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Differential reactivities of four homogeneous assays for LDL-cholesterol in serum to intermediate-density lipoproteins and small dense LDL: Comparisons with the Friedewald equation

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ABSTRACT

Background: In routine clinical laboratory testing and numerous epidemiological studies, LDL-cholesterol (LDL-C) has been estimated commonly using the Friedewald equation. We investigated the relationship between the Friedewald equation and 4 homogeneous assays for LDL-C.
Methods: LDL-C was determined by 4 homogeneous assays [liquid selective detergent method: LDL-C (L), selective solubilization method: LDL-C (S), elimination method: LDL-C (E), and enzyme selective protecting method: LDL-C (P)]. Samples with discrepancies between the Friedewald equation and the 4 homogeneous assays for LDL-C were subjected to polyacrylamide gel electrophoresis and the β-quantification method.
Results: The correlations between the Friedewald equation and the 4 homogeneous LDL-C assays were as follows: LDL-C (L) ($r=0.962$), LDL-C (S) ($r=0.986$), LDL-C (E) ($r=0.946$) and LDL-C (P) ($r=0.963$). Discrepancies were observed in sera from type III hyperlipoproteinemia patients and in sera containing large amounts of midband and small dense LDL on polyacrylamide gel electrophoresis. LDL-C (S) was most strongly correlated with the β-quantification method even in sera from patients with type III hyperlipoproteinemia.
Conclusions: Of the 4 homogeneous assays for LDL-C, LDL-C (S) exhibited the closest correlation with the Friedewald equation and the β-quantification method, thus reflecting the current clinical databases for coronary heart disease.

1. Introduction

Numerous clinical studies have shown an independent relationship between increases in LDL-cholesterol (LDL-C) concentrations and risk of coronary heart disease (CHD) [1,2]. According to the National Cholesterol Education Program (NCEP)-Adult Treatment Panel III, the diagnosis and management of adult patients with hypercholesterolemia is largely based on the concentration of LDL-C [3].

A wide variety of methods have been used for determining LDL-C in serum. These methods include sequential and density-gradient ultracentrifugation [4], β-quantification [5–8], Friedewald equation [9], electrophoresis [10], HPLC [11] and homogeneous assay [12]. As a reference procedure for LDL-C, the CDC has adopted a variation of the multi-step β-quantification procedure used by the Lipid Research Clinics [13], which combines separation by ultracentrifugation and chemical precipitation. The β-quantification procedure for LDL-C has been also recommended by the NCEP Lipoprotein Measurement Working Group [7]. However, the β-quantification method requires a relatively large volume of serum, special equipment, and is a time-consuming procedure; therefore, it is not well suited for routine testing in hospitals and clinics. Although the Friedewald equation is the most commonly used technique in clinical laboratories for the estimation of LDL-C, it cannot be accurately estimated when plasma triglycerides (TG) >4.52 mmol/l (400 mg/dl) or when specimens are

Abbreviations: LDL-C, LDL-cholesterol; CHD, coronary heart disease; NCEP, National Cholesterol Education Program; TG, triglycerides; IDL, intermediate-density lipoproteins; apo, apolipoprotein; CRMLN, Cholesterol Reference Method Laboratory Network; PAGE, polyacrylamide gel electrophoresis; TC, total cholesterol; HDL-C, HDL-cholesterol; LDL-C (F), LDL-C estimated by Friedewald equation; LDL-C (L), liquid selective detergent method; LDL-C (S), selective solubilization method; LDL-C (E), elimination method; LDL-C (P), enzyme selective protecting method; LDL-C (BQ), modified β-quantification method; LDL-MI, LDL migration index.

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collected in the non-fasting state [5,14]. In most clinical studies investigating the relationship between LDL-C and CHD, including the Framingham Heart Study and the study of the prediction of CHD, LDL-C was estimated by the Friedewald equation [15–17]. In the NCEP-Adult Treatment Panel III study, the recommended level of LDL-C for the prevention of CHD was also estimated by the Friedewald equation. Like the β-quantification method, LDL-C estimated by the Friedewald equation includes cholesterols in lipoprotein (a) and intermediate-density lipoproteins (IDL) [5,7,18], which are atherogenic apolipoproptein (apo) B-containing lipoproteins, and there is a close correlation between the β-quantification method and the Friedewald equation [5,19].

In recent years, convenient homogeneous assays for LDL-C that are less influenced by TG have been developed and are widely performed at clinical laboratories. As mentioned above, LDL-C, which has been shown to be a risk factor for CHD by clinical studies, is estimated by the Friedewald equation, and some studies have stated that LDL-C measured by homogeneous assays for LDL-C need to reflect the current clinical databases for CHD risks as assessed by the Friedewald equation [7]. Clinicians have also expressed the need for a homogeneous assay for LDL-C reflecting LDL-C as estimated by the Friedewald equation [7]. Most homogeneous assays for LDL-C have received CDC certification from the Cholesterol Reference Method Laboratory Network (CRMLN) [12]. Because different homogeneous assays for LDL-C employ different measurement principles, discrepancies have been reported with samples having IDL, lipoprotein (a) and abnormal lipoproteins associated with liver dysfunction, such as lipoprotein X or lipoprotein Y [20–22].

We investigated the correlations between the Friedewald equation and 4 homogeneous assays developed in Japan and to determine whether the homogeneous assays for LDL-C reflect the clinical databases for CHD where LDL-C was estimated by the Friedewald equation. Samples with discrepancies between the Friedewald equation and homogeneous assays for LDL-C were analyzed by polyacrylamide gel electrophoresis (PAGE) and the β-quantification method.

2. Materials and methods

2.1. Samples

With approval from the Ethics Review Board of Osaka University Hospital, human serum samples were from 156 patients (age: 30–65 years) with <4.52 mmol/l (400 mg/dl) TG and from 5 patients (age: 58–75 years) with type 2 diabetes mellitus. Informed consent was obtained from all patients. After 12 h of fasting, blood samples were collected in tubes without anticoagulant. Blood samples were stored at 4 °C and were measured within 3 days using a Hitachi 7170 analyzer.

2.2. Procedures

2.2.1. Total cholesterol (TC), TG and HDL-cholesterol (HDL-C) assays

TC and TG were measured by an enzymatic method (Kyowa Medex) according to the manufacturer's protocols. The CVs for TC and TG were <1.5 and 2%, respectively. HDL-C was measured by a modified enzymatic method (Kyowa Medex) [12] according to the manufacturer's protocols. The CV for HDL-C was <1.5%.

2.2.2. Friedewald equation [9]

Using samples with <4.52 mmol/l (400 mg/dl) TG, LDL-C in the Friedewald equation was estimated using the following formula: [LDL-C (F)] = [TC] − [HDL-C] − [TG]/2.22. HDL-C concentrations in the Friedewald equation were measured by a modified enzymatic method (Kyowa Medex) [12].

Table 1
Lipid levels, PAGE[a] and apoE phenotype of discrepancy samples and samples from diabetic patients.

Sample	Total cholesterol	Triglycerides	PAGE		apo E phenotype
	mmol/l (mg/dl)	mmol/l (mg/dl)	midband[b]	LDL-MI[c]	
Discrepancy samples					
A	4.43 (171)	1.11 (99)	+	0.24	E2/2
B	4.76 (184)	1.42 (127)	+	0.23	E2/2
C	4.60 (178)	0.93 (83)	+	0.31	E2/2
D	6.11 (236)	1.86 (166)	+	0.36	E2/2
E	4.74 (183)	1.36 (121)	+	0.29	E2/2
F	4.45 (172)	1.04 (93)	+	0.31	E2/2
G	5.27 (204)	3.40 (303)	+	0.41	E2/2
H	4.76 (184)	3.38 (302)	+	0.48	E3/3
I	7.86 (304)	4.40 (393)	+	0.40	E3/3
Diabetic patients					
J	5.63 (218)	2.03 (181)	+	0.38	E3/3
K	5.27 (204)	4.18 (373)	+	0.41	E3/2
L	6.92 (268)	6.38 (569)	+	0.44	E3/2
M	4.65 (180)	3.06 (273)	+	0.41	E3/3
N	5.77 (223)	1.36 (121)	+	0.43	E3/3

[a] PAGE, polyacrylamide gel electrophoresis.
[b] When an independent peak was observed between the β and pre-β lipoprotein bands or when a shoulder was observed on the pre-β side of the β lipoprotein, the midband was interpreted as positive.
[c] LDL-MI, LDL migration index. The LDL-MI was obtained by dividing the distance from the LDL peak to VLDL peak (LVp) by the distance from the HDL peak to VLDL peak (HVp), and when LVp/HVp was ≥0.40, the existence of small dense LDL is inferred.

2.2.3. LDL-C homogeneous assays

LDL-C was determined by 4 homogeneous assays [liquid selective detergent method (Sekisui Medical): LDL-C (L), selective solubilization method (Kyowa Medex): LDL-C (S), elimination method (Denka Seiken): LDL-C (E), and enzyme selective protecting method (Wako Pure Chemical): LDL-C (P)] [12]. LDL-C was measured according to the manufacturers' protocols. The CV was <2% for LDL-C (S), <2% for LDL-C (L), <2% for LDL-C (E), and <2% for LDL-C (P).

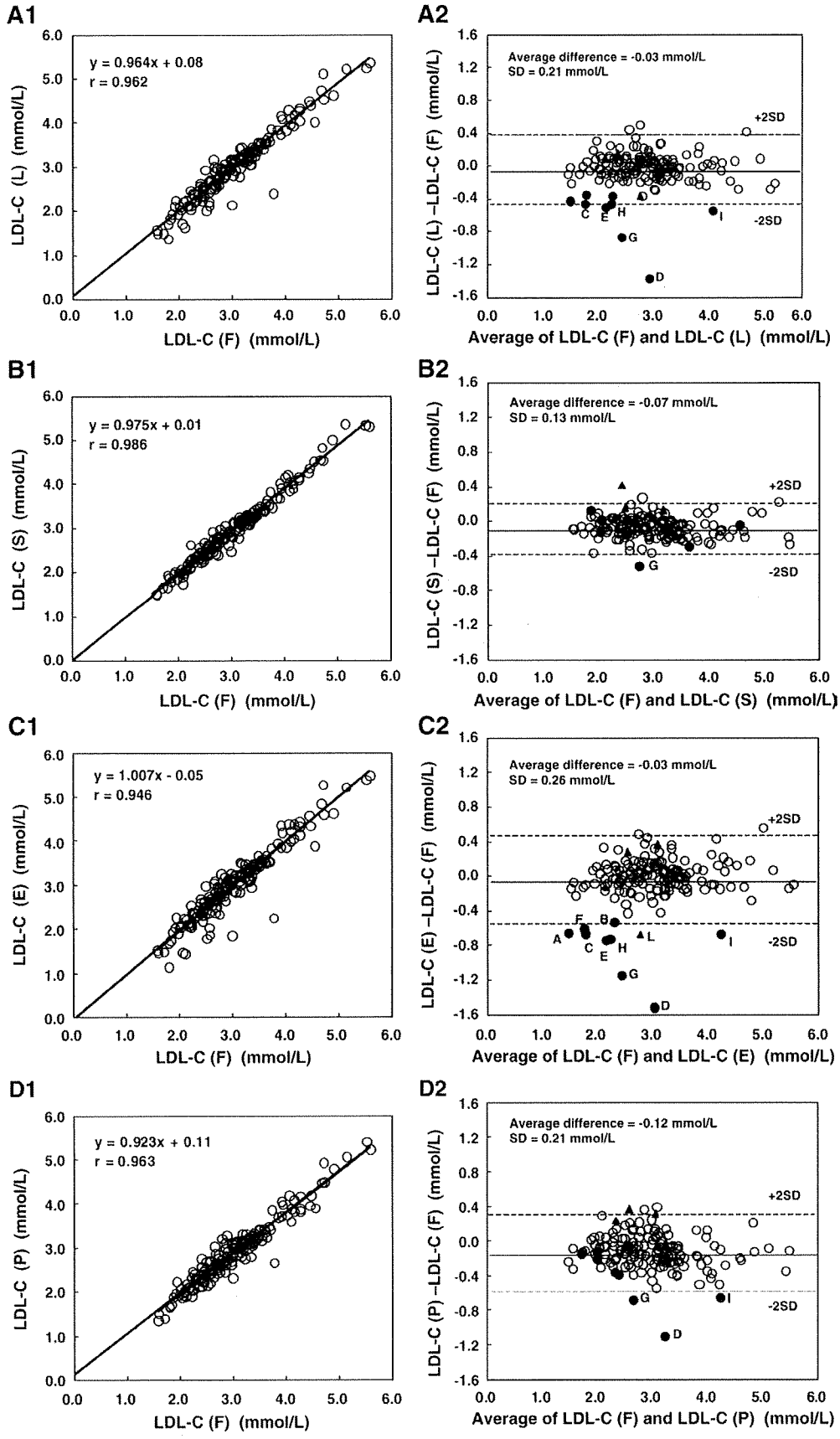
2.2.4. Modified β-quantification method [LDL-C (BQ)]

We employed the method described in the Handbook of Lipoprotein Testing, 2nd ed. [5,6]. After ultracentrifugation at a density of 1.006 kg/l with 0.8 ml serum, the bottom fraction was obtained by the heparin-Mn^{2+} precipitation method, and the TC for the bottom fraction and after fractionation was measured by the enzymatic method (Kyowa Medex). To check the accuracy of the LDL-C (BQ), the values obtained by LDL-C (BQ) were compared with those of the β-quantification method at the CRMLN Laboratory of Osaka Medical Center for Health Science and Promotion in Osaka, Japan, which uses a heparin-Mn^{2+} precipitation with subsequent cholesterol determination by Abell–Kendall method [6], in fasting serum samples from 11 healthy volunteers. LDL-C (BQ) was found to be highly correlated with the β-quantification method of CRMLN (see Supplemental data Table 1).

