonography (EUB-405X; Hitachi, Tokyo, Japan) with a 10-MHz probe according to a technique developed by Pignoli *et al.*[29]. The IMT measurement protocol has been previously described[19]. All measurements were obtained by one physician using the same equipment.

Statistical Analysis

The data are expressed as the mean value and standard deviation or median (25th-75th percentile), depending on the data distribution. For variables with a skewed distribution, log-transformation was performed prior to the analysis. This transformation was applied to the body mass index (BMI), fasting IRI (FIRI), two-hour IRI after oral glucose loading (2-h IRI), total area under the curve (AUC) for IRI during OGTT ($OGTT_{AUC}$ IRI), incremental AUC for IRI during OGTT ($OGTT_{IAUC}$ IRI), HOMA-IR, TG, CRP and IMT values. First, based on the results of the 75-g OGTT, the serum cholesterol efflux capacity was examined in three subgroups defined according to the glucose tolerance status: NGT, IGT and DM. Next, in order to examine the influence of the presence of glucose intolerance on the serum cholesterol efflux capacity, we combined the IGT and DM groups into a single glucose intolerance group. We then examined the serum cholesterol efflux capacity in the NGT group and the single glucose intolerance group. Continuous variables were compared using an age- and sex-adjusted analysis of covariance (ANCOVA) for comparison depending on the category of the glucose-tolerance status. Categorized variables were analyzed according to a χ^2 analysis. The total AUC for serum glucose during the OGTT ($OGTT_{AUC}$ glucose) and $OGTT_{AUC}$ IRI values were determined according to the trapezoidal method based on the FSG, 1-h SG,

2-h SG, FIRI, 1-h IRI and 2-h IRI measurements. The incremental AUC for serum glucose during the OGTT (OGTT$_{IAUC}$ glucose) and OGTT$_{IAUC}$ IRI values were calculated using the method recommended by Wolever[30]. The correlation between the AUC values and the serum cholesterol efflux capacity was evaluated using a Spearman's rank correlation analysis. We also performed a regression analysis, before and after adjusting for age and sex, to assess the relationships between the serum cholesterol efflux capacity and the metabolic parameters. In each regression model, either the glucose-tolerance status (NGT=0, glucose intolerance=1) or smoking status (never=0, past=1, current=2) was entered as a categorical variable. *P*-values of less than 0.05 were considered to be statistically significant. We used the SPSS version 19 software package (IBM Corp., Armonk, NY) for the statistical analysis.

Results

The clinical characteristics of the study subjects, separated into three groups according to the glucose tolerance status as defined by the 75-g OGTT results, are indicated in **Table 1**. The NGT, IGT and DM groups comprised 330, 71 and 38 subjects, respectively. No significant differences were observed regarding the smoking status (never, past or current). However, the subjects in the IGT and DM groups were significantly older than those in the NGT group. In addition, the incidence of IGT and DM tended to be higher in men than in women, although the difference was not significant. We therefore analyzed the following metabolic parameters after adjusting for age and sex. The BMI, systolic blood pressure (SBP), FSG, 2-h SG, OGTT$_{AUC}$ glucose, OGTT$_{IAUC}$ glucose, FIRI, 2-h IRI, OGTT$_{AUC}$ IRI, OGTT$_{IAUC}$ IRI, HOMA-IR, HbA1c, TG, CRP and IMT values were significantly higher in the IGT and DM groups than in the NGT group. The Apo-B values were also significantly higher in the IGT group than in the NGT group. In contrast, the HDL-C values were, as expected, significantly lower in the IGT and DM groups. The serum cholesterol efflux capacity was 33.2±6.1% in the NGT group, 31.3±6.2% in the IGT group and 31.6±6.2% in the DM group. The serum cholesterol efflux capacity tended to be lower in the subjects with IGT or DM than in those with NGT, although the difference was not significant ($P=0.061$). We subsequently analyzed the efflux capacity in men and women separately. There were no significant differences in the serum cholesterol efflux capacity between men and women (31.9±5.3% vs. 32.8±5.8%; $P=0.157$). The serum cholesterol efflux capacity was 32.6±5.7% in the men in the NGT group, 31.6±6.0% in the men in the IGT group and 31.3±7.2% in the men in the DM group, compared to 33.6±6.4% in the women in the NGT group, 31.0±6.4% in the women in the IGT group and 32.0±5.2% in the women in the DM group. In both sexes, the serum cholesterol efflux capacity tended to be lower in the subjects with IGT or DM than in those with NGT, although the difference was not significant ($P=0.224$ for men, $P=0.062$ for women). In order to examine the influence of the presence of glucose intolerance, we divided the study subjects into an NGT group and glucose intolerance group. Consequently, the serum cholesterol efflux capacity was 33.2±6.1% in the NGT group and 31.4±6.2% in the glucose intolerance group. A significantly low cholesterol efflux capacity was observed in the glucose intolerance group compared with that noted in the NGT group ($P=0.012$) (**Fig. 1**). In addition, there were significant negative correlations between the serum cholesterol efflux capacity and the OGTT$_{AUC}$ glucose ($r=-0.141$, $P=0.025$), OGTT$_{IAUC}$ glucose ($r=-0.185$, $P<0.001$) and 2-h SG ($r=-0.118$, $P=0.013$) values (**Fig. 2A-C**).

