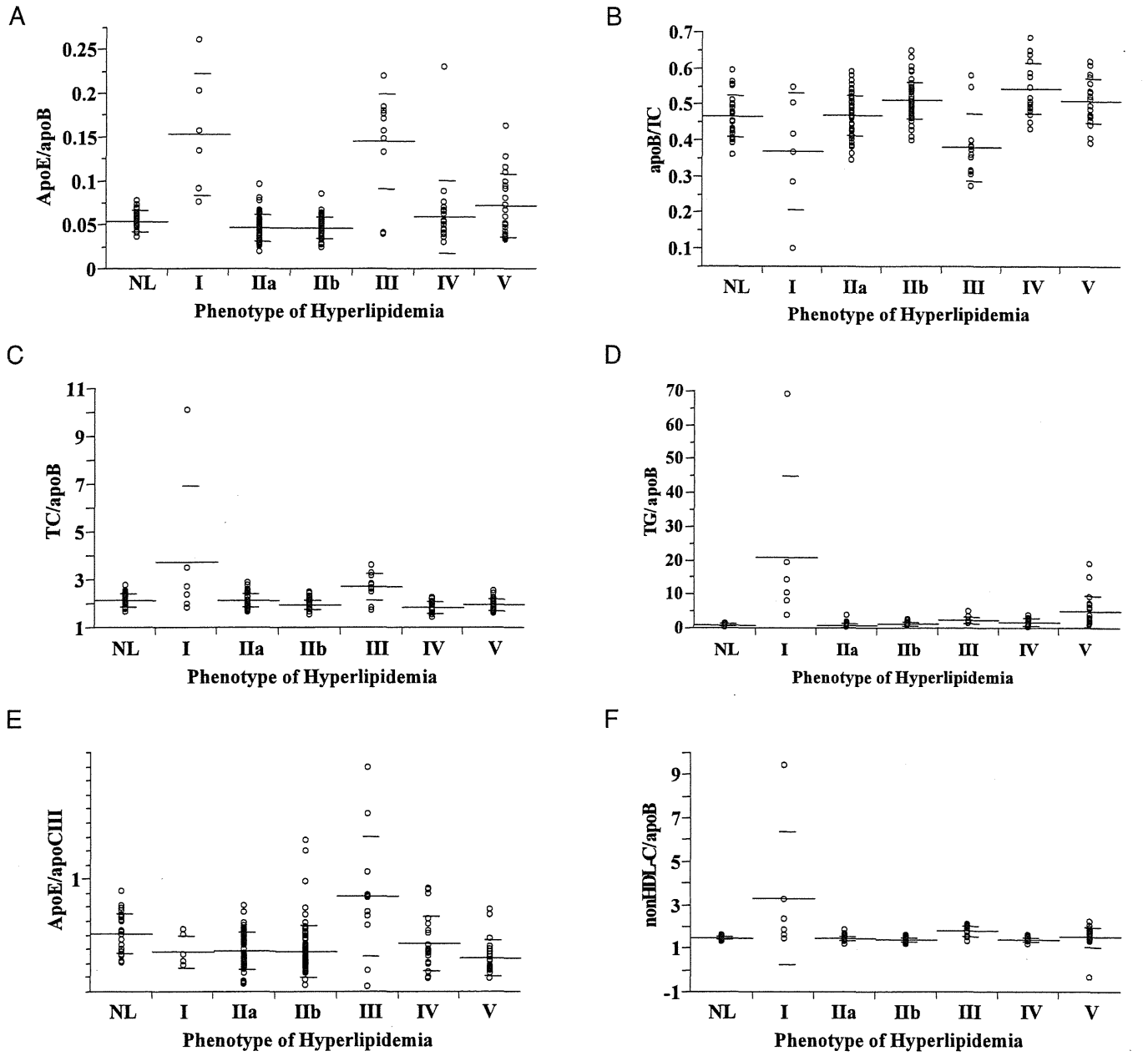

Fig. 3. Clinical indexes for screening type III hyperlipidemia.

The apo E/apo B (A), apo B/TC (B), TC/apo B (C), TG/apo B (D), non HDL-C/apo B (E) and apo E/apo C-III ratios (F) were determined in 25 subjects with NL, and type I (n=6), type IIa (n=62), type IIb (n=66), type III (n=12), type IV (n=22), and type V (n=23) HL patients. Data are expressed as a scatter plot using the mean ± SD.

LPL activity may decrease VLDL-TG and CM-TG but not the number of VLDL and CM remnants, because liver uptake of them is impaired due to genetic apo E abnormality. Apo E and apo CIII were significantly higher in patients with type I, III, and V HL (**Table 1**), which might also be due to impaired liver uptake of remnant lipoproteins. LPL activity is genetically impaired in patients with type I HL, and fibrates might not be able to decrease TG and apoB-48 levels. As a result, high apo B-48 levels and low TG levels were observed in patients with type III HL after the administration of fibrates.