2.2.5. PAGE and apoE phenotyping

We performed PAGE using Lipophor system (Jokoh) according to the manufacturer's instructions. The lipoprotein bands were

Fig. 1. Correlations between the Friedewald equation and the 4 homogeneous assays for LDL-C. Both the linear regression and the Bland–Altman difference plot are shown. *(A)*, LDL-C (F) vs. LDL-C (L). *(B)*, LDL-C (F) vs. LDL-C (S). *(C)*, LDL-C (F) vs. LDL-C (E). *(D)*, LDL-C (F) vs. LDL-C (P). Fresh fasting serum samples ($n = 160$) including samples from diabetic patients with <4.52 mmol/l TG were used. The closed circles indicate samples being smaller than the lower limit of 2 SD of LDL-C (E), which showed the largest SD among the homogenous assays for LDL-C. The closed triangles indicate samples from diabetic patients.


A1
y = 0.964x + 0.08
r = 0.962
LDL-C (L) (mmol/L)
LDL-C (F) (mmol/L)
A2
Average difference = -0.03 mmol/L
SD = 0.21 mmol/L
+2SD
-2SD
LDL-C (L) –LDL-C (F) (mmol/L)
Average of LDL-C (F) and LDL-C (L) (mmol/L)
B1
y = 0.975x + 0.01
r = 0.986
LDL-C (S) (mmol/L)
LDL-C (F) (mmol/L)
B2
Average difference = -0.07 mmol/L
SD = 0.13 mmol/L
+2SD
-2SD
LDL-C (S) –LDL-C (F) (mmol/L)
Average of LDL-C (F) and LDL-C (S) (mmol/L)
C1
y = 1.007x - 0.05
r = 0.946
LDL-C (E) (mmol/L)
LDL-C (F) (mmol/L)
C2
Average difference = -0.03 mmol/L
SD = 0.26 mmol/L
+2SD
-2SD
LDL-C (E) –LDL-C (F) (mmol/L)
Average of LDL-C (F) and LDL-C (E) (mmol/L)
D1
y = 0.923x + 0.11
r = 0.963
LDL-C (P) (mmol/L)
LDL-C (F) (mmol/L)
D2
Average difference = -0.12 mmol/L
SD = 0.21 mmol/L
+2SD
-2SD
LDL-C (P) –LDL-C (F) (mmol/L)
Average of LDL-C (F) and LDL-C (P) (mmol/L)

densitometrically determined. When an independent peak was observed between the β and pre-β lipoprotein bands or when a shoulder was observed on the pre-β side of the β lipoprotein, the midband was interpreted as positive [23]. LDL particle size was also simply estimated based on an LDL migration index (LDL-MI) [24,25]. The LDL-MI was obtained by dividing the distance from the LDL peak to VLDL peak (LVp) by the distance from the HDL peak to VLDL peak (HVp), and when LVp/HVp was ≥0.40, the existence of small dense LDL is inferred. We identified the apoE phenotype of samples with a Phenotyping ApoE Kit (Jokoh) using the immunoblotting method.

2.2.6. Statistics

Pearson correlation coefficient analysis, simple regression, the Bland–Altman plot [26] and the Boxplot were used to assess the relation between the Friedewald equation, the β-quantification, and the 4 homogenous assays for LDL-C. *P* values were obtained from the Kruskal–Wallis test followed by Dunn's multiple comparison test. Statistical analysis was performed using Excel 2003 (Microsoft) with the Statcel 2 software plug-in [27] or GraphPad Prism software with Windows XP.

3. Results

3.1. Comparison between the Friedewald equation and homogeneous assays for LDL-C

The 4 homogeneous assays for LDL-C were compared with the Friedewald equation in fasting serum samples from 156 patients and 4 diabetic patients with <4.52 mmol/l TG. As shown in Fig. 1, LDL-C (S) was correlated most closely with LDL-C (F), with a correlation coefficient of 0.986 ($P<0.001$), and a regression line of $y=0.975x+0.01$ mmol/l, the extent of agreement decreasing in the order of LDL-C (P) ($r=0.963$, $P<0.001$, $y=0.923x+0.11$ mmol/l) > LDL-C (L) ($r=0.962$, $P<0.001$, $y=0.964x+0.08$ mmol/l) > LDL-C (E) ($r=0.946$, $P<0.001$, $y=1.007x-0.05$ mmol/l). Fig. 1 also shows the Bland–Altman bias plot of the comparison between LDL-C (F) and the 4 homogeneous assays for LDL-C. The SD were 0.21 mmol/l (8.2 mg/dl) for LDL-C (L), 0.13 mmol/l (4.9 mg/dl) for LDL-C (S), 0.26 mmol/l (10.2 mg/dl) for LDL-C (E) and 0.21 mmol/l (7.9 mg/dl) for LDL-C (P). The average differences (*y*-axis) were −0.03 mmol/l (−1.2 mg/dl) for LDL-C (L), −0.07 mmol/l (−2.7 mg/dl) for LDL-C (S), −0.03 mmol/l (−1.0 mg/dl) for LDL-C (E) and −0.12 mmol/l (−4.7 mg/dl) for LDL-C (P). The difference between the Friedewald equation and the homogeneous assays for LDL-C (S) and LDL-C (P) was significantly smaller than that for LDL-C (L) and LDL-C (E) (Fig. 2). It should be noted that the homogenous assays for LDL-C occasionally exhibited lower concentrations of LDL-C than the Friedewald equation. The closed circles in Fig. 1 were the values that are smaller than the lower limit of 2 SD of LDL-C (E), which showed the largest SD among the homogenous assays for LDL-C.

3.2. Analysis by PAGE and identification of apoE phenotypes by immunoblotting of discrepancy samples

The 9 samples with discrepancies (samples A, B, C, D, E, F, G, H and I with closed circles in Fig. 1 and the 5 samples from diabetic patients (samples J, K, L, M and N with closed triangle in Fig. 1) were analyzed by PAGE (Fig. 3), and apoE phenotypes were identified by immunoblotting (Table 1). With samples A through G [TC: 4.43–6.11 mmol/l (171–236 mg/dl), TG: 0.93–3.40 mmol/l (83–303 mg/dl)], a midband was observed and the apoE phenotype in all the seven samples was E2/2, indicating type III hyperlipoproteinemia. With samples G through I [TC: 4.76–7.86 mmol/l (184–304 mg/dl), TG: 3.38–4.40 mmol/l (302–393 mg/dl)], PAGE showed a midband, and their LDL-MI values were ≥0.40, which indicate the existence of small dense LDL [24,25]. With samples J through N [TC: 4.65–6.92 mmol/l (180–268 mg/dl), TG: 1.36–6.38 mmol/l (121–569 mg/dl)], PAGE showed a midband, and the LDL-MI values of samples K through N were ≥0.40. The apoE phenotypes of samples J through N were E3/3 or E3/2.

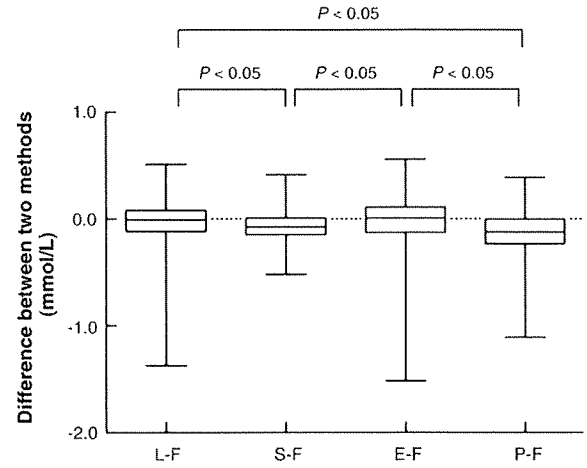


Fig. 2. Boxplots of the difference between the homogeneous assays and the Friedewald equation. Values are represented as median ± interquartile range with upper and lower limits. L − F, the difference between LDL-C (L) and LDL-C (F). S − F, the difference between LDL-C (S) and LDL-C (F). E − F, the difference between LDL-C (E) and LDL-C (F). P − F, the difference between LDL-C (P) and LDL-C (F).

3.3. Analysis by LDL-C (BQ) of discrepancy samples and samples from diabetic patients

The 7 type III hyperlipoproteinemia samples (samples A through G) and the five samples from diabetic patients (samples J through N) were analyzed by LDL-C (BQ). Fig. 4 shows the Bland–Altman bias plot of the comparison between LDL-C (BQ) and 4 homogeneous assays for LDL-C. Judging by the average difference and SD, LDL-C (S) [average difference 0.08 mmol/l (3.1 mg/dl), SD 0.18 mmol/l (7.0 mg/dl)] mostly agreed with LDL-C (BQ) as well as LDL-C (F) [average difference 0.06 mmol/l (2.3 mg/dl), SD 0.23 mmol/l (9.0 mg/dl)], the extent of agreement decreasing in the order of LDL-C (P) [average difference −0.06 mmol/l (−2.5 mg/dl), SD 0.28 mmol/l (10.9 mg/dl)] > LDL-C (L) [average difference −0.27 mmol/l (−10.4 mg/dl), SD 0.33 mmol/l (12.8 mg/dl)] > LDL-C (E) [average difference −0.40 mmol/l (−15.4 mg/dl), SD 0.48 mmol/l (18.5 mg/dl)]. Of the 5 methods tested, the difference from the β-quantification method for LDL-C (S) and the Friedewald equation was significantly smaller than that for LDL-C (E) (Fig. 5). It should be noted that downward deviations in LDL-C (L) and LDL-C (E) against LDL-C (BQ) were observed for the samples from the patients with type III hyperlipoproteinemia, unlike those from the diabetic patients (Fig. 4).