The associations between the serum cholesterol efflux capacity and the metabolic parameters assessed using a single regression analysis and a multiple regression analysis adjusting for age and sex are shown in **Table 2**. In the single regression analysis, we found significant positive associations in all subjects between the serum cholesterol efflux capacity and the levels of T-Cho, HDL-C and apo-AI. Meanwhile, negative associations were found between the serum cholesterol efflux capacity and the BMI, CRP, 2-h SG and OGTT$_{IAUC}$ glucose values and glucose intolerance. After adjusting for age and sex, we found significant positive associations between the serum cholesterol efflux capacity and the T-Cho, HDL-C and apo-AI levels. The serum cholesterol efflux capacity was also negatively associated with the CRP and OGTT$_{IAUC}$ glucose values and glucose intolerance. However, no significant relationships were observed between the serum cholesterol efflux capacity and the smoking status, IRI level or IMT values in the single regression analysis or multiple regression analysis after adjusting for age and sex.

Finally, in order to clarify the relationship between cholesterol efflux and glucose intolerance, we added the potential confounders exhibiting significant relationships with the serum cholesterol efflux capacity, as shown in **Table 2**, and glucose intolerance to the multiple regression models after adjusting for age and sex (**Table 3**). Among the lipid parameters, after

Table 1. Clinical characteristics of study subjects

	NGT	IGT	DM
Indv. characteristic			
N (men/women)	330 (132/198)	71 (34/37)	38 (20/18)
Age (y)	57.0 ± 15.1	66.6 ± 12.0*	64.6 ± 8.0*
BMI† (kg/m^2)	22.5 (20.3-24.7)	24.0 (22.3-26.6)*	25.3 (23.4-27.0)*
SBP (mmHg)	128 ± 17	139 ± 19*	138 ± 19*
DBP (mmHg)	86 ± 10	95 ± 10	94 ± 7
Smoking status (never/past/current)	201/79/50	42/22/7	15/19/4
Glucose metabolic parameters			
FSG (mg/dl)	87.0 ± 8.2	95.8 ± 10.6*	122.1 ± 42.9*, **
2-h SG (mg/dl)	98.0 ± 21.3	159.1 ± 22.5*	246.0 ± 77.8*, **
OGTT$_{AUC}$ glucose (mg/dl·120min)	13506 ± 2741	19220 ± 2720*	26631 ± 6861*, **
OGTT$_{IAUC}$ glucose (mg/dl·120min)	3230 ± 2283	7807 ± 2266*	11982 ± 2927*, **
FIRI† (μU/ml)	4.46 (3.46-7.63)	6.50 (3.69-12.13)*	6.69 (4.31-12.72)*
2-h IRI† (μU/ml)	43.74 (25.34-66.81)	81.81 (57.80-111.33)*	81.83 (43.38-119.42)*
OGTT$_{AUC}$ IRI† (μU/ml·120min)	6111 (4150-8672)	7563 (5009-10711)*	6866 (3036-10441)*
OGTT$_{IAUC}$ IRI† (μU/ml·120min)	5331 (3520-7890)	6707 (4275-9627)*	5578 (2219-9729)*
HOMA-IR†	0.95 (0.73-1.66)	1.72 (0.80-3.06)*	2.11 (1.12-3.96)*
HbA1c (%)	5.5 ± 0.3	5.7 ± 0.3*	6.6 ± 1.2*
Lipid parameters			
T-Cho (mg/dl)	209.1 ± 37.2	221.5 ± 50.2	210.3 ± 36.0
HDL-C (mg/dl)	62.2 ± 15.4	56.6 ± 17.1*	58.4 ± 17.6*
TG† (mg/dl)	104 (74-145)	131 (93-167)*	126 (86-189)*
LDL-C (mg/dl)	123.0 ± 33.5	136.1 ± 48.3	122.3 ± 31.6
Apo-AI (mg/dl)	165.2 ± 28.0	158.0 ± 31.6	161.8 ± 29.9
Apo-B (mg/dl)	100.8 ± 27.0	115.3 ± 34.0*	109.3 ± 28.6
Inflammatory marker			
CRP† (mg/dl)	0.045 (0.023-0.094)	0.104 (0.051-0.193)*	0.082 (0.045-0.221)*
Atherosclerotic parameters			
IMT† (mm)	0.77 (0.66-0.91)	0.87 (0.75-1.03)*	0.92 (0.74-1.10)*
Cholesterol efflux (%)	33.2 ± 6.1	31.3 ± 6.2	31.6 ± 6.2