Clinical Indices for Screening Type Ⅲ HL after Lipid-Lowering Interventions

Five groups of investigators have developed and evaluated clinical indices for screening type Ⅲ HL before the initiation of lipid-lowering interventions[12-16]. We evaluated whether their clinical indices including the apoB-48/TG ratio were also useful for screening type Ⅲ HL when lipid and lipoprotein profiles were already improved by lipid-lowering agents (**Table 3** and **Fig. 3**). A high apo E/apo B ratio was based on the concept that the accumulation of remnant lipoproteins increases apo E but not apo B levels[12]; fibrates reduce apo B levels in patients with type I HL resulting in a high apo E/apo B ratio in patients with type I HL as well as type Ⅲ HL. A low apo B/TC ratio[13] was based on the concept that remnant lipoproteins are larger and contain more lipids than LDL particles; this increases serum cholesterol levels and decreases apo B levels. Since statins and fibrates effectively decrease both TC and apo B levels, low apo B/TC levels are observed in patients with type I HL as well as type Ⅲ HL (**Table 1**). In an algorithm by Sniderman *et al.*, a high TC/apo B ratio discriminated patients with type I, Ⅲ, and V HL, and a low TG/apo B ratio discriminated patients with type Ⅲ HL among them[14]. This might be based on the concept that the accumulation of remnant lipoproteins results in greater cholesterol content than that of LDL but a lower TG content than that of CM or VLDL. Statins and fibrates effectively decrease both TC and apo B levels, high TC/apo B levels were also observed in patients with type I as well as type Ⅲ HL and the TG/apo B ratio was significantly lower in patients with type Ⅲ HL than in those with type I HL (**Table 1**). This algorithm may be useful even after the administration of lipid-lowering agents; however, the cut-off value that divided type I or Ⅲ HL from the other types of HL was lower than that of the TC/apo B ratio reported by Sniderman *et al.* (TC/apo B >6.2)[14]. The non-HDL-C/apo B ratio relates to the cholesterol content in one particle of remnant lipoproteins and LDL, and a high non-HDL-C/apo B ratio indicates the accumulation of remnant lipoprotein and LDL[15]. Statins and fibrates decrease the cholesterol content of remnant lipoproteins in patients with type Ⅲ HL, and the non-HDL-C/apo B ratio was significantly highest in patients with not only type Ⅲ HL but also type I HL (**Table 3** and **Fig. 3**). The high non-HDL-C/apo B ratio in patients with type I HL may be caused by impaired LPL activity, which could not decrease CM and CM remnants even after lipid-lowering intervention with fibrates. A high apo E/apo C-Ⅲ ratio was based on the lipoprotein profile in which apo E levels were 3-fold higher but apo C-Ⅲ levels were 2-fold higher than in NL subjects, which might be due to the marked accumulation of remnant lipoproteins associated with apo E[16]. After lipid-lowering intervention, high apo E levels were still observed in patients with type I, Ⅲ, and V HL, and high apo C-Ⅲ levels were observed in patients with type I and V HL (**Table 1**); however, high apo E/apo C-Ⅲ ratios were observed in patients with not only type Ⅲ but also type Ⅱb, Ⅳ, and V HL (**Table 3** and **Fig. 3**). Both statins and fibrates significantly decreased VLDL-apo C-Ⅲ levels in patients with dyslipidemia by reducing the production and increasing the fractional catabolic rate of VLDL-apo C-Ⅲ[28, 29].

The apo B-48/TG ratio was the only index that could distinguish patients with type Ⅲ HL from those with other types of HL after the initiation of lipid-lowering interventions (**Table 1** and **3**). Fibrates strongly decrease TG levels in patients with accumulated TG-rich lipoproteins by increasing LPL activity; however, they only minimize the size of remnant lipoproteins and cannot improve hepatic uptake of these remnants because of the genetic dysfunction of apo E2/E2. We estimated that the apo B48/TG ratio yields an AUC-ROC value of 0.895 (**Fig. 2**), which indicates that the ratio has a high accuracy rate in detecting type Ⅲ HL (cut-off value, 0.110; error rate vs. type Ⅲ HL, 2.8%). Using an automated chemical analyzer in our CLEIA system (the results are available within 2 h), the apo B-48/TG ratio will provide a simple index for screening type Ⅲ HL before as well as after the administration of lipid-lowering agents.

Atherogenicity of Type Ⅲ HL after Lipid-Lowering Intervention

Emerging evidence has shown that CM remnants might be responsible for the initiation and development of atherosclerotic plaques *in vitro* and *in vivo*[2]. High fasting apo B-48 is correlated with premature carotid artery stenosis[21, 22] and the existence of CHD among other metabolic biomarkers related to coronary risk, and the combination with other impaired biomarkers represents a stronger risk state for CHD (Masuda D *et al.*, unpublished observation, 2011). Many *in vitro* and *in vivo* studies have shown that small CM remnants may play an important role in the initiation and development of atherosclerotic plaques[2]. The size of CMs and CM remnant particles produced from the intestine during the postprandial period has varied from a large CM to small LDL or HDL[2, 30]. A high apo B-48/TG ratio indicates the accumulation of small CM remnants that contain one apo B-48 molecule and lower TG content than in large CM particles.

In patients with type Ⅲ HL, the apo E2/E2 phenotype may continuously prevent the liver uptake of small CM remnants, resulting in the accumulation of small CM remnants, and lipid-lowering drugs do not improve this uptake.