4. Discussion

In most clinical studies investigating the relationship between LDL-C and CHD, LDL-C was estimated by the Friedewald equation or the β-quantification method [15–17]. Although LDL-C is generally considered to be a major risk factor for progression of atherosclerosis, the traditional measurement of LDL-C includes measurement of IDL [18]. Like the β-quantification method, LDL-C estimated by the Friedewald equation includes cholesterols in lipoprotein (a) and IDL [5,7,18], which are atherogenic apoB-containing lipoproteins. In recent years, homogeneous assays for LDL-C have been widely used, but when referring to the clinical databases for CHD risks, as assessed based on LDL-C estimated by the Friedewald equation or the β-quantification method, homogeneous assays for LDL-C should reflect

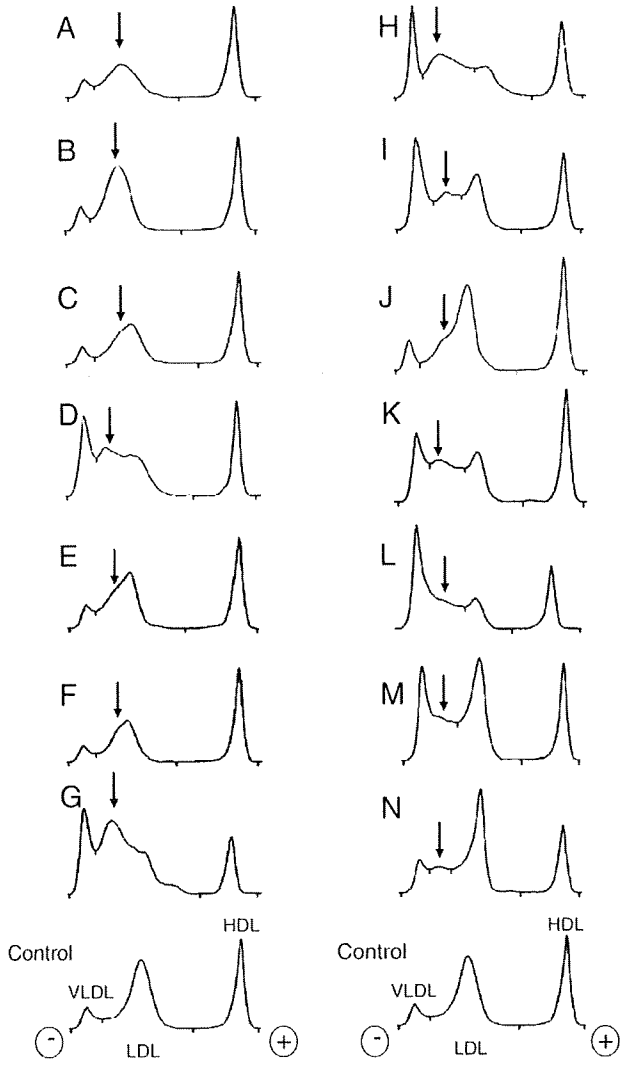


Fig. 3. Densitometric patterns of PAGE (polyacrylamide gel electrophoresis) of discrepancy samples and samples from diabetic patients. Control, normal serum. (A), sample A. (B), sample B. (C), sample C. (D), sample D. (E), sample E. (F), sample F. (G), sample G. (H), sample H. (I), sample I. (J), sample J. (K), sample K. (L), sample L. (M), sample M. (N), sample N.; downward closed arrows, location of midband.

the LDL-C measurements from the Friedewald equation or the β-quantification method [7]. We investigated the correlations between the Friedewald equation and 4 homogeneous assays developed in Japan to determine whether the homogeneous assays for LDL-C reflect the clinical databases for CHD where LDL-C was estimated by the Friedewald equation.

Using 156 fasting serum samples and 4 samples from diabetic patients with <4.52 mmol/l (400 mg/dl) TG, the correlations between the Friedewald equation and the 4 homogeneous assays for LDL-C were investigated because the Friedewald equation cannot be used to estimated LDL-C in samples with ≥4.52 mmol/l (400 mg/dl) TG [5,14]. Of the homogenous assays evaluated, LDL-C (S) was correlated most closely with LDL-C (F). However, the homogenous assays for LDL-C occasionally exhibited lower concentrations of LDL-C than the Friedewald equation (Fig. 1). When the samples showing lower concentrations were analyzed by PAGE, a large amount of midband was detected in samples A through G, indicating increased concentrations of IDL (Fig. 3) [23]. Furthermore, when these samples were analyzed by immunoblotting, apoE2/2 was identified (Table 1). It was suggested that samples A through G are from patients with type III hyperlipoproteinemia, indicating increased levels of IDL or β-VLDL [28,29]. When samples G through I were analyzed by PAGE, a midband was detected (Fig. 3), and their LDL-MI values were ≥0.40 (Table 1) [24,25]. This suggests that samples G through I contain a large volume of small dense LDL. When samples J through N from diabetic patients were analyzed by PAGE, a midband was detected (Fig. 3), and the LDL-MI values of sample K through N were ≥0.40 (Table 1), suggesting that the samples contain IDL and small dense LDL.

LDL-C (F) and the 4 homogeneous assays for LDL-C were compared with LDL-C (BQ), using 7 samples from the patients with type III hyperlipoproteinemia (samples A through G) and 5 samples from diabetic patients (samples J through N). Of the method tested, LDL-C (S) and the Friedewald equation mostly agreed with LDL-C (BQ), whereas a consistent mean negative bias of LDL-C (E), LDL-C (L) and LDL-C (P) against LDL-C (BQ) was observed more apparent in the Bland–Altman plot (Fig. 4). In the samples, sample D exhibited the largest difference among the measurements methods. LDL-C (S) mostly agreed with LDL-C (BQ) in sample D, whereas LDL-C (F) exhibited slightly higher values and other LDL-C homogeneous measurements exhibited markedly lower values. In general, using type III hyperlipoproteinemia samples, upward deviations in the Friedewald equation against LDL-C (BQ) have been reported [5,14]. Nevertheless, in the present study, there was no significant difference between the 2 methods (Fig. 4). Based on the medical records of the patients (A to F) with type III hyperlipoproteinemia, these patients were managed with lipid-lowering drugs, such as fenofibrate or bezafibrate, and the serum concentrations of TC and TG in the patients were maintained at low levels. In such a situation, upward deviations in the Friedewald equation against the LDL-C (BQ) might not be able to be caused even in the patients with type III hyperlipoproteinemia.

As shown in Figs. 1 and 4, marked downward deviations were observed in LDL-C (L) and LDL-C (E) methods against the Friedewald equation and β-quantification method, especially in the samples from the patients with type III hyperlipoproteinemia, not in those from diabetic patients. The composition or amounts of IDL and triglyceride-rich lipoprotein remnants in the samples from the patients with type III hyperlipoproteinemia seem to differ from those from the diabetic patients. In fact, the cholesterol content and the cholesterol–triglycerides ratio in IDL from the patients with type III hyperlipoproteinemia are reported to be larger than those from the diabetic patients [30,31]. Such a difference seems to be reflected in the difference in the reactivities for the homogenous assays for LDL-C [17].

In this study, 4 homogeneous assays with different measurement principles were used for LDL-C [LDL-C (L), LDL-C (S), LDL-C (E), and LDL-C (P)] [12]. However, the measurement principles for the homogeneous assays for LDL-C can be divided into two groups: 1) method I in which cholesterols in lipoproteins, other than LDL, are eliminated in the first reaction and cholesterol in LDL is detected in the second reaction [LDL-C (L), LDL-C (E) and LDL-C (P)]; and 2) method II in which LDL is selectively modified by surfactants, making its component cholesterol available for enzymatic assay [LDL-C (S)]. In type III hyperlipoproteinemia, the results tended to be low for method I [LDL-C (L), LDL-C (E) and LDL-C (P)] (Fig. 4B, D and E). With the method I, cholesterols in HDL, VLDL and chylomicron are eliminated in the first reaction, and LDL-C is measured in the second reaction. Unlike LDL, IDL from the patients with type III hyperlipoproteinemia has not only apoB but also apoE and apoCs, which are relatively hydrophilic. Therefore, the surface structure of such lipoproteins differs from that of the LDL in terms of charge, while the apolipoprotein composition of such lipoproteins is similar to that of the VLDL [32]. In the case of samples with higher IDL levels from the patients with type III hyperlipoproteinemia, therefore, with the method I some portion of cholesterol in such IDL may also be eliminated

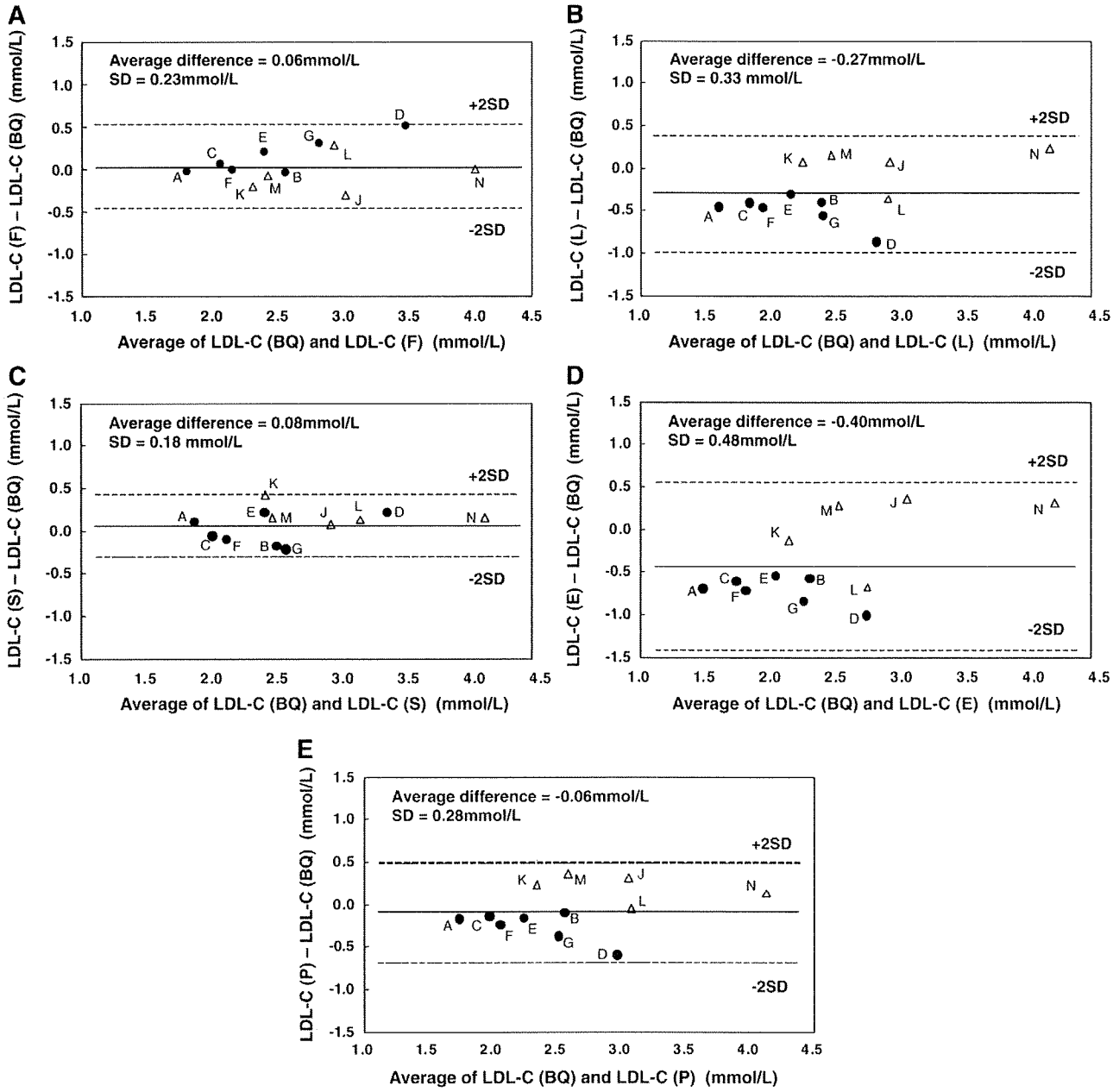


Fig. 4. Correlations between LDL-C (BQ), LDL-C (F) and 4 homogeneous assays for LDL-C in discrepancy samples and samples from diabetic patients. The Bland–Altman difference plot is shown. (A), LDL-C (BQ) vs. LDL-C (F). (B), LDL-C (BQ) vs. LDL-C (L). (C), LDL-C (BQ) vs. LDL-C (S). (D), LDL-C (BQ) vs. LDL-C. (E), LDL-C (BQ) vs. LDL-C (P). Seven type III hyperlipoproteinemia samples (samples A, B, C, D, E, F and G with closed circles) and five samples from diabetic patients (samples J, K, L, M and N with open triangles) were used.

during the first reaction. Accordingly, LDL-C measurements with the method I may lead to lower values than those of the β-quantification method and the Friedewald equation. With the method II, it has been reported that the nonionic surfactant, polyoxyethylene-polyoxypropylene block copolyether, may be able to recognize differences in hydrated density, net charge, or size of the various lipoprotein fractions [33]. It is most likely that such specific surfactant used in LDL-C (S) method shows the specificity toward IDL from the patients with type III hyperlipoproteinemia as well as LDL. On the other hand, Miller et al. [34] reported that the selective solubilization method for LDL-C measurement, corresponding to LDL-C (S) in this study, gave the big difference against the β-quantification method with the specimens from type III hyperlipoproteinemia. In this study, we used a currently commercially available reagent purchased from the manufacturer, in which the non-specific reactivity toward cholesterol in triglyceride-rich lipoprotein remnants from type III hyperlipoproteinemia are sure to be improved. It has been reported that IDL concentrations increase in serum samples from patients with type III hyperlipoproteinemia, diabetic mellitus [35], or myocardial infarction [23]. Furthermore, accumulating evidence indicates that IDL is associated with the presence, severity, and progression of coronary artery atherosclerosis [18,36,37]. In serum samples from the patients with increased IDL from type III hyperlipoproteinemia, the method I may

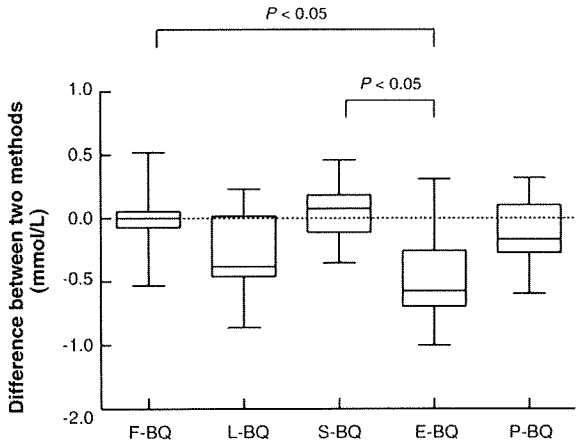


Fig. 5. Boxplots of the difference between LDL-C (BQ), LDL-C (F) and 4 homogeneous assays. Values are represented as median ± interquartile range with upper and lower limits. F − BQ, the difference between LDL-C (BQ) and LDL-C (F). L − BQ, the difference between LDL-C (L) and LDL-C (BQ). S − BQ, the difference between LDL-C (S) and LDL-C (BQ). E − BQ, the difference between LDL-C (E) and LDL-C (BQ). P − BQ, the difference between LDL-C (P) and LDL-C (BQ).

underestimate the LDL-C values compared with the Friedewald equation and the β-quantification method.