Data are expressed as the means ± SD, median (inter-quartile range), or number. †Parameters were transformed logarithmically before analysis. NGT, Normal glucose tolerance; IGT, Impaired glucose tolerance; DM, diabetes mellitus; BMI, Body mass index; SBP, Systolic blood pressure; DBP, Diastolic blood pressure; FSG, Fasting serum glucose; 2-h SG, 2 h serum glucose after an oral glucose load; OGTT$_{AUC}$ glucose, AUC for serum glucose during an oral glucose tolerance test; OGTT$_{IAUC}$ glucose, Incremental AUC for serum glucose during an oral glucose tolerance test; FIRI, Fasting immunoreactive insulin; 2-h IRI, 2 h immunoreactive insulin after an oral glucose load; OGTT$_{AUC}$ IRI, AUC for immunoreactive insulin during an oral glucose tolerance test; OGTT$_{IAUC}$ IRI, Incremental AUC for immunoreactive insulin during an oral glucose tolerance test; HOMA-IR, Homeostasis model assessment for insulin resistance; HbA1c, Hemoglobin A1c; T-Cho, Total cholesterol; HDL-C, High-density lipoprotein cholesterol; TG, Triglyceride; LDL-C, Low-density lipoprotein cholesterol; Apo-AI, Apolipoprotein AI; Apo-B, Apolipoprotein B; CRP, C-reactive protein; IMT, Intima-media wall thickness. Data were analyzed using the Chi-squared test or by analysis of covariance adjusted for age and sex. *$P<0.05$, compared to NGT group. **$P<0.05$, compared to IGT group.

further adjusting for glucose intolerance, the serum cholesterol efflux capacity was found to be positively associated with the T-Cho levels only (model 1: $\beta=0.130$, $P=0.010$ and model 4: $\beta=0.137$, $P=0.006$). In addition, a significant negative association was observed between the serum cholesterol efflux capacity and the CRP levels (model 4: $\beta=-0.115$, $P=0.023$). Importantly, a significant negative association was detected between the serum cholesterol efflux capacity and glucose intolerance, even after additional adjustment for the T-Cho (model 1: $\beta=-0.128$, $P=0.010$), HDL-C (model 2: $\beta=-0.113$, $P=0.024$), apo-AI (model 3: $\beta=-0.116$, $P=0.021$), T-Cho and CRP (model 4: $\beta=-0.103$, $P=0.041$) levels.

Discussion

In this study, among subjects with glucose intol-

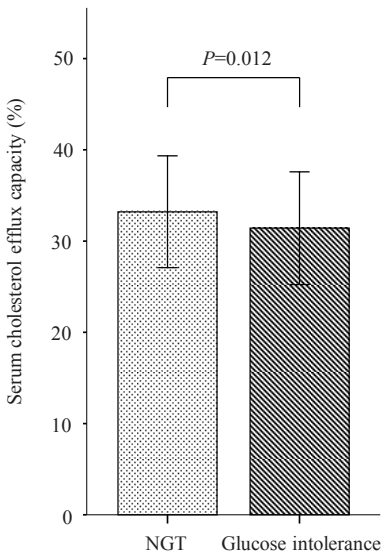
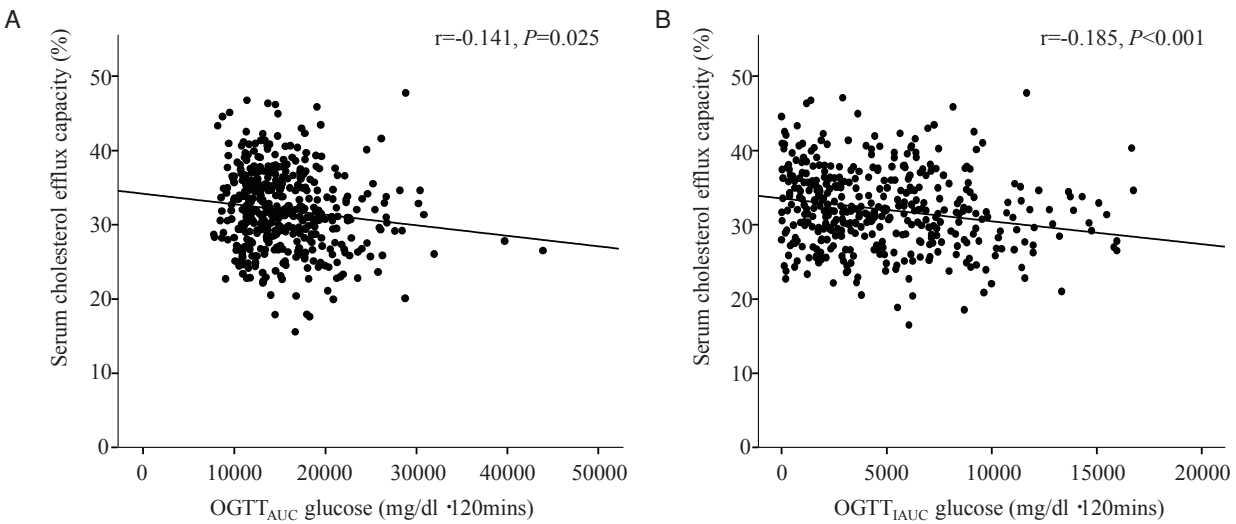


Fig. 1. Serum cholesterol efflux capacity in subjects with NGT and glucose intolerance

Bar graphs showing the serum cholesterol efflux capacity in the subjects with NGT and those with glucose intolerance. NGT, normal glucose tolerance. The column and error bars indicate the mean ± S.D.