High fasting apo B-48 and the accumulation of small CM remnants should be treated carefully and reduced using a variety of nutritional and pharmacological approaches. It was reported that atorvastatin markedly improved the postprandial increase in apo B-48-containing lipoproteins in patients with type Ⅲ HL[1]. The apo B-48/TG ratio remained high in patients with type Ⅲ HL in the current study despite appropriate lipid-lowering treatment with fibrates or statins (**Table 1**, **2**, and **3**), which implied that fibrate and statin therapies could not decrease small CM remnants that are clearly atherogenic. For a sufficient decrease in CMs and CM remnants in patients with type Ⅲ HL, the intestinal production of CM must be suppressed along with the increase in LPL activity. In our recent study, we reported that an intestinal cholesterol transporter inhibitor, ezetimibe, improves postprandial HL in patients with type Ⅱb HL[30], possibly due to a reduction in intestinal cholesterol uptake and the formation of apoB-48 and CMs[31]. Combination therapy with fibrates and ezetimibe may more effectively decrease CMs and small CM remnants and may prevent the development of atherosclerotic plaques in patients with type Ⅲ HL.

Limitation of the Study

The present study was performed with a small number of patients with type Ⅲ HL ($n=12$) because it is a very rare familial disorder. It may be necessary to examine whether the apo B-48/TG ratio is useful for detecting type Ⅲ HL with a large-scale study.

Conclusion

In conclusion, these data suggest that the apo B-48/TG ratio is a novel and useful marker for detecting type Ⅲ HL, even after the initiation of lipid-lowering therapy.

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Disclosure

S. Yamashita is a member of Fujirebio's Advisory Boards (Tokyo, Japan). He has received compensation for travel expenses for Conference Lectures from this company. The co-authors do not have anything to disclose.

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Original Article

Effect of body mass index-z score on adverse levels of cardiovascular disease risk factors

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Abstract ***Background***: Cardiovascular disease (CVD) risk factors are associated with body mass index z-score (BMISD) and/or insulin resistance (IR). However, the correlation between adverse levels of these risk factors and BMISD, and the effect of IR on these associations are not fully understood in children. The aim of this study was to evaluate the association between adverse levels of CVD risk factors and BMISD, and the effect of IR on these associations in schoolchildren.
Methods: Conventional CVD risk factors, C-reactive protein (CRP), uric acid (UA) and adiponectin were determined in 757 boys and 494 girls aged between 7 and 12 years. IR was assessed by the homeostasis model approximation index.
Results: BMISD were linearly associated with relative risks having adverse levels of all factors, except for glucose and low-density lipoprotein cholesterol (LDL-C) in boys, and except for glucose, LDL-C and adiponectin in girls ($P < 0.01$–0.001). These associations were weakened after adjustment for IR, but still significant in cases of UA and CRP in boys and UA, high-density lipoprotein cholesterol and CRP in girls ($P < 0.01$–0.001).
Conclusion: The relative risk of having adverse levels of most CVD risk factors in school children increased across the entire range of BMISD. IR contributed to most of these relative risks, but BMISD itself also contributed to these relative risks. To prevent future development of CVD, it might be important for schoolchildren to maintain BMISD within normal range. However, in cases of hyper LDL-cholesterolemia, we should consider causes other than BMISD.

Key words adipocytokine, cardiovascular disease risk factors, hypercholesterolemia, insulin resistance, obesity.

Many epidemiological studies have shown that overweight and obesity are increasing globally in both children and adults.[1] In Japan, the prevalence of obesity in schoolchildren has steadily increased in recent decades, possibly due to changes in dietary patterns and lifestyles among these children.[2] Must *et al.* reported that the risk of morbidity from coronary heart disease and atherosclerosis was increased among men and women who had been overweight as adolescents of 13–18 years old.[3] Given this finding, it seems rational to consider that the incidence of atherosclerotic cardiovascular disease (CVD) in Japan could increase dramatically in the near future. In contrast, a recent study reported that the overweight and obese show no increased risk for total mortality and cardiovascular mortality compared with those with a normal body mass index (BMI):[4] severely obese patients did not have increased total mortality, but they had the highest risk for cardiovascular mortality. These results suggest that the metabolic aberrations that coexisted with overweight and obesity may be more important than overweight and obesity themselves. In this regard, Barter *et al.* reported that overweight people with normal plasma lipids might be at relatively low risk for developing diabetes and cardiovascular disease.[5]

In our previous studies, we showed that abnormal CVD risk factors, such as small dense low-density lipoproteins, dyslipidemia, hyperinsulinemia, high levels of inflammatory markers and low levels of adiponectin, were found in schoolchildren.[6–9] In addition, low-density lipoprotein particle size and serum concentrations of these CVD risk factors were closely associated with BMI.[6–9] However, these abnormal CVD risk factors may occur regardless of BMI.[7] As reported previously, genetic predispositions appear to contribute more to dyslipidemia in children than they do in adults.[7,10] Thus, it is important to clarify whether abnormal CVD risk factors are merely complications of overweight or obesity. It is generally accepted that many comorbidities with obesity, such as diabetes, dyslipidemia and hypertension, are attributed to insulin resistance.[11] Thus, in the present study, we investigated the correlations between adverse levels of CVD risk factors and BMI z-score (BMISD), and the effect of insulin resistance on these associations in Japanese schoolchildren.