On the other hand, in this study, the significant differences among LDL-C methods have not been observed in serum samples from the diabetic patients with increased IDL. Further studies are needed to clarify such differential reactivity of IDL from type III hyperlipoproteinemia and diabetic patients in LDL-C homogeneous assays.

Using some samples with higher small dense LDL, the homogeneous assays for LDL-C exhibited lower values compared to LDL-C (F) (Fig. 1). Compared to normal LDL, small dense LDL has a different overall three-dimensional structure of apoB and immunoreactivity at the apoB receptor recognition domain [38]. In small dense LDL, being smaller than LDL, there is significant loss of phospholipids from the surface, loss of cholesterol esters from the core, gain of TG into the core, and most importantly, loss of free cholesterol from both the surface and the core [39]. Furthermore, compared to normal size LDL, small dense LDL has intrinsically increased susceptibility to LDL oxidation and aggregation [40,41]. As described above, small dense LDL is different from normal LDL in terms of structure and the property on lipoprotein. Therefore, unlike LDL, small dense LDL might not be fully recognized to the LDL-C homogeneous assays, thereby resulting in lower estimations of LDL-C. Further studies must examine the reactivity to small dense LDL in LDL-C homogeneous assays.

When investigating the correlations of the 4 homogenous assays for LDL-C to the Friedewald equation, LDL-C (S), which is selective solubilization method for LDL-C, correlated most closely to the Friedewald equation and even with type III hyperlipoproteinemia samples, a favorable correlation was observed between LDL-C (S) and LDL-C (BQ), thus suggesting that LDL-C (S) closely reflects the clinical databases for CHD where LDL-C was estimated by the Friedewald equation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cca.2009.09.010.

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Hormone-sensitive lipase deficiency suppresses insulin secretion from pancreatic islets of *Lep*$^{ob/ob}$ mice

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ABSTRACT

It has long been a matter of debate whether the hormone-sensitive lipase (HSL)-mediated lipolysis in pancreatic β-cells can affect insulin secretion through the alteration of lipotoxicity. We generated mice lacking both leptin and HSL (*Lep*$^{ob/ob}$/*HSL*$^{-/-}$) and explored the role of HSL in pancreatic β-cells in the setting of obesity. *Lep*$^{ob/ob}$/*HSL*$^{-/-}$ developed elevated blood glucose levels and reduced plasma insulin levels compared with *Lep*$^{ob/ob}$/*HSL*$^{+/+}$ in a fed state, while the deficiency of HSL did not affect glucose homeostasis in *Lep*$^{+/+}$ background. The deficiency of HSL exacerbated the accumulation of triglycerides in *Lep*$^{ob/ob}$ islets, leading to reduced glucose-stimulated insulin secretion. The deficiency of HSL also diminished the islet mass in *Lep*$^{ob/ob}$ mice due to decreased cell proliferation. In conclusion, HSL affects insulin secretary capacity especially in the setting of obesity.

Introduction

Obesity and type 2 diabetes represent a growing threat to the health of the population of almost every country in the world. Accumulation of lipids in non-adipose tissues can lead to cell dysfunction, a phenomenon known as lipotoxicity as a molecular link between obesity and glucose homeostasis dysregulation [1]. Pancreatic β-cells are known to be highly susceptible to lipotoxicity, and both exogenous and endogenous sources of free fatty acids (FFAs) are believed to be involved in insulin secretory machinery [2,3]. The liberation of FFA by intracellular hydrolysis of acylglycerols, which is referred to as lipolysis, is observed in the pancreatic islets [4,5], and the lipolytic reaction supplies the endogenous FFA.

Hormone-sensitive lipase (HSL) is an intracellular neutral lipase that is capable of hydrolyzing triglycerides (TGs), diglycerides, monoglycerides, and cholesterol esters as well as other lipids [6–9]. Although HSL was initially identified as an adipose-specific lipase, it has been clarified that HSL is also expressed and functions in a wide variety of organs and cells, including heart, skeletal muscle, adrenal glands, testes, ovaries, intestines and liver [7,8,10,11]. HSL was also identified in pancreatic β-cells [12], and several laboratories reported the relationship between the HSL-mediated lipolysis and insulin secretory machinery using rodent models without coming to consensus [13–17].

We reported the phenotypes caused by the combined deficiency of leptin and HSL (*Lep*$^{ob/ob}$/*HSL*$^{-/-}$) to explore the role of HSL in the setting of obesity [18]. Here we further explored the potential role of HSL in the insulin secretion using the leptin-deficient model. *Lep*$^{ob/ob}$/*HSL*$^{-/-}$ showed elevated plasma glucose levels and decreased plasma insulin levels compared with *Lep*$^{ob/ob}$/*HSL*$^{+/+}$ in a fed state, while the deficiency of HSL did not affect both plasma glucose levels and plasma insulin levels in a fasted state. In the Lep$^{+/+}$ background, the glucose homeostasis was not affected by HSL. The impaired insulin secretion of *Lep*$^{ob/ob}$/*HSL*$^{-/-}$ was further supported by glucose-stimulated insulin secretion (GSIS) from isolated islets. In addition, the deficiency of HSL decreased the enlarged islet mass in *Lep*$^{ob/ob}$ mice due to decreased cell proliferation.

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Materials and methods

Animals. $Lep^{+/+}/HSL^{-/-}$ and $Lep^{ob/ob}/HSL^{-/-}$ mice were generated, and genotyping was performed as described previously [18,19]. Mice were housed in a temperature-controlled environment with a 12-h light/dark cycle and allowed free access to water and a standard chow diet (Oriental MF, Oriental Yeast, Tokyo, Japan). Mice were euthanized at 16 weeks after a 6-h fast. Blood was collected from the retro-orbital venous plexus after a 6-h fast (fasted state, at 8 and 16 weeks of age) or at the beginning of the light cycle (fed state, at 9 and 15 weeks of age). Plasma glucose was measured by ANTSENSE II (Bayer Medical, Tokyo, Japan), and plasma insulin was measured by the mouse insulin ELISA kit (Morinaga, Tokyo, Japan).

Pancreatic islets isolation and insulin secretion experiment. Islet isolation was carried out using the collagenase technique from 16-week-old non-fasted mice. In brief, 2.5 ml of collagenase (Type XI, Sigma, St. Louis, MO) solution at 4 mg/ml were introduced into the common bile duct after occlusion of the distal end just proximal to the duodenum. The distended pancreas was excised and the digestion was performed in a water bath at 37 °C for 3.5 min. The islets were washed and purified with a hand pick-up. Glucose stimulation tests were performed on isolated pancreatic islets essentially according to Sutton et al. [20]. Six groups (each group comprising 8 islets) were prepared from each mouse and three groups were used for low glucose (2.8 mM) stimulation and the other three were for high glucose (20 mM) stimulation. After preincubation at 37 °C for 30 min in KRBH buffer (130 mM NaCl, 5.2 mM KCl, 1.3 mM KH_2PO_4, 2.7 mM $CaCl_2$, 1.3 mM $MgSO_4$, 24.8 mM $NaHCO_3$, and 10 mM Hepes at pH 7.4) containing 2.8 mM glucose and 0.5% bovine serum albumin (BSA; Fraction V; Sigma, St. Louis, MO), groups of islets were incubated at 37 °C in KRBH containing 0.5% bovine serum albumin and either 2.8 mM glucose or 20 mM glucose for 30 min, then the media were withdrawn for insulin measurement. Lipids were extracted from ~100 islets/each mouse by chloroform–methanol method and TG content was measured by a kit (GPO-Trinder, Sigma, St. Louis, MO) (n = 8–11). Lipid extracted islets were dissolved in phosphate buffered saline by sonication. DNA content was measured by fluorescent method with Hoechst 33258 [21]. The size of islets under these experiments was controlled visually to be similar between $HSL^{+/+}$ and $HSL^{-/-}$ group.

Histology and quantification of islet mass. Sixteen-week-old mice were euthanized, and the pancreases were excised, fixed in 10% neutral buffered formalin, embedded in paraffin, and stained with hematoxylin–eosin. Three non-sequential sections from each pancreas were scanned (three mice for each genotype) by NIH image. At least 50 islets from each pancreas were quantified and total number of islets scanned was from 195 to 260 for each genotype.

Proliferating cells were detected by bromodeoxyuridine (BrdU) (Sigma, St. Louis, MO) immunostaining. Two hours after administration of BrdU intraperitoneally at 50 mg/kg body weight in phosphate buffered saline, mice were euthanized, and the pancreases were excised and embedded in paraffin. Immunostaining was performed by a BrdU *in situ* detection kit (PharMingen, San Diego, CA) and counterstained with Mayer's hematoxylin (Wako Pure Chemicals). Apoptotic cells were detected by terminal deoxynucleotidyl-transferase-mediated dUTP nick end-labeling (TUNEL) using an *in situ* apoptosis detection kit (Takara Biomedicals, Otsu, Japan) according to the manufacturer's instructions, with counterstaining with methyl green (Wako Pure Chemicals).

Statistics. Statistical differences between groups were analyzed by the Student's t-test or the one-way analysis of variance and a *post hoc* Tukey–Kramer test, unless otherwise stated.

Results and discussion

Levels of plasma insulin and glucose suggested the impaired insulin secretion in $Lep^{ob/ob}/HSL^{-/-}$ mice

To gain insight into the physiological roles of HSL especially in the setting of obesity, we generated $Lep^{ob/ob}/HSL^{-/-}$ mice. In this study, we attempted to characterize the roles of HSL in the pancreatic β-cells in the setting of obesity. In these mice, fasting plasma glucose and insulin levels were not affected by the HSL deficiency in both $Lep^{+/+}$ and $Lep^{ob/ob}$ backgrounds (Fig. 1). The results suggested the limited contribution of HSL to the whole-body insulin sensitivity, and in support of this finding, intraperitoneal insulin tolerance test was not affected by the HSL deficiency (data not shown). On the other hand, $Lep^{ob/ob}/HSL^{-/-}$ mice showed elevated plasma glucose levels (26% increase for males and 72% increase for females) and decreased plasma insulin levels (45% decrease for males and 53% decrease for females) compared with $Lep^{ob/ob}/HSL^{+/+}$ mice under fed conditions (Fig. 1), which suggested impaired insulin secretion in $Lep^{ob/ob}/HSL^{-/-}$ mice. In the $Lep^{+/+}$ background, there was no significant difference between $Lep^{+/+}/HSL^{+/+}$ and $Lep^{+/+}/HSL^{-/-}$ (Fig. 1). Since it was reported that the impaired insulin secretion of $HSL^{-/-}$ mice was age- and gender-dependent [15], we examined both gender at two time points (8 weeks and 16 weeks for fasted conditions, and 9 weeks and 15 weeks for fed conditions. Data shown are representative of the two time points. It is also known that almost all the gender differences disappear in $Lep^{ob/ob}$ mice). The $Lep^{ob/ob}/HSL^{-/-}$ had defective insulin secretion even at an earlier age. We previously reported that the food intake was reduced in $Lep^{ob/ob}/HSL^{-/-}$ mice [18], and the impaired insulin secretion of $Lep^{ob/ob}/HSL^{-/-}$ might be underestimated under free-feeding conditions. In accordance with the impaired insulin secretion, the hepatic expression of gluconeogenetic enzymes such as phospho-enol-pyruvate carboxykinase (PEPCK) was reduced in $Lep^{ob/ob}/HSL^{-/-}$ (data not shown).