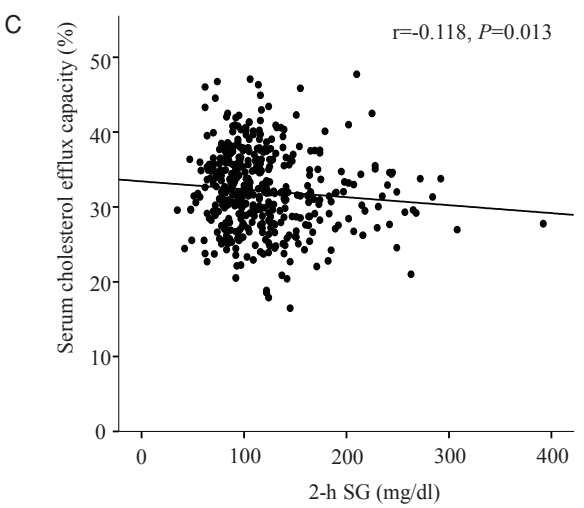


Fig. 2. Correlation of the serum cholesterol efflux capacity with glucose metabolic parameters in all subjects

OGTT$_{AUC}$ glucose (A), OGTT$_{IAUC}$ glucose (B) and 2-h SG (C) plotted against the serum cholesterol efflux capacity in each subject. Spearman's correlation coefficients (r) and *P*-values are given. OGTTAUC glucose, total area under the curve (AUC) for serum glucose during the 75-g OGTT; OGTTIAUC glucose, incremental AUC for serum glucose during the 75-g OGTT; 2-h SG, two-hour serum glucose after oral glucose loading.

Table 2. Relationships between cholesterol efflux and metabolic parameters in all subjects

	Crude		Age- and sex- adjusted	
	β	P	β	P
Indv. characteristic				
BMI† (kg/m^2)	−0.098	0.041	−0.081	0.097
SBP (mmHg)	−0.010	0.828	0.003	0.947
DBP (mmHg)	0.052	0.236	0.048	0.333
Smoking status (never/past/current)	−0.029	0.541	−0.004	0.934
Lipid parameters				
T-Cho (mg/dl)	0.152	0.001	0.127	0.012
HDL-C (mg/dl)	0.112	0.018	0.107	0.034
TG† (mg/dl)	0.041	0.388	0.046	0.341
LDL-C (mg/dl)	0.095	0.148	0.069	0.162
Apo-AI (mg/dl)	0.129	0.007	0.102	0.047
Apo-B (mg/dl)	0.073	0.128	0.057	0.250
Inflammatory marker				
CRP† (mg/dl)	−0.124	0.010	−0.129	0.010
Atherosclerotic parameter				
IMT† (mm)	0.008	0.874	0.025	0.657
Glucose metabolic parameters				
FSG (mg/dl)	−0.076	0.119	−0.079	0.121
2-h SG (mg/dl)	−0.105	0.044	−0.091	0.067
$OGTT_{IAUC}$ glucose (mg/dl·120min)	−0.174	<0.001	−0.175	<0.001
FIRI† (μU/ml)	0.026	0.585	0.026	0.592
2-h IRI† (μU/ml)	−0.040	0.405	−0.043	0.382
$OGTT_{IAUC}$ IRI† (μU/ml·120min)	−0.032	0.505	−0.034	0.474
Glucose tolerance status				
Glucose intolerance (ref. NGT)	−0.126	0.008	−0.125	0.012

β, Standardized regression coefficients. †Parameters were transformed logarithmically before analysis. Data were analyzed by a single regression analysis and by a multiple regression analysis adjusted for age and sex.