Methods

Subjects

We studied 1251 Japanese children (757 boys and 494 girls) aged 7–12 years, who underwent screening and were enrolled in a care

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program for lifestyle-related diseases in Okinawa and Kumamoto, Japan. Sex-maturity stages in the children studied were equal or less than Tanner stage 3 (Tanner stage was evaluated by inspection of mammary development in girls, and by asking condition of pubic hair in boys and this evaluation was performed by a pediatrician). BMI was calculated as weight [kg]/height^2 [m^2]. BMISD adjusted for age and sex were obtained based on data on Japanese schoolchildren provided in 2000 by the Ministry of Education, Culture, Sports, Science and Technology (unpublished data). We employed BMISD to continuously evaluate BMI in the studied schoolchildren. None of the children was receiving therapy for weight reduction, or drugs that might affect lipid metabolism, and none had a smoking habit. Venous blood was drawn after an overnight fast. Informed consent was obtained from the parents of all of the children. This study was approved by the ethics committee of the Ryukyu University.

Laboratory measurements

The serum concentration of C-reactive protein (hCRP) was measured by a highly sensitive immunoturbidimetric assay using reagents and calibrators from Dade Behring Marbura GmbH (Marburg, Germany). The lower limit of detection for serum CRP concentration was 0.05 mg/L. Adiponectin was measured by enzyme-linked immunosorbent assays (R&D Systems, Minneapolis, MN, USA). Serum insulin was measured by two-step sandwich enzyme-linked immunosorbent assay (ELISA) (SRL Inc., Hachioji, Japan). Routine chemical methods were used to determine the serum concentrations of total cholesterol (TC), high-density-lipoprotein cholesterol (HDL-C), triglycerides (TG), uric acid and glucose. Low-density-lipoprotein cholesterol (LDL-C) was calculated as [TC – HDL-C – TG/5]. Apolipoprotein B (apoB) was measured by the turbidity immunoassay method.[12] Insulin resistance was calculated using the homeostasis model approximation index (HOMA-IR).[13]

Statistical evaluation

The significance of differences between boys and girls was determined by the Mann–Whitney *U*-test. Serum concentrations of insulin, TG and hCRP were markedly skewed. Thus, these parameters were normalized by log-transformation. Pearson and partial correlation coefficients were then computed to assess the associations between BMISD and various parameters. The logistic model was used to evaluate linear associations between adverse levels of variables and BMISD (continuous). The relative risks to have adverse variables (odds ratio) were adjusted for age by a multiple logistic regression analysis.

Results

Several indexes of overweight and/or abdominal obesity have been proposed for children, as in the case for adults. Among these, waist–height ratio is more strongly associated with CVD risk factors than is BMI;[14] however, a recent report found that BMISD and waist–height ratio did not differ in their ability to identify adverse risk factors.[15] Because waist circumference was not measured in our schoolchildren, we employed BMISD to evaluate the correlation between adverse levels of CVD risk factors and BMI.

As shown in Table 1, significant sex differences were found for several parameters; thus, we separated the data for boys and girls in the following analysis. Because age was significantly correlated with BMISD (boys: r = 0138, $P < 0.001$; and girls: r = 0.139, $P < 0.01$), age was adjusted by partial correlation. Table 2 shows age-adjusted correlations between BMISD and 10 parameters. All parameters except glucose were significantly associated with BMISD in both boys and girls ($P < 0.01$-0.001). BMISD showed a positive correlation with LDL-C and a negative correlation with HDL-C; therefore, we did not examine its correlation with TC. HOMA-IR and serum concentrations of insulin showed stronger correlations with BMISD than those of other factors in both boys and girls. HOMA-IR has recently been validated as a surrogate maker of insulin resistance, even in children.[16,17]

We then evaluated the correlation between adverse levels of CVD risk factors (except for glucose) and BMISD with a multiple logistic regression analysis. To date, there are no criteria to define adverse levels of these CVD risk factors in Japanese

Table 1 Clinical and chemical data

	Boys (n = 757)	P-value	Girls (n = 494)
Age (years)	10.0 ± 1.1	(NS)	10.0 ± 1.1
BMI SD	1.64 ± 1.12	($P < 0.01$)	1.46 ± 1.12
TC (mg/dL)‡	182 ± 29	($P < 0.01$)	176 ± 28
TG (mg/dL)§	79 ± 59	(NS)	80 ± 46
LDL-C (mg/dL)‡	107 ± 25	(NS)	104 ± 25
HDL-C (mg/dL)‡	59 ± 12	($P < 0.01$)	56 ± 11
ApoB (mg/dL)	79 ± 18	(NS)	77 ± 18
Glucose (mg/dL)†	90 ± 6	($P < 0.01$)	89 ± 7
Insulin (μU/mL)	12.21 ± 8.96	($P < 0.01$)	14.10 ± 9.79
HOMA-IR	2.75 ± 2.24	($P < 0.01$)	3.12 ± 2.36
Uric acid (mg/dL)	4.9 ± 1.0	(NS)	4.8 ± 1.0
Adiponectin (μg/mL)	8.7 ± 3.6	(NS)	8.6 ± 3.7
hCRP (mg/L)	1.65 ± 4.56	(NS)	1.24 ± 3.12

Values are expressed as mean ± standard deviation. †To convert to mmol/L, divided by 18. ‡To convert to mmol/L, multiply by 0.0259. §To convert to mmol/L, multiply by 0.0113. ApoB, apolipoprotein B; BMI, body mass index; hCRP, serum concentration of C-reactive protein; HDL-C, high-density-lipoprotein cholesterol; HOMA-IR, homeostasis model approximation index; LDL-C, low-density-lipoprotein cholesterol; NS, not significant; TG, triglycerides; TC, total cholesterol.