Islets isolated from $Lep^{ob/ob}/HSL^{-/-}$ mice displayed a decreased GSIS response

To eliminate the indirect effect, we isolated islets from each genotype, and GSIS assay was performed. In $Lep^{ob/ob}$ background, the response of $Lep^{ob/ob}/HSL^{-/-}$ islets to high concentration of glucose was significantly reduced by 36% compared with that of $Lep^{ob/ob}/HSL^{+/+}$ islets (Fig. 2). In $Lep^{+/+}$ background, the deficiency of HSL caused a milder decrease in GSIS, which did not reach statistical significance. The discrepancy between the earlier report [13] and our results needs to be studied further.

The deficiency of HSL led to marked accumulation of TG in $Lep^{ob/ob}$ islets

Leptin deficiency may confer vulnerability to the lipotoxicity, because leptin causes TG depletion in islets via stimulating β-oxidation [22]. Furthermore leptin-treatment of $Lep^{ob/ob}$ mice is known to reverse their glucose intolerance and is reported to upregulate HSL expression in islets [23], which imply protective function of HSL against lipotoxicity. Therefore, we examined whether and to what extent the deficiency of HSL influenced the islet TG content. There was a striking increase (78%) in islet TG content in $Lep^{ob/ob}/HSL^{-/-}$ (Fig. 3A and C). The size of islets used for the GSIS experiment and lipid extraction was controlled visually to be similar between $HSL^{+/+}$ and $HSL^{-/-}$ group. To assure the precision, islet DNA content was measured fluorometrically (Fig. 3B), and the marked accumulation of islet TG in $Lep^{ob/ob}/HSL^{-/-}$ was also supported by the data normalized with respect to DNA content

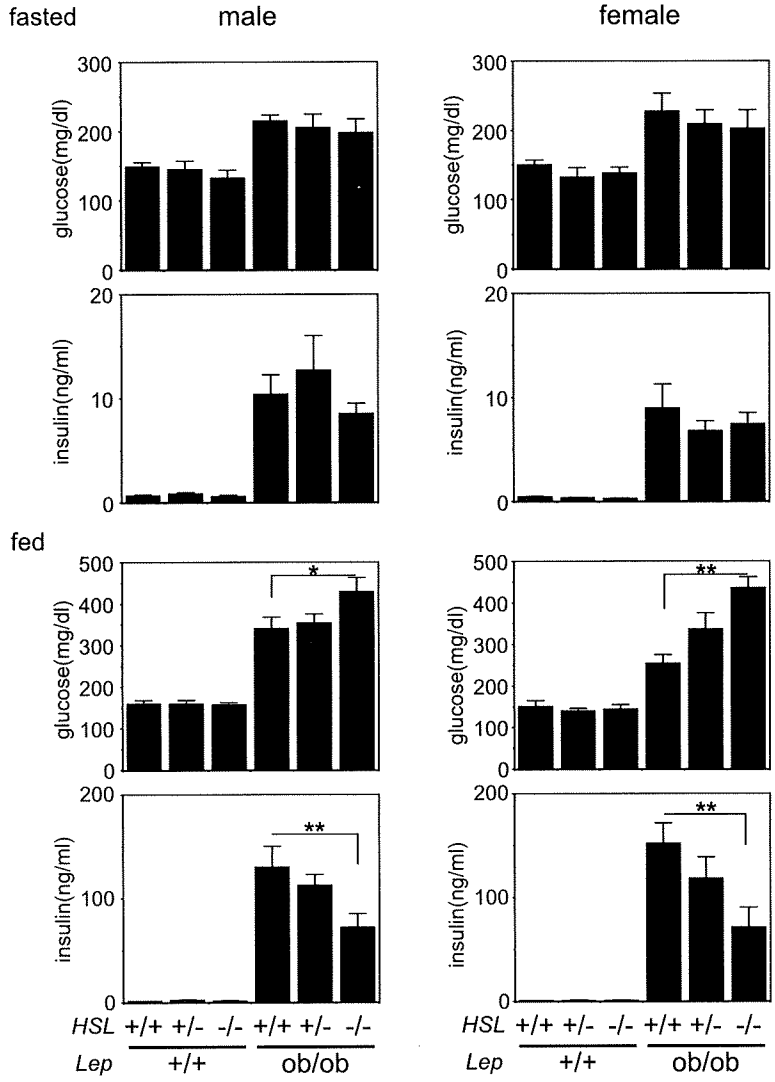

Fig. 1. *Lep*$^{ob/ob}$/*HSL*$^{-/-}$ mice exhibited impaired insulin secretion *in vivo*. Blood samples were collected from either fasted (after a 6-h fast, at the age of 8 weeks, displayed in the upper four panels) or fed (at the beginning of light cycle, at the age of 15 weeks, displayed in the lower four panels) mice (n = 10). Each value represents the mean ± SE. *And **denote significance at $P < 0.05$ and $P < 0.01$, respectively, vs *HSL*$^{+/+}$ mice in the same *Lep* background determined by ANOVA followed by the Tukey–Kramer *post hoc* test. Similar pattern was observed in both younger (at the age of 8–9 weeks) and adult (at the age of 15–16 weeks) mice, and data shown are representative of the two time points.

(Fig. 3C). On the other hand, there was no significant difference between *Lep*$^{+/+}$/*HSL*$^{+/+}$ and *Lep*$^{+/+}$/*HSL*$^{-/-}$, which is inconsistent with previous report [13]. Although it is necessary to further elucidate whether or not there might be a difference that was undetectable by our methods between *Lep*$^{+/+}$/*HSL*$^{+/+}$ and *Lep*$^{+/+}$/*HSL*$^{-/-}$, the contribution of HSL to islet TG store was certainly clarified by the co-ablation of the leptin gene. Since excessive TG accumulation in islets is reported to require hyperglycemic background [24], HSL deficiency might induce lipotoxic impairment of insulin secretion only in the *Lep*$^{ob/ob}$ background.

Islet mass was significantly reduced in Lep$^{ob/ob}$/*HSL*$^{-/-}$

Examination of pancreatic sections revealed that the islet mass was reduced by 25% in *Lep*$^{ob/ob}$/*HSL*$^{-/-}$ compared with *Lep*$^{ob/ob}$/*HSL*$^{+/+}$, whereas there was no significant difference between *Lep*$^{+/+}$/*HSL*$^{+/+}$ and *Lep*$^{+/+}$/*HSL*$^{-/-}$ (Fig. 4A). Previous reports which addressed the reduced insulin secretory capacity in HSL deficient mice showed no specific data regarding islet mass [13,15], while Holm and her colleagues reported the increased islet mass in HSL deficient mice accompanied by the whole-body insulin resistance [16]. Taken together with the findings in previous reports, the role of HSL in islet function would be limited, and the experiments using HSL deficient mice could sometimes produce confusing results. Our investigation using co-ablation of leptin gene would facilitate our understanding of the role of HSL in pancreatic β-cells. The combined effects of the reduction of insulin secretion per same size islet and reduced islet mass in *Lep*$^{ob/ob}$/*HSL*$^{-/-}$ would impair insulin secretion *in vivo*.

Reduction in islet mass in Lep$^{ob/ob}$/*HSL*$^{-/-}$ *occurred via defective cell proliferation*

To determine whether the decreased islet mass is due to defective proliferation or increased apoptosis, pancreatic sections were analyzed by immunohistochemistry for apoptosis by TUNEL

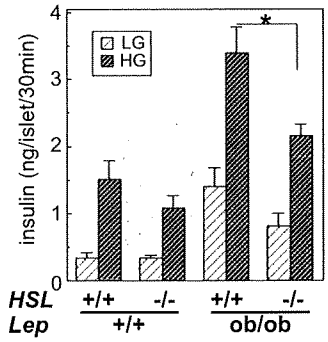

Fig. 2. Glucose-stimulated insulin secretion (GSIS) from $Lep^{ob/ob}/HSL^{-/-}$ islets was impaired. Six groups (each group comprising 8 islets) were prepared from each mouse and three groups were used for low glucose (LG, 2.8 mM) stimulation and the other three were used for high glucose (HG, 20 mM) stimulation ($n = 5$ for each genotype). After preincubation at 37 °C for 30 min in KRBH containing 2.8 mM glucose, groups of islets were incubated at 37 °C in KRBH containing either 2.8 mM glucose or 20 mM glucose for 30 min, then the media were withdrawn for insulin measurement. Each value represents the mean ± SE. *Denotes significance at $P < 0.05$, vs $HSL^{+/+}$ mice in the same *Lep* background determined by the Student's *t*-test.

(Fig. 4B) and for cell proliferation by BrdU incorporation (Fig. 4C). No TUNEL positive nuclei were detected in pancreatic sections of all genotypes. On the other hand, BrdU incorporation study revealed that increased proliferative activity of $Lep^{ob/ob}/HSL^{+/+}$ islets completely disappeared in $Lep^{ob/ob}/HSL^{-/-}$ islets. The mechanism of upregulated proliferation of islet cells of $Lep^{ob/ob}$ mice is not established [25], but this is believed to be due to the adaptation to the increased insulin demand [26]. The promoted islet cell proliferation in $Lep^{ob/ob}/HSL^{+/+}$ was completely abolished and no BrdU positive cells were detected in the sections of $Lep^{ob/ob}/HSL^{-/-}$ as well as $Lep^{+/+}/HSL^{+/+}$ and $Lep^{+/+}/HSL^{-/-}$. Although the precise mechanism should be elucidated, cAMP/cAMP-dependent protein kinase A (PKA) pathway might be involved. Glucagon-like peptide (GLP)-1 increases cell survival via cAMP-dependent stimulation of cAMP response element binding protein (CREB) activity, and subsequent enhancement of the insulin receptor substrate (IRS)-2 regulated pathway [27]. The decreased feeding behavior in $Lep^{ob/ob}/HSL^{-/-}$ might reduce the serum Glucagon-like peptide (GLP)-1 levels.

The long-term exposure to FFA leads to disruption of GSIS [28], and genetic deletion of HSL decreases plasma FFA levels mobilized from adipose tissue in both $Lep^{+/+}$ and $Lep^{ob/ob}$ background. Since deficiency of HSL raises numerable changes caused from phenotype of adipose tissue or the other tissues, tissue-specific genetic deletion will be required to further determine the precise role of HSL on pancreatic β-cells.

It was reported that lipids generated by acylglycerol hydrolysis play a critical role in insulin secretion using a chemical inhibition of β-cell lipolysis [4]. However, it still remained to be determined whether the lipase(s) responsible for the regulation of insulin secretion would be HSL or the other lipase(s). It was reported recently that adipose triglyceride lipase is another promising candidate [29]. In the present study, we demonstrated that HSL is at least one of the key molecules involved in the insulin secretory machinery.

In summary, we clarified the role of HSL in pancreatic β-cells by the co-ablation of leptin gene. It was demonstrated that the deficiency of HSL in $Lep^{ob/ob}$ mice causes impaired insulin secretion and reduces the islet mass due to the defective cell proliferation. These findings provide the basis for understanding the pathophysiology of obesity and diabetes.

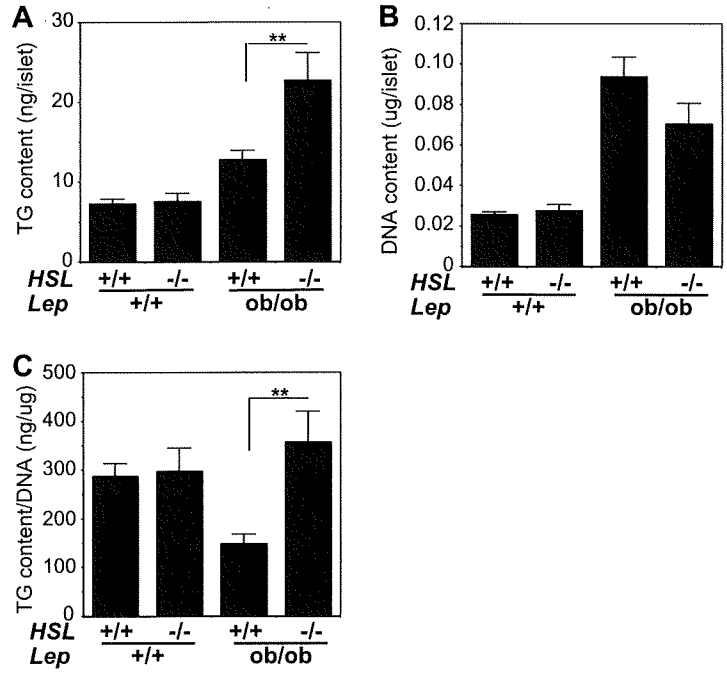

Fig. 3. Excessive triglyceride accumulation was observed in $Lep^{ob/ob}/HSL^{-/-}$ islets ($n = 9$–11). (A) Lipids were extracted from ~100 islets/each mouse by the chloroform–methanol method. (B) Lipid extracted islets were dissolved in phosphate buffered saline by sonication, and the DNA content was measured by fluorescent method. (C) TG content of islets was normalized with respect to DNA content. Each value represents the mean ± SE. **Denotes significance at $P < 0.01$, vs $HSL^{+/+}$ mice in the same *Lep* background determined by the Student's *t*-test.