Table 3. Multiple regression analysis of the relationship between cholesterol efflux and glucose intolerance in all subjects

Independent variables	Model 1		Model 2		Model 3		Model 4	
	β	P	β	P	β	P	β	P
Lipid parameters								
T-Cho (mg/dl)	0.130	0.010					0.137	0.006
HDL-C (mg/dl)			0.091	0.073				
Apo-AI (mg/dl)					0.089	0.085		
Inflammatory marker								
CRP† (mg/dl)							−0.115	0.023
Glucose tolerance status								
Glucose intolerance (ref. NGT)	−0.128	0.010	−0.113	0.024	−0.116	0.021	−0.103	0.041

β, Standardized regression coefficients. †Parameters were transformed logarithmically before Analysis. Data were analyzed by each multiple regression model. Model 1: age, sex, glucose intolerance, and T-Cho; Model 2: age, sex, glucose intolerance, and HDL-C; Model 3: age, sex, glucose intolerance, and Apo-AI; Model 4: age, sex, glucose intolerance, T-Cho, and CRP. Model 1: $R^2=0.025$, $P=0.004$; Model 2: $R^2=0.018$, $P=0.020$; Model 3: $R^2=0.017$, $P=0.022$; Model 4: $R^2=0.035$, $P=0.001$.

erance, we identified the serum cholesterol efflux capacity, an anti-atherogenic function of the RCT, as being modestly but significantly impaired. In addition, glucose intolerance was found to be independently and negatively associated with the cholesterol efflux capacity. To the best of our knowledge, this is the first report to identify a reduction in the serum cholesterol efflux capacity in patients with newly diagnosed glucose intolerance detected on 75-g OGTTs.

Possible mechanisms of the reduction in serum cholesterol efflux capacity observed in patients with glucose intolerance include the following. First, in the IGT or DM groups, insulin resistance may reduce the number of HDL particles in the serum able to act as direct and efficient cholesterol acceptors[5]. In patients with insulin resistance, the lipoprotein lipase activity is reduced[31], thus prolonging the rate of degradation of TG components in very-low-density lipoprotein (VLDL) produced by the liver[32]. As a result, the CETP activity is increased, which leads to the production of TG-rich HDL particles and a reduction in the HDL-C level[5]. TG-rich HDL readily hydrolyzes[33], resulting in a reduced number of HDL particles[34]. A reduction in the number of HDL particles has been reported to be associated with a diminished cholesterol efflux capacity in a healthy volunteer cohort[9]. Second, in patients with glucose intolerance, the abnormal secretion of adipokines, such as tumor necrosis factor-alpha (TNF-α) and leptin, induces chronic inflammation[35]. As a result, the level of the inflammatory marker CRP is elevated. CRP has been reported to inhibit cholesterol efflux from THP-1 macrophages *in vitro* via the activation of extracellular signal-regulated kinase (ERK) 1/2 and downregulation of the ATP-binding cassette transporter A1 and G1 (ABCA1/G1) expression in a dose-dependent manner[36]. Third, glucose intolerance also promotes the expression of advanced glycation end-products (AGEs)[37], which can lead to the degradation of HDL into a dysfunctional state[38], thus resulting in a decrease in the ability of HDL in the serum to promote cholesterol efflux[27, 39].

In addition to the finding of a reduced cholesterol efflux capacity in patients with glucose intolerance, the AUC values for the serum glucose concentration observed during the 75-g OGTTs were negatively correlated with the serum cholesterol efflux capacity. These results indicate the presence of adverse effects of glucose intolerance on cholesterol efflux. On the other hand, the insulin level was not observed to correlate directly with the efflux capacity in this study. Insulin has been reported to suppress cholesterol efflux from macrophages at pharmacologic doses by inhibiting the ABCG1 expression *in vitro*[40]. However, in the present study of humans, serum cholesterol efflux may have been more strongly affected by alteration of the serum composition related to insulin resistance, such as the production of degenerated lipoproteins, than by the insulin level itself.

In this study, among all lipid parameters tested, the T-Cho level was observed to have the strongest positive association with the serum cholesterol efflux capacity. This observation is consistent with other recent findings indicating that determinants of serum cholesterol efflux include HDL and apo-AI, which act directly as acceptors of cholesterol, and that efflux depends on the ability of HDL to pass cholesterol acquired from cells to cholesterol pools within apo-B-containing lipoproteins, such as VLDL and LDL via CETP[41-44]. On the other hand, in the present study, HDL-C and apo-AI were each observed to have a significant positive association with the serum cholesterol efflux capacity based on the results of a multiple regression analysis adjusted for age and sex, but not a multiple regression analysis further adjusted for glucose intolerance. These results suggest a strong relationship between glucose intolerance and both HDL-C and apo-AI due to the modification of HDL by hyperglycemia[39], a state that diminished the direct relationships observed between serum cholesterol efflux and both HDL-C and apo-AI after adjusting glucose intolerance.

In the present study, we found no significant associations between the IMT and cholesterol efflux values. This result is inconsistent with the findings of a previous report that indicated an inverse relationship between apo-B depleted serum-mediated cholesterol efflux from J774 cells and the IMT[9]. This discrepancy may be due to the fact that the efflux capacity may have not decreased to the point of influencing IMT thickening, as subjects with NGT or newly diagnosed glucose intolerance were enrolled in our study. Moreover, to our knowledge, no previous reports have examined the relationship between IMT and cholesterol efflux using whole serum or plasma as a cholesterol acceptor. Therefore, the discrepancy between our results and those of the previous literature may also arise from the setting of efflux experiments, namely, the differences in cholesterol acceptors and species of cell lines used as cholesterol donors.