Table 2 Age-adjusted correlations between body mass index z-score and cardiovascular disease risk factors

	Boys		Girls	
	r	*P*	r	*P*
Log TG	**0.177**	**<0.001**	**0.218**	**<0.001**
LDL-C	**0.107**	**<0.01**	**0.150**	**<0.01**
HDL-C	**−0.277**	**<0.001**	**−0.399**	**<0.001**
ApoB	**0.178**	**<0.001**	**0.239**	**<0.001**
Glucose	0.045	0.213	0.068	0.135
Log insulin	**0.568**	**<0.001**	**0.647**	**<0.001**
Log HOMA-IR	**0.561**	**<0.001**	**0.634**	**<0.001**
Uric acid	**0.370**	**<0.001**	**0.437**	**<0.001**
Adiponectin	**−0.264**	**<0.001**	**−0.303**	**<0.001**
Log hCRP	**0.464**	**<0.001**	**0.333**	**<0.001**

Bold indicates significant associations ($P < 0.05$). ApoB, apolipoprotein B; hCRP, serum concentration of C-reactive protein; HDL-C, high-density-lipoprotein cholesterol; HOMA-IR, homeostasis model approximation index; LDL-C, low-density-lipoprotein cholesterol; TG, triglycerides.

school children. Thus, when levels of CVD risk factors were greater than those of the 90th percentiles of our subjects, we tentatively considered the children to have adverse levels, except for HDL-C and adiponectin (boys: insulin > 20.8 μU/mL, HOMA-IR > 4.39, TG > 145 mg/dL, LDL-C > 138 mg/dL, apoB > 101 mg/dL, uric acid > 6.3 mg/dL and hCRP > 3.41 mg/L; girls: insulin > 26.64 μU/mL, HOMA-IR > 5.62, TG > 148 mg/dL, LDL-C 133 mg/dL, apoB > 98 mg/dL, uric acid > 5.9 mg/dL and hCRP > 2.39 mg/L). HDL-C and adiponectin were considered to be adverse levels when their levels were less than those of the 10th percentiles (boys: HDL < 44 mg/dL and adiponectin < 4.2 μg/mL; girls: HDL-C < 43 mg/dL and adiponectin < 4.1 μg/mL). As shown in Table 3, we observed no linear association of BMISD with adverse levels of LDL-C in boys. The relative risk of having adverse levels of other CVD risk factors increased with increasing BMISD. Table 4 shows the case of girls. In contrast to the case of boys, BMISD did not show a linear correlation with adverse levels of adiponectin. As shown in Table 2, HOMA-IR showed stronger correlations with BMISD than those of other CVD risk factors in both boys and girls. Thus, to examine whether the correlations of adverse levels of CVD risk factors with BMISD were independent of insulin resistance, the findings were adjusted for HOMA-IR, in addition to age. After adjustment for HOMA-IR and age (Table 5), the relative risk of having adverse levels of uric acid and hCRP increased with increasing BMISD in boys. Significant associations of adverse levels of HDL-C, TG, apoB and adiponectin with BMISD were eliminated in boys after adjustment. In girls, the relative risk of having adverse levels of uric acid, HDL-C and

Table 3 Age-adjusted associations between body mass index z-score and adverse levels (above the 90th percentile or below the 10th percentile; HDL-C and adiponectin) in boys as assessed by a multiple logistic regression analysis

Dependent variable	β	Wald χ^2	*P*-value	Exp (β)	95%CI
LDL-C	0.133	1.64	0.201	1.14	0.93–1.40
HDL-C	**0.351**	**11.56**	**<0.001**	**1.42**	**1.16–1.74**
TG	**0.230**	**4.87**	**0.027**	**1.26**	**1.03–1.54**
ApoB	**0.228**	**4.85**	**0.028**	**1.26**	**1.03–1.54**
Insulin	**1.009**	**68.55**	**<0.001**	**2.74**	**2.16–3.48**
HOMA-IR	**0.894**	**33.84**	**<0.001**	**2.44**	**1.95–3.07**
Uric acid	**0.680**	**40.99**	**<0.001**	**1.97**	**1.60–2.43**
Adiponectin	**0.387**	**14.49**	**<0.001**	**1.47**	**1.21–1.80**
hCRP	**0.745**	**45.05**	**<0.001**	**2.11**	**1.69–2.62**

Bold type indicates a significant correlation ($P < 0.05$). ApoB, apolipoprotein B; CI, confidence interval; hCRP, serum concentration of C-reactive protein; HDL-C, high-density-lipoprotein cholesterol; HOMA-IR, homeostasis model approximation index; LDL-C, low-density-lipoprotein cholesterol; TG, triglycerides.