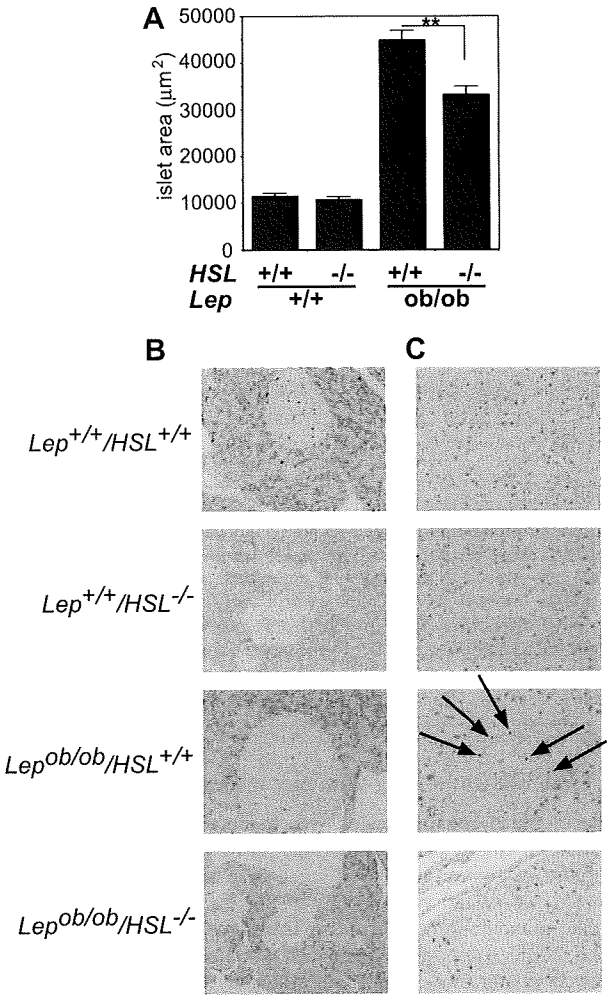

Fig. 4. The deficiency of HSL reduced the pancreatic islet mass of $Lep^{ob/ob}$ mice. (A) Islet mass was quantified using NIH image. At least 50 islets from each pancreas were quantified and total number of islets scanned was 195–260 for each genotype ($n = 3$). Each value represents the mean ± SE. **Denotes significance at $P < 0.01$, respectively, vs $HSL^{+/+}$ mice in the same *Lep* background determined by the Student's *t*-test. (B, C) Immunohistochemical analysis of islets. (B) TUNEL staining and (C) BrdU labeling. Arrows point to the labeled cells (C).

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Geriatrics in the most aged country, Japan

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ABSTRACT

The aging of society is a common problem in many developed countries. To tackle the problems related to an aging society, the role of geriatricians, as well as government support, is becoming more and more important. There is need to recruit young physicians with the skills required to care for elderly patients, and to establish an education system which encourages more young physicians to undertake training in geriatrics. Additionally, there is a need for improvement of our insurance systems to support such care. Our approach to the aging society will pave the way for other countries in Asia and the west.

1. Aging of society in Japan

The aging of society is a phenomenon affecting many developed countries in the west and Asia (Khaw, 1999; Imuta et al., 2001). Among them, Japan has the highest proportion of over-65s in the world: 22.1% in 2008. Further, 10.4% of the total population is over 75, making Japan the first country to have over 10% of the population in this very elderly range. In addition, we have also the longest average life span in the world: the 2008 life span being approximately 79 and 86, in men and women respectively. The aging rate will continue to rise in the future, yet fewer babies are born each year. Accordingly, Japan is inexorably aging. Our population has already peaked just short of 130 million, and we will face a population decline on a scale unprecedented in the developed world during this century. Although we have obtained this astonishing longevity, relatively inexpensively and within a short period of time, compared with other countries, we are facing a lot of problems associated with aging of our society, such as shortage of health-care professionals. Another problem is the increase of people with cognitive decline or dementia. The number of such people is estimated to be approximately 2 million and will definitely increase in the future (Meguro et al., 2007; Ikejima et al., 2009). Therefore, the support for dementia patients and their family becomes more and more important.

Our economic growth rate and technological achievements held the world in awe after World War II. However, in the future, Asian, as well as Western countries should watch Japan for another reason. The choices we make can affect the way in which the rest of the aging world tackles the same problem in years to come. Therefore, in this editorial I will describe what we have done for our society and what we should do as geriatricians and as members of the Japan Geriatrics Society, to meet the health-care needs of our aging population.

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2. Establishment of long-term care insurance (LTCI)

One of the problems tackled was to develop a social insurance system for elderly people, especially for frail elderly, mainly for the purpose of community-based caregiving. Japan moved decisively toward "socialization of care" for the frail elderly by initiating public, mandatory long-term care insurance (LTCI) on April 1, 2000 (Arai, 2001). Those aged 65 and over who need nursing care in their daily lives and those who require long-term care due to illnesses between the ages of 40 and 64 (specified illnesses) are eligible to receive the care services of LTCI. Consumers can choose the services and providers that they want. However, the increase in the number of elderly people requiring LTCI, and the associated costs of these services, are imposing a burden on our society. The Ministry of Health, Labor and Welfare estimated that the number of elderly people requiring LTCI will increase from 1.5 million in 2000 to 5.2 million by 2025. This increase in LTCI demand due to an increase in the elderly population will jeopardize our society, as well as the LTCI service itself. It has been reported that most institutions and businesses related to LTCI face difficulties in their operation. Most such care facilities employ care workers bettering their running. As care workers in the LTCI service receive insufficient rewards for their efforts, there is a high turnover rate, with many such workers leaving their jobs. Although the relationships between the economy and labor situation, and increases in LTCI demand are not clear, stable management of LTCI, including the efficient running of related facilities, and high job satisfaction among care workers, is required for the sustainability of the LTCI system. Since 2006, LTCI regional comprehensive support centers have been introduced to deal with counseling related to elderly care and care management. Nurses, care workers, and physical and occupational therapists are running these centers. In spite of tough economic situations, I hope the Japanese government will decide to put more money into this system, although we should maintain our efforts to improve the cost-effectiveness of health care for the elderly, and decrease the number of frail elderly by preventing cardiovascular

disease, osteoporosis, and geriatric syndrome. Many studies have been done by doctors, nurses, physical or occupational therapists, care workers, and the government to assess and improve the LTCI system (Ikegami et al., 2003; Ozawa and Nakayama, 2005; Kikuchi et al., 2006). We should try our best to improve this system in our country by supporting caregivers with professional training in improvement of long-term care for elderly patients, evaluation of long-term care services, and further assistance for care workers.

3. The health insurance system for elderly 75 and over and comprehensive geriatric assessment

The second problem tackled was the health insurance system specifically for elderly people aged 75 and over, which began on April 1, 2008. This has been unpopular due to several problems in the insurance system. However, the notion that people aged 75 and over should have their own primary-care physicians is useful for the transition to the home medical care. Some people are concerned about this system, because the "free-access" maintained in the Japanese medical system is no longer guaranteed for the very elderly. In spite of this concern, the idea should be generally implemented to avoid adverse drug effects related to polypharmacy, because elderly patients tend to see many doctors. This is also a good chance for our society to appreciate the role of geriatricians. Along with the enforcement of this insurance system, the Japan Geriatrics Society started a new education program for geriatricians and general practitioners to teach care of elderly patients. In the program, the Japan Geriatrics Society is trying to educate in the practical aspects of geriatric medicine required for the care of elderly patients and how to deal with clinical problems, especially geriatric syndrome. In this program the role of comprehensive geriatric assessment (CGA) has been stressed to maintain activities of daily living and quality of life of elderly patients. CGA is an integral part of geriatric care throughout the world, where the assessment of physiological, psychosocial, and cognitive aspects in elderly is essential (Rubenstein and Wieland, 1989). The frail elderly often have multiple chronic illnesses, functional disabilities, and psychosocial problems. Therefore, their needs obviously extend far beyond the treatment of any single medical condition. CGA also requires a multidisciplinary team approach and the use of guidelines and procedures to identify and address potentially reversible problems. The ultimate goal is to systematically restore and maintain the functions essential to preserving quality of life. Assessing how we offer medical examinations, treatment plans, rehabilitation, and the care services on the basis of a result of CGA is important. Among Japanese geriatricians, CGA is becoming more and more popular and many physicians are becoming aware of the importance of CGA. The applicability and effect of CGA on in-patient care, as well as community health care, have been reported (Onishi et al., 2004; Iizaka et al., 2008). Thus, the medical care system for the very elderly is a touchstone of our society.

4. Role of geriatricians in the aged society

To ensure that every older person receives high-quality, patient-centered health care, the role of geriatricians and the Japan Geriatrics Society is enormous. The first department of geriatric medicine in Japan was founded in the University of Tokyo in 1962. Our department of geriatric medicine at the Kyoto University Graduate School of Medicine is the second oldest and was established in 1967. Since then, many medical schools have established departments of geriatric medicine. However, at the moment less than 30% of Japan's 79 medical schools have departments of geriatric medicine. Therefore, not all the medical students receive the education in geriatric medicine and many graduate without even learning what the geriatric medicine is. Another sad fact is that some departments of geriatric medicine are managed without full professors. Generally speaking, undergraduate education in geriatrics in Japan emphasizes the theoretical aspects of the aging process and the features of disease in the elderly, but tends to omit attention to the practical aspects of care. In contrast, education in the United Kingdom and other countries places emphasis on practical aspects of elderly care. Thus, the education of geriatric medicine in our country is still remarkably poor in spite of the demand from society. I personally think that training in geriatric medicine should be required in all medical schools, and be taken into the medical-training system of young residents. Therefore, to expand the geriatrics knowledge base, increase the number of health-care professionals who employ the principles of geriatric medicine in caring for older persons, and recruit physicians and other health-care professionals into careers in geriatric medicine, the Japan Geriatrics Society is a key leader of change to achieve the goals of geriatric medicine and optimize the health of our aging population. At the moment the Japan Geriatrics Society has 6,134 members, and has approved 1,487 specialists of geriatrics. I hope that more and more young physicians are interested in geriatric medicine and are involved in the care of elderly people along with other health-care professionals. I do hope that our efforts will bring a brighter future for the Asian countries that follow Japan in terms of an aging society.

5. Conclusions

We have made significant progress in geriatrics and gerontology in the last two decades in Japan, as well as establishing our own health-care systems. Because Japan is the most aged country in the world – the second and third also being Asian countries – collaboration among Asian geriatric societies is important for the improvement of elderly health care in Asia.

Conflict of interest statement

The author has no conflicts of interest to report.

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Mulberry leaf ameliorates the expression profile of adipocytokines by inhibiting oxidative stress in white adipose tissue in db/db mice

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ABSTRACT

Previous study showed that mulberry (*Morus Alba* L.) leaf (ML) ameliorates atherosclerosis in apoE$^{-/-}$ mice. Although the adipocytokine dysregulation is an important risk factor for atherosclerotic cardiovascular disease, the effect of ML on metabolic disorders related to adipocytokine dysregulation and inflammation has not been studied. Therefore, we studied the effects of ML in metabolic disorders and examined the mechanisms by which ML ameliorates metabolic disorders in db/db mice. We treated db/db mice with ML, pioglitazone, or both for 12 weeks and found that ML decreased blood glucose and plasma triglyceride. Co-treatment with ML and pioglitazone showed additive effects compared with pioglitazone. Moreover, their co-treatment attenuated the body weight increase observed under the pioglitazone treatment. ML treatment also increased the expression of adiponectin, and decreased the expression of TNF-α, MCP-1, and macrophage markers in white adipose tissue (WAT). Furthermore, ML decreased lipid peroxides and the expression of NADPH oxidase subunits in WAT and liver. Their co-treatment enhanced these effects. Thus, ML ameliorates adipocytokine dysregulation at least in part through inhibiting oxidative stress in WAT of db/db mice, and that ML may be a basis for a pharmaceutical for the treatment of the metabolic syndrome as well as reducing adverse effects of pioglitazone.