In this study, we evaluated the cholesterol efflux capacity using whole serum as a cholesterol acceptor for a physiological assessment in the human body, as the ability of serum to remove cholesterol from macrophages depends on multiple factors, such as lipoproteins and enzymes. In this context, the composition

and functional capacity of HDL itself were not evaluated in our set of experiments. Because efflux to HDL or apo-B depleted serum differs from efflux to whole serum, there is a possibility that the results of our study may have differed if HDL or apo-B depleted serum had been used as a cholesterol acceptor.

Several limitations of this study must be acknowledged and addressed. First, this investigation was designed as an observational study. In addition, our results did not confirm whether the opposite findings - that improved glucose tolerance leads to an improvement in the serum cholesterol efflux capacity - is true. Second, subjects who received diabetes and/or lipid medications were excluded, while those using other drugs were not. Finally, when we divided the subjects into three groups (NGT, IGT and DM) according to the 75-g OGTT results, the serum cholesterol efflux capacity tended to be lower in the IGT and DM groups than in the NGT group; however, the difference lacked significance. In addition, when we analyzed the serum cholesterol efflux capacity in men and women separately, similar results were observed, most likely due to a lack of statistical power. Taking this observation into account, we used sex as an adjustment factor in the statistical analysis. Further studies are needed to examine whether the effects of glucose intolerance on the serum cholesterol efflux capacity differ between men and women.

In conclusion, we identified a reduced serum cholesterol efflux capacity in patients with glucose intolerance, as detected using 75-g OGTTs, in Japanese-Americans. Clinical physicians may therefore need to consider not only conventional lipid parameters, but also the anti-atherogenic function, including the efflux capacity, in the whole serum in patients with glucose intolerance as a complementary assessment of the atherosclerotic disease risk.

Acknowledgements

We wish to thank the members of the Hiroshima Kenjin-Kai Association in Los Angeles for their participation in this study. We are also indebted to Drs. Kazufumi Ishida and Yuji Usui from the Hiroshima General Hospital for conducting the sample assays.

Sources of Funding

This work was supported by the Japan Heart Foundation & Astellas/Pfizer Grant for Research on Atherosclerosis Update, the Japan Diabetes Foundation and a Tsuchiya Memorial Medical Foundation (Hiroshima, Japan) Grant. These funding sources had no involvement in the study design, the collection, analysis or interpretation of the data, the writing of this report or the decision to submit this article for publication.

Conflicts of Interest

None.

References

1) Kannel WB, McGee DL: Diabetes and glucose tolerance as risk factors for cardiovascular disease: The Framingham Study. Diabetes Care, 1979; 2: 120-126
2) De Marco R, Locatelli F, Zoppini G, Verlato G, Bonora E, Muggeo M: Cause-specific mortality in type 2 diabetes. The Verona Diabetes Study. Diabetes Care, 1999; 22: 756-761
3) DECODE Study Group, European Diabetes Epidemiology Group: Is the current definition for diabetes relevant to mortality risk from all causes and cardiovascular and noncardiovascular disease? Diabetes Care, 2003; 26: 688-696
4) Tominaga M, Eguchi H, Manaka H, Igarashi K, Kato T, Sekikawa A: Impaired glucose tolerance is a risk factor for cardiovascular disease, but not impaired fasting glucose. The Funagata Diabetes Study. Diabetes Care, 1999; 22: 920-924
5) Vergès BL: Dyslipidaemia in diabetes mellitus. Review of the main lipoprotein abnormalities and their consequences on the development of atherogenesis. Diabetes Metab, 1999; 25: 32-40
6) Maeda S, Nakanishi S, Yoneda M, Awaya T, Yamane K, Hirano T, Kohno N: Associations between small dense LDL, HDL subfractions (HDL2, HDL3) and risk of atherosclerosis in Japanese-Americans. J Atheroscler Thromb, 2012; 19: 444-452
7) Cuchel M, Rader DJ: Macrophage reverse cholesterol transport: key to the regression of atherosclerosis? Circulation, 2006; 113: 2548-2555
8) Rothbalat GH, de la Llera-Moya M, Atger V, Kellner-Weibel G, Williams DL, Phillips MC: Cell cholesterol efflux: integration of old and new observations provides new insights. J Lipid Res, 1999; 40: 781-796
9) Khera AV, Cuchel M, de la Llera-Moya M, Rodrigues A, Burke MF, Jafri K, French BC, Phillips JA, Mucksavage ML, Wilensky RL, Mohler ER, Rothblat GH, Rader DJ: Cholesterol efflux capacity, high-density lipoprotein function, and atherosclerosis. N Engl J Med, 2011; 364: 127-135
10) Miyamoto-Sasaki M, Yasuda T, Monguchi T, Nakajima H, Mori K, Toh R, Ishida T, Hirata K: Pitavastatin increases HDL particles functionally preserved with cholesterol efflux capacity and antioxidative actions in dyslipidemic patients. J Atheroscler Thromb, 2013; 20: 708-716
11) Zanotti I, Favari E, Bernini F: Cellular cholesterol efflux pathways: impact on intracellular lipid trafficking and