Table 4 Age-adjusted associations between body mass index z-score and adverse levels (above the 90th percentile or below the 10th percentile; HDL-C and adiponectin) in girls as assessed by a multiple logistic regression analysis

Dependent variable	β	Wald χ^2	*P*-value	Exp (β)	95%CI
LDL-C	0.216	2.65	0.103	1.24	0.96–1.61
HDL-C	**0.831**	**32.98**	**<0.001**	**2.30**	**1.73–3.05**
TG	**0.451**	**11.21**	**<0.001**	**1.57**	**1.21–2.04**
ApoB	**0.392**	**8.62**	**<0.01**	**1.48**	**1.14–1.92**
Insulin	**0.846**	**33.28**	**<0.001**	**2.33**	**1.75–3.11**
HOMA-IR	**0.947**	**39.92**	**<0.001**	**2.58**	**1.92–3.46**
Uric acid	**0.931**	**42.75**	**<0.001**	**2.54**	**1.92–3.36**
Adiponectin	0.203	2.53	0.112	1.23	0.95–1.57
hCRP	**0.643**	**15.73**	**<0.001**	**1.90**	**1.39–2.62**

Bold type indicates a significant correlation ($P < 0.05$). ApoB, apolipoprotein B; CI, confidence interval; hCRP, serum concentration of C-reactive protein; HDL-C, high-density-lipoprotein cholesterol; HOMA-IR, homeostasis model approximation index; LDL-C, low-density-lipoprotein cholesterol; TG, triglycerides.

Table 5 Age- and homeostasis model approximation index-adjusted significant associations between body mass index z-score and adverse levels (above the 90th percentile or below the 10th percentile; HDL-C and adiponectin) in schoolchildren as assessed by a multiple logistic regression analysis

Dependent variable	β	Wald χ^2	*P*-value	Exp (β)	95%CI
Boys					
hCRP	0.666	27.46	<0.001	1.95	1.52–2.50
Uric Acid	0.559	20.85	<0.001	1.75	1.38–2.22
Girls					
Uric acid	0.827	25.28	<0.001	2.29	1.66–3.16
HDL-C	0.591	12.49	<0.001	1.81	1.30–2.51
hCRP	0.530	9.48	<0.01	1.70	1.21–2.38

CI, confidence interval; hCRP, serum concentration of C-reactive protein; HDL-C, high-density-lipoprotein cholesterol.

hCRP showed an increase with increasing BMISD. Significant associations of adverse levels of TG and apoB with BMISD were eliminated in girls after adjustment.

Discussion

Based on the findings of the present study, adverse levels of CVD risk factors can be divided into three groups (Table 6): (i) independent of BMISD (boys: glucose and LDL-C; girls: glucose, LDL-C and adiponectin); (ii) dependent on BMISD and independent of insulin resistance (boys: uric acid and hCRP; girls: uric acid, HDL-C and hCRP); and (iii) dependent on BMISD and insulin resistance (boys: insulin, HOMA-IR, HDL-C, TG, apoB and adiponectin; girls: insulin, HOMA-IR, TG and apoB).

It is generally accepted that many comorbidities with obesity, such as diabetes, dyslipidemia and hypertension, are attributed to insulin resistance.[11] In the present study, BMISD was strongly correlated with insulin resistance. The relative risk of having an adverse level of insulin resistance was linearly increased across the normal range. The risk of an adverse level of insulin resistance was significantly higher for children at BMISD 1.0 compared with those at BMISD 0.0, with an odds ratio of adverse level of insulin resistance ranging from 2.44 to 2.58 (Tables 3 and 4). The present findings suggest that in schoolchildren, a slight shift of BMISD from normal ranges affects insulin resistance.

Correlations of BMISD and adverse levels of CVD risk factors are generally reported only in studies regarding hypertension,[18,19] in which the risk of hypertension has been found to be significantly higher in obese children than in non-obese children, with an odds ratio of hypertension ranging from 2.4 to 2.5; however, it has been noted that the prevalence of hypertension in children increases across the entire range of BMI values and cannot be defined by a simple threshold effect. The effects of BMISD on adverse levels of LDL-C, HDL-C, TG and apoB are not yet clear. Unexpectedly, LDL-C was not associated with BMISD in both boys and girls. However, adverse levels of TG and apoB were associated with BMISD in both boys and girls. These significant associations were lost after adjustment for insulin resistance. Different findings of LDL-C and apoB were consistent with our previous report that LDL size was inversely associated with BMI in school children.[6] In the case of HDL-C, a strong association between BMISD and adverse level of HDL-C was found in both boys and girls; however, after adjustment for insulin resistance, a significant association was only retained in girls. As reported previously, hypercholesterolemia (hyper LDL-C) in school children commonly occurs regardless of BMISD.[6,7] Familial hypercholesterolemia and familial combined hyperlipidemia should not be overlooked in school children with overweight and obesity. Effect of genetic factors on hyper LDL-C may be greater than that of environmental factors. In addition to hyper LDL-C in school children, low HDL-C in schoolgirls should not be diagnosed as complications of overweight and obesity before clarifying the genetic background.