1. Introduction

Recent study showed that white adipose tissue (WAT) produces and secretes a variety of adipocytokines involved in metabolic syndrome [1,2]. Increased production of monocyte chemoattractant protein-1 (MCP-1) from WAT contributes to macrophage infiltration into WAT and causes inflammation [3], while tumor necrosis factor-α (TNF-α) causes insulin resistance [4]. In contrast, adiponectin, which is an adipocyte-specific endocrine protein, exhibits anti-atherogenic and anti-diabetic properties, and its plasma level is decreased in visceral obesity [5,6].

In addition to inflammation, oxidative stress also plays critical roles in the metabolic syndrome [7]. Oxidative stress is shown to be increased in obesity via NADPH oxidase activation [8]. NADPH oxidase is a major source of reactive oxygen species (ROS) in various organs, especially in WAT [8]. NADPH oxidase consists of membrane-associated flavocytochrome b558 family of proteins, which include gp91^{phox} and p22^{phox} as well as cytosolic components p47^{phox}, p67^{phox}, and p40^{phox} [9]. Because macrophages are also known to produce ROS in addition to inflammatory adipocytokines, such as TNF-α [10], infiltrated macrophages might be involved in augmented NADPH oxidase and elevate ROS production in WAT. Furthermore, in adipocytes ROS themselves have been shown to augment expression of NADPH oxidase subunits as well as PU.1, a member of the ETS family of transcription factors required for the development of multiple hematopoietic lineages [8]. Thus, increased oxidative stress in WAT might cause dysregulated production of adipocytokines, which induces macrophage infiltration into WAT, causing more inflammation, and induction of oxidative stress. Furthermore, previous study showed that anti-oxidants such

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as vitamin C, E, and α-lipoic acid ameliorate insulin resistance [11]. Thus anti-oxidant may be a potential agent for the metabolic syndrome.

We have studied the role of mulberry leaf (ML), because it contains various nutritional components, such as flavonoids. Dietary ML also shows hypoglycemic [12] and anti-atherogenic effects [13,14] in certain animal models. Recently, we demonstrated that ML treatment reduced atherosclerotic lesions in apoE$^{-/-}$ mice by inhibiting lipoprotein oxidation [13]. We also showed that ML-derived aqueous fractions (MLAF) inhibit TNF-α-induced nuclear factor κB activation and lectin-like oxidized low-density lipoprotein receptor-1 expression in vascular endothelial cells [15]. However, roles and mechanisms of ML in metabolic disorders and inflammation in WAT have not been investigated.

Therefore, in this study, we examined the effects of ML on the expression profile of adipocytokines and related metabolic disorders in obese diabetic db/db mice, and compared its effect with that of a PPAR-γ agonist, pioglitazone. We also investigated the mechanisms by which ML improves development of metabolic disorders.

2. Materials and methods

2.1. Mulberry leaves

Mulberry trees were cultured in mulberry plantation of Center for Bioresource Field Science, Kyoto Institute of Technology by a standard method in Japan. Mulberry (*Morus Alba* L.) race used was "Shin-Ichinose". Mulberry leaves were harvested in July 2006 and immediately dried by air flush at 180 °C for 7 s. The average diameter of the dried powder used in this experiment was 20 μm.

2.2. Animals and experimental protocol

All animals were obtained from Oriental Bio-Service (Kyoto, Japan) and housed in a temperature-, humidity-, and light-controlled room (14-h light and 10-h dark cycle) and had free access to water and chow. In db/db mice studies, male mice at 9 weeks of age were treated with each diet for 12 weeks (n = 5–6 in each group). Briefly, mice in the ML group were fed with regular chow containing 3% (w/w) ML powder, mice in the Pio group were fed with regular chow containing 0.01% (w/w) pioglitazone (Takeda Pharmaceutical, Osaka, Japan) and mice in the ML + Pio group were fed with regular chow containing both 3% ML powder and 0.01% pioglitazone. Mice at 21 weeks of age were euthanized, blood was collected, and epididymal WAT and liver tissue were dissected out and frozen in liquid nitrogen. Samples were stored at −80 °C until use. All animal experiments were performed according to the guidelines of Kyoto University Animal Research Committee.

2.3. Body fat composition analysis

For computed tomography (CT) analysis of body fat composition, mice were anesthetized and then scanned using a LaTheta (LCT-100 M) experimental animal CT system (Aloka, Tokyo, Japan). Body fat mass was analyzed quantitatively using LaTheta software (version 1.00), as previously described [16].

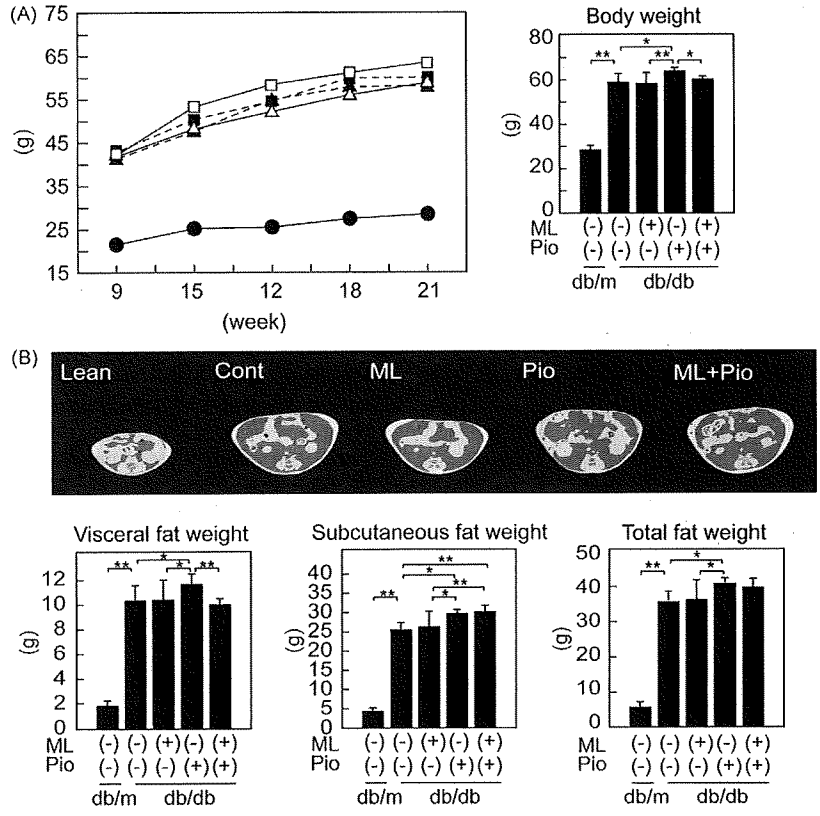

Fig. 1. Effect of mulberry leaf, pioglitazone, or both treatment for 12 weeks on body and fat weight. (A) Growth curve during experiment and body weight at the end of experiment of db/m (Lean) and db/db mice on control (Cont), 3% ML-supplemented (ML), 0.01% pioglitazone-supplemented (Pio), or co-supplemented (ML + Pio) diet for 12 weeks, respectively. Closed circle, Lean (n = 6); open triangle, Cont (n = 5); closed triangle, ML (n = 5); open square, Pio (n = 6); closed square, ML + Pio (n = 6). (B) Representative CT sections of abdominal regions and weight of visceral, subcutaneous, and total fat in db/m and db/db mice on each treatment calculated from CT scan data. Pink areas show visceral fat, while yellow areas show subcutaneous fat. Data are expressed as means ± SD. n = 5 or 6. $^{*}P < 0.05$; $^{**}P < 0.01$.

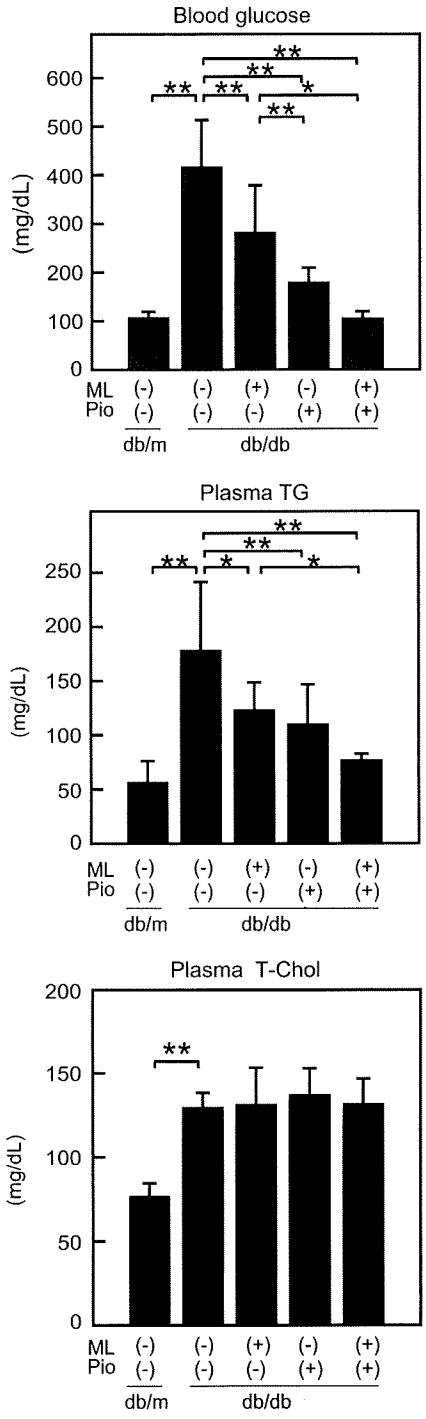

Fig. 2. Effect of mulberry leaf, pioglitazone, or their co-treatment for 12 weeks on glucose and lipid metabolism. Blood glucose levels and plasma concentrations of triglyceride and total cholesterol in each group of mice are shown. Data are expressed as means ± SD. $n = 5$ or 6. $^{*}P < 0.05$; $^{**}P < 0.01$.

2.4. Analysis of metabolic parameters

All blood samples were collected after overnight fasting. Blood glucose level and plasma concentrations of triglyceride (TG), total cholesterol (T-Chol) and adiponectin were measured by automatic glucometer (Glutest Ace, Sanwa Chemical, Hiratsuka, Japan),

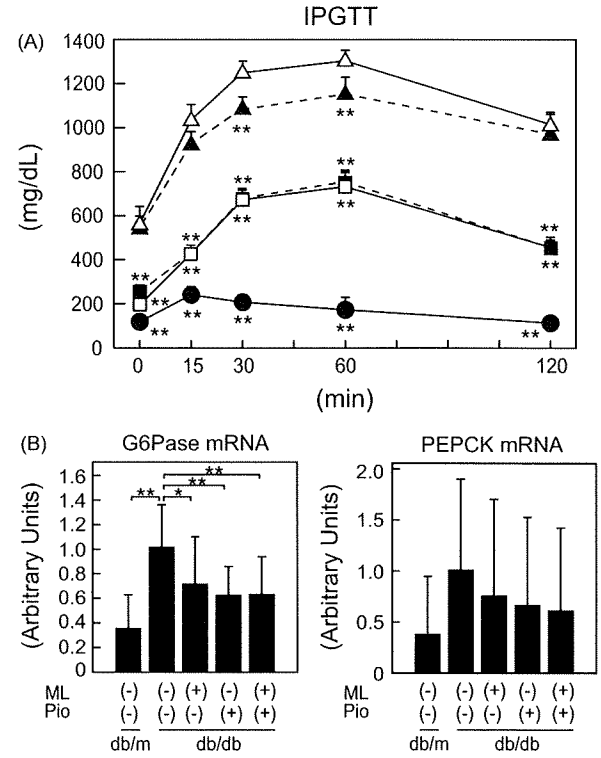

Fig. 3. Effect of mulberry leaf, pioglitazone, or their co-treatment for 12 weeks on abnormal glucose tolerance and the expression of genes related to gluconeogenesis. (A) Plasma glucose levels were determined in each group of mice are shown. $n = 5$ or 6. $^{**}P < 0.01$ versus db/db mice on control diet. (B) Expression of G6Pase and PEPCK in the liver of db/m and db/db mice on each diet after 12 weeks is shown. Data are expressed as means ± SD. $n = 5$ or 6. $^{**}P < 0.01$.

triglyceride E-test Wako, cholesterol E-test Wako (Wako Pure Chemical), and adiponectin ELISA kit (Otsuka Pharmaceutical, Tokyo, Japan), respectively. For intraperitoneal glucose tolerance tests (IPGTT), mice were starved for 16 h and then injected with 1.5 mg/kg body weight of glucose. Blood samples were collected before and after injection, and plasma glucose concentration was measured with an automatic glucometer.