methodological considerations. Curr Pharm Biotechnol, 2012; 13: 292-302
12) Villard EF, EI Khoury P, Frisdal E, Bruckert E, Clement K, Bonnefont-Rousselot D, Bittar R, Le Goff W, Guerin M: Genetic determination of plasma cholesterol efflux capacity is gender-specific and independent of HDL-cholesterol levels. Arterioscler Thromb Vasc Biol, 2013; 33: 822-828
13) Ohashi R, Mu H, Wang X, Yao Q, Chen C: Reverse cholesterol transport and cholesterol efflux in atherosclerosis. QJM, 2005; 98: 845-856
14) Syvänne M, Castro G, Dengremont C, De Geitere C, Jauhiainen M, Ehnholm C, Michelagnoli S, Franceschini G, Kahri J, Taskinen MR: Cholesterol efflux from Fu5AH hepatoma cells induced by plasma of subjects with or without coronary artery disease and non-insulin-dependent diabetes: importance of LpA-I: A-II particles and phospholipid transfer protein. Atherosclerosis, 1996; 127: 245-253
15) Zhou H, Shiu SW, Wong Y, Tan KC: Impaired serum capacity to induce cholesterol efflux is associated with endothelial dysfunction in type 2 diabetes mellitus. Diab Vasc Dis Res, 2009; 6: 238-243
16) Dullaart RP, Annema W, de Boer JF, Tietge UJ: Pancreatic β-cell function relates positively to HDL functionality in well-controlled type 2 diabetes mellitus. Atherosclerosis, 2012; 222: 567-573
17) Nakanishi S, Okubo M, Yoneda M, Jitsuiki K, Yamane K, Kohno N: A comparison between Japanese-Americans living in Hawaii and Los Angeles and native Japanese: the impact of lifestyle westernization on diabetes mellitus. Biomed Pharmacother, 2004; 58: 571-577
18) Yoneda M, Yamane K, Jitsuiki K, Nakanishi S, Kamei N, Watanabe H, Kohno N: Prevalence of metabolic syndrome compared between native Japanese and Japanese-Americans. Diabetes Res Clin Pract, 2008; 79: 518-522
19) Watanabe H, Yamane K, Fujikawa R, Okubo M, Egusa G, Kohno N: Westernization of lifestyle markedly increases carotid intima-media wall thickness (IMT) in Japanese people. Atherosclerosis, 2003; 166: 67-72
20) Egusa G, Murakami F, Ito C, Matsumoto Y, Kado S, Okamura M, Mori H, Yamane K, Hara H, Yamakido M: Westernized food habits and concentrations of serum lipids in the Japanese. Atherosclerosis, 1993; 100: 249-255
21) Friedewald WT, Levy RI, Fredrickson DS: Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem, 1972; 18: 499-502
22) Expert Committee on the Diagnosis and Classification of Diabetes Mellitus: Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. Diabetes Care, 1997; 20: 1183-1197
23) Matthews DR, Hosker JP, Rundenski AS, Naylor BA, Treacher DF, Turner RC: Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia, 1985; 28: 412-419
24) Kashiwagi A, Kasuga M, Araki E, Oka Y, Hanafusa T, Ito H, Tominaga M, Oikawa S, Noda M, Kawamura T, Sanke T, Namba M, Hashiramoto M, Sasahara T, Nishio Y, Kuwa K, Ueki K, Takei I, Umemoto M, Murakami M, Yamakado M, Yatomi Y, Ohashi H, Committee on the Standardization of Diabetes Mellitus-Related Laboratory Testing of Japan Diabetes Society: International clinical harmonization of glycated hemoglobin in Japan: From Japan Diabetes Society to National Glycohemoglobin Standardization Program values. J Diabetes Invest, 2012; 3: 39-40
25) Nakanishi S, Vikstedt R, Söderlund S, Lee-Rueckert M, Hiukka A, Muilu M, Metso J, Naukkarinen J, Palotie L, Kovanen PT, Jauhiainen M, Taskinen MR: Serum, but not monocyte macrophage foam cells derived from low HDL-C subjects, displays reduced cholesterol efflux capacity. J Lipid Res, 2009; 50: 183-192
26) Ozasa H, Ayaori M, Iizuka M, Terao Y, Uto-Kondo H, Yakushiji E, Takiguchi S, Nakaya K, Hisada T, Uehara Y, Ogura M, Sasaki M, Komatsu T, Horii S, Mochizuki S, Yoshimura M, Ikewaki K: Pioglitazone enhances cholesterol efflux from macrophages by increasing ABCA1/ABCG1 expressions via PPARγ/LXRα pathway: findings from in vitro and ex vivo studies. Atherosclerosis, 2011; 219: 141-150
27) Matsuki K, Tamasawa N, Yamashita M, Tanabe J, Murakami H, Matsui J, Imaizumi T, Satoh K, Suda T: Metformin restores impaired HDL-mediated cholesterol efflux due to glycation. Atherosclerosis, 2009; 206: 434-438
28) Marcil M, Yu L, Krimbou L, Boucher B, Oram JF, Cohn JS, Genest J Jr: Cellular cholesterol transport and efflux in fibroblasts are abnormal in subjects with familial HDL deficiency. Arterioscler Thromb Vasc Biol, 1999; 19: 159-169
29) Pignoli P, Tremoli E, Poli A, Oreste P, Paoletti R: Intimal plus medial thickness of the arterial wall: a direct measurement with ultrasound imaging. Circulation, 1986; 74: 1399-1406
30) Wolever TM: Effect of blood sampling schedule and method of calculating the area under the curve on validity and precision of glycaemic index values. Br J Nutr, 2004; 91: 295-301
31) Kobayashi J, Nohara A, Kawashiri MA, Inazu A, Koizumi J, Nakajima K, Mabuchi H: Serum lipoprotein lipase mass: clinical significance of its measurement. Clin Chim Acta, 2007; 378: 7-12
32) Adiels M, Westerbacka J, Soro-Paavonen A, Hakkinen AM, Vehkavaara S, Caslake J, Packard C, Olofsson SO, Yki-Jarvinen H, Taskinen MR, Boren J: Acute suppression of VLDL1 secretion rate by insulin is associated with hepatic fat content and insulin resistance. Diabetologia, 2007; 50: 2356-2365
33) Syvänne M, Ahola M, Lahdenperä S, Kahri J, Kuusi T, Virtanen KS, Taskinen MR: High density lipoprotein subfractions in non-insulin-dependent diabetes mellitus and coronary artery disease. J Lipid Res, 1995; 36: 573-582
34) Golay A, Zech L, Shi MZ, Chiou YA, Reaven GM, Chen YD: High density lipoprotein (HDL) metabolism in non-insulin-dependent diabetes mellitus: measurement of HDL turnover using tritiated HDL. J Clin Endocrinol Metab, 1987; 65: 512-518