Serum concentrations of adiponectin were inversely correlated with BMISD in both boys and girls. However, the association between the relative risk of having an adverse level of adiponectin and BMISD was only significant in boys. This association was completely dependent on insulin resistance. In other words, relative risk of an adverse level of adiponectin is not increased in obese boys without insulin resistance, thereby indicating a close correlation between adverse adiponectin level and insulin resistance in schoolboys. In girls, factors other than BMISD and insulin resistance seemed to regulate adverse levels

Table 6 Correlation between BMISD and adverse levels of cardiovascular disease risk factors

	Boys	Girls
Independent of BMISD	Glucose, LDL-C	Glucose, LDL-C, Adiponectin
Dependent on BMISD		
Independent on IR	Uric acid, hCRP	Uric acid, HDL-C, hCRP
Dependent of IR	Insulin, HOMA-IR, HDL-C, TG, apoB, adiponectin	Insulin, HOMA-IR, TG, apoB

apoB, apolipoprotein B; BMISD, body mass index z-score; hCRP, serum concentration of C-reactive protein; HDL-C, high-density-lipoprotein cholesterol; HOMA-IR, homeostasis model approximation index; IR, insulin resistance; LDL-C, low-density-lipoprotein cholesterol; TG, triglycerides.

of adiponectin. Recently, Magge *et al.* also reported similar findings that adiponectin levels are independent of insulin resistance in adolescents.[20]

With respect to hCRP, the association between the relative risk of adverse levels of hCRP and BMISD was unaffected by the adjustment for insulin resistance in both boys and girls. The underlying mechanism behind the correlation between hCRP and BMISD has not been clarified in the present study; however, our data suggest that subclinical inflammation as expressed by hCRP did occur even in school children, and that the degree of inflammation was associated with BMISD. According to a recent report, serum concentrations of uric acid are associated with all-cause and cardiovascular disease mortality.[21] Association between an adverse level of uric acid and BMISD was unexpectedly high and was unaffected by insulin resistance in both boys and girls. In addition, the association was unaffected by hCRP (data not shown). Although further studies are needed to clarify the physiological role of uric acid in children, the strong association between the relative risk of having adverse levels of uric acid and BMISD should be highlighted as a complication of overweight and obesity.

Conclusion

In the present study, hyper LDL-cholesterolemia in school children cannot be explained by BMISD. However, the relative risk of having adverse levels of other CVD risk factors in school children increases across the entire range of BMISD. Relative risks of adverse levels of UA and CRP in boys, and those of UA, HDL-C and CRP in girls are independent of insulin resistance. Not only obese children but also overweight children seem to be high-risk for the future development of CVD. To prevent future development of CVD, it is quite important for school children to maintain BMISD within normal range. However, we should also consider causes other than BMISD, especially in cases of hyper LDL-cholesterolemia in school children.

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Ceiling culture-derived proliferative adipocytes retain high adipogenic potential suitable for use as a vehicle for gene transduction therapy

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[1]*Center for Advanced Medicine, Chiba University Hospital, Chiba University;* [2]*Department of Genome Research and Clinical Application,* [3]*Department of Plastic and Reconstructive Surgery, and* [4]*Department of Immunology, Graduate School of Medicine,* [5]*Chiba University; and* [6]*CellGenTech, Chiba, Japan*

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Asada S, Kuroda M, Aoyagi Y, Fukaya Y, Tanaka S, Konno S, Tanio M, Aso M, Satoh K, Okamoto Y, Nakayama T, Saito Y, Bujo H. Ceiling culture-derived proliferative adipocytes retain high adipogenic potential suitable for use as a vehicle for gene transduction therapy. *Am J Physiol Cell Physiol* 301: C181–C185, 2011. First published April 6, 2011; doi:10.1152/ajpcell.00080.2011.—Adipose tissue is expected to provide a source of proliferative cells for regenerative medicine and cell-transplantation therapies using gene transfer manipulation. We have recently identified ceiling culture-derived proliferative adipocytes (ccdPAs) from the mature adipocyte fraction as cells suitable as a therapeutic gene vehicle because of their stable proliferative capacity. In this study, we examined the capability of adipogenic differentiation of the ccdPAs compared with stromal vascular fraction (SVF)-derived progenitor cells (adipose-derived stem cells, ASCs) with regard to their multipotential ability to be converted to another lineage and therefore their potential to be used for regenerative medicine research. After in vitro passaging, the surface antigen profile and the basal levels of adipogenic marker genes of the ccdPAs were not obviously different from those of the ASCs. However, the ccdPAs showed increased lipid-droplet accumulation accompanied with higher adipogenic marker gene expression after stimulation of differentiation compared with the ASCs. The higher adipogenic potential of the ccdPAs than the ASCs from the SVF was maintained for 42 days in culture. Furthermore, the difference in the adipogenic response was enhanced after partial stimulation without indomethacin. These results indicate that the ccdPAs retain a high adipogenic potential even after in vitro passaging, thus suggesting the commitment of ccdPAs to stable mature adipocytes after autotransplantation, indicating that they may have potential for use in regenerative and gene-manipulated medicine.

gene therapy; adipose tissue-derived stem cells; adipogenesis

ADIPOSE TISSUE is now recognized as a source of proliferative cells for cell-based gene therapy (2) and for regenerative therapy (4, 5). The cells propagated from aspirated fat tissue have been shown to proliferate rapidly and differentiate into mature adipocytes both in vitro and in vivo (2, 4, 5). Although the prepared cells are highly heterogeneous with regard to differentiation and adipogenecity, two types of preparations have been methodologically reported to be sources of adipose tissue-derived proliferative cells. One is the stromal vascular fractions (SVFs), which can be obtained as a sediment by the centrifugation of collagenase-digested fat tissue (15). Numerous studies have reported that adherent cells obtained from SVFs can differentiate into not only adipocytes, but also other cell lineages, and these cells are recognized as adipose-derived stem cells (ASCs) (11). The other cell preparation is obtained from the floating mature adipocytes fraction obtained from the centrifugation, followed by a ceiling culture (13). These cells have mainly been used for the culture of mature adipocytes after proper differentiation stimulation, although their limited abilities to differentiate into other lineages have been demonstrated to be maintained in vitro (9, 10).