2.5. Quantitative real time polymerase chain reaction (PCR)

Total RNA was extracted from WAT and liver tissue using RNeasy lipid tissue kit (Qiagen, Valencia, CA). Real time PCR was performed on an ABI PRISM 7700 (Applied Biosystems, Foster City, CA) using the SYBR GREEN PCR Master Mix (Applied Biosystems). Primer sets used for quantitative real time PCR are shown in Supplementary Table. mRNA levels were normalized relative to the amount of 18S rRNA and expressed in arbitrary units.

2.6. Lipid peroxide concentration

WAT and liver tissue were homogenized in lysis buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1% Nondient-P40, 0.25% SDS). Tissue suspension was centrifuged at 1600 × g for 10 min at 4 °C, and the supernatants were collected and used for assay. The levels of lipid peroxide in tissue homogenate were measured as thiobarbituric acid reactive substance (TBARS) using the TBARS Assay kit (Cayman Chemical, Ann Arbor, MI) according to the manufacture's recommendation.

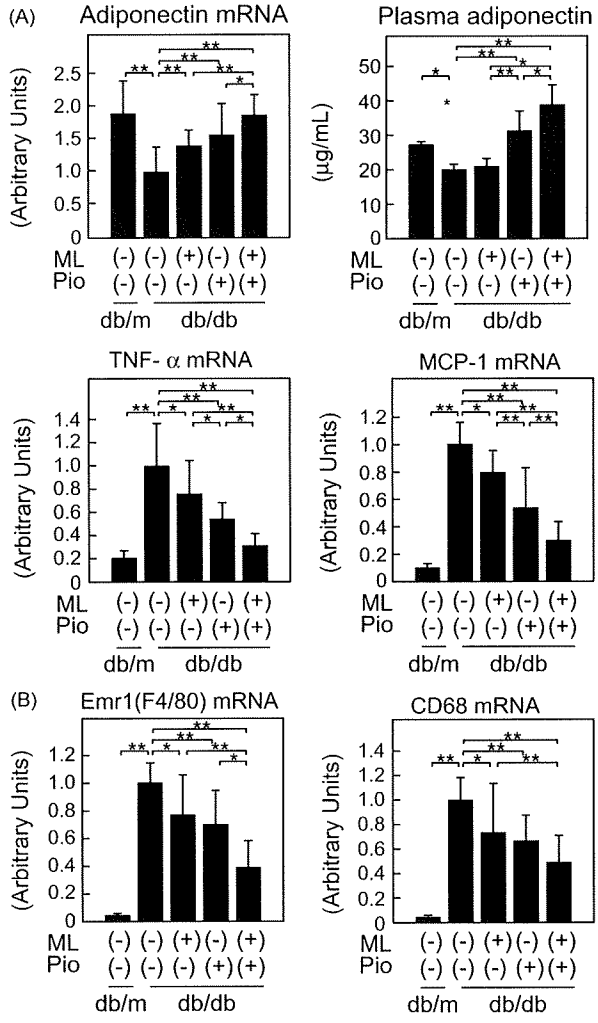

Fig. 4. Effect of mulberry leaf, pioglitazone, or their co-treatment for 12 weeks on the expression of adipocytokines and macrophage infiltration in white adipose tissue. (A) Levels of adiponectin in WAT and in plasma in each group of mice are shown. (B) Expression of TNF-α and MCP-1 mRNAs and Emr1 (F4/80) and CD68 mRNAs in WAT in each group of mice are shown. Data are expressed as means ± SD. $n = 5$ or 6. $^{*}P < 0.05$; $^{**}P < 0.01$.

2.7. Hepatic TG content

Hepatic TG content was measured as previously described [17].

2.8. NADPH oxidase activity

Liver tissue was homogenized on ice in ice-cold Tris–sucrose buffer (10 mM Tris (pH 7.6), 340 mM sucrose, 1 mM EDTA, 1 mM PMSF, 0.5% Protease inhibitor cocktail (Sigma–Aldrich)). The tissue suspension was centrifuged at 15,000 × g for 20 min at 4 °C, and the supernatant was collected and used for assay. NADPH oxidase activity was measured as previously described [18]. NADPH oxidase activity was expressed as relative NADPH oxidase activity versus the rate of NADPH consumption of non-treated db/db mice.

2.9. Statistical analysis

The results are expressed as means ± SD. The statistical significances of differences among multiple groups were evaluated using ANOVA and post hoc Fischer's PLSD tests. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Effect of ML, pioglitazone, and their co-treatment on body weight and body fat mass

Db/db mice (9 weeks of age) were treated with ML, pioglitazone, or both for 12 weeks and the changes of body weight were examined. ML did not affect the body weight gain of db/db mice, whereas pioglitazone slightly but significantly increased it by 7% compared with non-treated db/db mice. Their co-treatment significantly attenuated the body weight gain induced by pioglitazone (Fig. 1A). Next, we analyzed the body fat composition by CT scan. As previously shown, pioglitazone significantly increases visceral, subcutaneous, and total fat mass. Interestingly, the addition of ML to pioglitazone inhibited the increase of visceral fat mass induced by pioglitazone, while ML did not affect the visceral, subcutaneous, or total fat mass (Fig. 1B).

3.2. Effect of ML, pioglitazone, and their co-treatment on energy homeostasis and lipolysis

To investigate the effect of ML on energy homeostasis and lipolysis, we next measured the expression of uncoupling protein (UCP)-1, 2, and β3-adorenoceptor (β3AR), which regulate energy expenditure and lipolysis [19] in WAT and liver. However, ML had no effect on the expression of UCP-1, 2 or β3AR in WAT, or UCP-2 in the liver (Supplementary Fig. 1). In addition, co-treatment of ML and pioglitazone did not affect total fat mass.

3.3. Effect of ML, pioglitazone, and their co-treatment on blood glucose, plasma TG and T-Chol

Next, we measured the changes in blood glucose, plasma TG and T-Chol levels. Although all these blood parameters were higher in db/db mice than in db/m mice, ML decreased blood glucose level by 32% and plasma TG level by 30% compared with non-treated db/db mice. Furthermore, co-treatment of ML and pioglitazone showed further 40% reduction in glucose level, and 30% reduction in TG level compared with pioglitazone alone. On the other hand, any treatment did not affect plasma T-Chol levels (Fig. 2).

3.4. Effect of ML, pioglitazone, and their co-treatment on glucose homeostasis

To investigate the effect of ML on glucose homeostasis, we performed IPGTT. ML significantly improved abnormal glucose tolerance, while pioglitazone, or their co-treatment markedly improved it (Fig. 3A). We also measured the expression of glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK), both of which regulate gluconeogenesis in the liver. Although the expression of G6Pase was significantly higher in db/db mice than in db/m mice, ML, pioglitazone, or their co-treatment significantly decreased the expression of G6Pase by 24, 37, and 31%, respectively. However, any treatment did not affect the expression of PEPCK (Fig. 3B).

3.5. Effect of ML, pioglitazone, and their co-treatment on adipocytokine expression

We next measured the levels of adipocytokines in epididymal WAT and plasma. Adiponectin levels in WAT and in plasma were

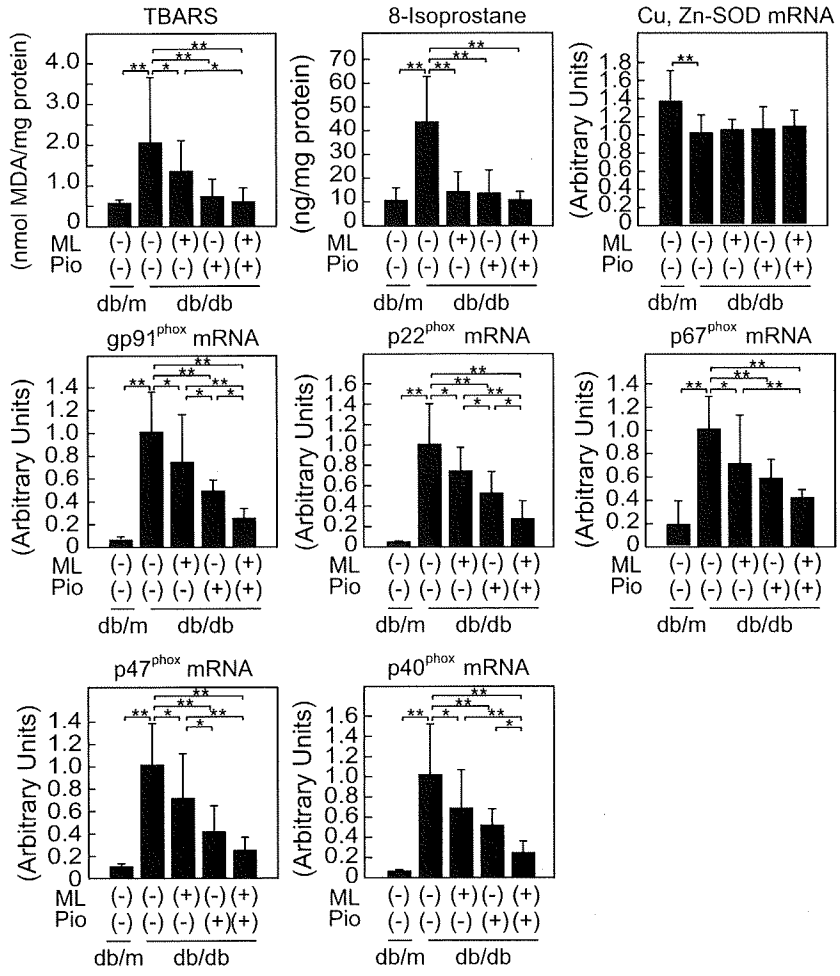

Fig. 5. Effect of mulberry leaf, pioglitazone, or their co-treatment for 12 weeks on oxidative stress in white adipose tissue. Levels of TBARS, Cu, Zn-SOD mRNA, and NADPH oxidase subunits, gp91^{phox}, p22^{phox}, p67^{phox}, p47^{phox} and p40^{phox} and PU.1 mRNAs in WAT from each group of mice are shown. Data are expressed as means ± SD. $n = 5$ or 6. $^{*}P < 0.05$; $^{**}P < 0.01$.

significantly lower in db/db mice than in db/m mice. ML significantly increased adiponectin levels in WAT by 40% compared with non-treated db/db mice, but not in plasma. Co-treatment further increased adiponectin levels by 17% in WAT and by 25% in plasma compared with pioglitazone alone. In contrast, the expression of inflammatory adipocytokines, such as TNF-α and MCP-1 in WAT was markedly increased in db/db mice than in db/m mice. However, ML decreased the expression of TNF-α and MCP-1 by 25 and 20% in db/db mice, respectively. In addition, co-treatment resulted in a further decrease by approximately 45% compared with pioglitazone alone (Fig. 4A).

We also measured the expression of two macrophage markers, F4/80 antigen, Emr1, and CD68 in WAT. Expression of Emr1 and CD68 was markedly increased in db/db mice than in db/m mice. However, ML significantly decreased the expression of Emr1 and CD68 by 13 and 16% in WAT, respectively. Co-treatment further decreased the expression of Emr1 and CD68 by 46 and 26%, respectively, compared with pioglitazone alone (Fig. 4B).

3.6. Effect of ML, pioglitazone, and their co-treatment on oxidative stress in WAT and liver

We next measured adipose TBARS concentrations to investigate the effect of ML on oxidative stress. Although adipose TBARS concentrations were markedly higher in db/db mice than in db/m mice, treatment with ML, pioglitazone, or both significantly decreased them in db/db mice by 43, 62, and 72%, respectively.

We also investigated the effects of ML, pioglitazone, or their co-treatment on gene expression related to the production and removal of ROS in WAT. Expression of genes related to the production of ROS, including all NADPH oxidase subunits and PU.1 was markedly increased in epididymal WAT of db/db mice, but ML significantly decreased them. Further, co-treatment consistently decreased the expression of these genes compared with pioglitazone alone. On the other hand, expression of Cu, Zn-SOD, the ROS-elimination system, was decreased in db/db mice compared with that in db/m mice. However, any treatment did not affect the expression of Cu, Zn-SOD (Fig. 5).

In the liver TG accumulation was higher in db/db mice than in db/m mice. ML significantly decreased hepatic TG content by 44% in db/db mice. TBARS concentrations and NADPH oxidase activity were also higher in db/db mice than in db/m mice. Treatment with ML, pioglitazone, or both markedly decreased hepatic TBARS concentrations by 35, 33, and 59%, respectively, and NADPH oxidase activity by 37, 65, and 74%, respectively in db/db mice. Furthermore, although we could not show a significant effect of each treatment on the expression of NADPH oxidase subunits, gp91^{phox} and p47^{phox}, and Cu, Zn-SOD, tendencies were