35) Matsuzawa Y, Funahashi T, Kihara S, Shimomura I: Adiponectin and metabolic syndrome. Arterioscler Thromb Vasc Biol, 2004; 24: 29-33
36) Wang X, Liao D, Bharadwaj U, Li M, Yao Q, Chen C: C-reactive protein inhibits cholesterol efflux from human macrophage-derived foam cells. Arterioscler Thromb Vasc Biol, 2008; 28: 519-526
37) Brownlee M: The pathobiology of diabetic complications. A unifying mechanism. Diabetes, 2005; 54: 1615-1625
38) Hedrick CC, Thorpe SR, Fu MX, Harper CM, Yoo J, Kim SM, Wong H, Peters AL: Glycation impairs high-density lipoprotein function. Diabetologia, 2000; 43: 312-320
39) Duell PB, Oram JF, Bierman EL: Nonenzymatic glycosylation of HDL and impaired HDL-receptor-mediated cholesterol efflux. Diabetes, 1991; 40: 377-384
40) Yamashita M, Tamasawa N, Matsuki K, Tanabe J, Murakami H, Matsui J, Suda T: Insulin suppresses HDL-mediated cholesterol efflux from macrophages through inhabitation of neutral cholesteryl ester hydrolase and ATP-binding cassette transporter G1 expressions. J Atheroscler Thromb, 2010; 17: 1183-1189
41) Chan DC, Hoang A, Barrett PH, Wong AT, Nestel PJ, Sviridov D, Watts GF: Apolipoprotein B-100 and ApoA-II kinetics as determinants of cellular cholesterol efflux. J Clin Endocrinol Metab, 2012; 97: 1658-1666
42) Borggreve SE, De Vries R, Dullaart RP: Alterations in high-density lipoprotein metabolism and reverse cholesterol transport in insulin resistance and type 2 diabetes mellitus: role of lipolytic enzymes, lecithin cholesterol acyltransferase and lipid transfer proteins. Eur J Clin Invest, 2003; 33: 1051-1069
43) Hoang A, Drew BG, Low H, Remaley AT, Nestel P, Kingwell BA, Sviridov D: Mechanism of cholesterol efflux in humans after infusion of reconstituted high-density lipoprotein. Eur Heart J, 2012; 33: 657-665
44) Vasudevan M, Tchoua U, Gillard BK, Jones PH, Ballantyne CM, Pownall HJ: Modest diet-induced weight loss reduces macrophage cholesterol efflux to plasma of patients with metabolic syndrome. J Clin Lipidol, 2013; 7: 661-670

09 24315 1087–1097(F–2)0925