In the clinical application of cell-based medicine using preadipocytes to patients, it is required that the transplanted cells reside stably at the subcutaneous adipose space without unexpected proliferation or migration and that they differentiate into adipocytes to reconstruct adipose tissue. We have previously shown the transplantation of gene-transduced adipocytes to be a candidate therapy for patients lacking insulin, growth hormone, or lecithin:cholesterol acyltransferase (1, 6, 7). We have recently identified proliferative cells with a higher adipogenic differentiation potential adequate for this strategy. The proliferative adipocytes obtained immediately after a 7-day primary culture (ceiling culture-derived proliferative adipocytes, ccdPAs) have suitable gene transduction characteristics for gene therapy applications (8). The ccdPAs are expected to provide vehicle cells for protein replacement therapy using autotransplantation of exogenous gene-transduced cells. However, little is known with respect to the differences in the differentiation potential between ccdPAs and SVF-derived ASCs, and a comparison of the adipogenic status between ccdPAs and ASCs would provide insight that would be relevant for plastic and reconstructive surgery, as well as future strategies using adipose tissue-based gene therapy combined with regenerative medicine. In this study, the adipogenic potential of ccdPAs was examined compared with ASCs from SVFs as multipotential adipose tissue-derived cells.

MATERIALS AND METHODS

Cell culture and adipogenic differentiation. The study was approved by the Ethics Committee of Chiba University School of Medicine, and informed consent was obtained from the healthy volunteers. Experiments were performed with the adipose tissue specimens obtained from four different volunteers, and representative data are described in the paper. ccdPAs and ASCs were prepared according to our previous report (8). Essentially, the floating fraction and the sediment after collagenese digestion followed by centrifugation were utilized for source of ccdPAs and ASCs, respectively. The floating fraction was subjected to ceiling culture (13). The sediment was cultured by regular method to obtain adherent proliferative cells

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(ASCs). DMEM/F12-HAM (Sigma-Aldrich, St. Louis, MO) containing 20% fetal bovine serum (FBS, SAFC Biosciences, Lenexa, KS) and 40 μg/ml gentamicin (Gentacin, Schering-Plough, Kenilworth, NJ) was used for both preparations. After 7 days primary culture, ccdPAs and ASCs were passaged twice a week with MesenPRO medium (Life Technologies, Carlsbad, CA) and used for further experiment. Bone marrow derived-mesenchymal stem cells (BM-MSC) were purchased from Lonza (Basel, Switzerland). For adipogenic induction, cells were seeded on 48-well or 6-well plates and then were incubated for 3 days to confluence. Next, growth medium was changed to adipogenic induction medium (Lonza) and cultured for 2 wk and then lipids were stained with Oil Red O.

FACS analysis. The cells cultured in MesenPRO medium for 14 days after the preparation were subjected to analysis of surface antigen as described previously (8). Fluorescein isocyanate (FITC) or phycoerythrin (PE)-conjugated antibodies were purchased from BD Farmingen (San Diego, CA), Beckman Coulter (Fullerton, CA), or Ancell (Bayport, MN). Five thousand events were acquired for each antibody on a FACS Calibur apparatus using the CELLQuest acquisition software program (Becton Dickinson, Flanklin Lakes, NJ).

Gene expression analysis. Total RNA was prepared at each time point by RNeasy kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. One microgram of total RNA was subjected to cDNA synthesis by ReverTraAce qPCR RT kit (Toyobo, Osaka, Japan). The amounts of mRNA were quantified by TaqMan methodology using ABI7500 real-time PCR apparatus. Probe and primer sets for CCAAT/enhancer binding protein δ (C/EBPδ), peroxisome proliferator-activated receptor γ2 (PPARγ2), adipocyte protein 2 (aP2), and leptin genes were purchased from Applied Biosystems (Life Technologies). A C_t value of 35 was considered as detection limit.

Statistical analysis. Data are presented as the means ± SD. Statistical comparisons were made by either Student's *t*-test or by ANOVA followed by the post hoc Dunnett test using the SPSS software program. In all cases, P values of <0.05 were considered to be statistically significant.

RESULTS

The ccdPAs express adipogenic markers and cell surface antigens similar to ASC cells in culture. We obtained ccdPAs after a 7-day ceiling culture as described previously (8). We first examined the expression of adipogenic markers (C/EBPδ, PPARγ2, aP2, and leptin genes) in these cells compared with the ASCs obtained from the SVF of the same fat origin after 7 days of regular plating culture in the same growth medium as the ceiling culture and also to BM-MSCs that were not related to adipocyte lineage. The messenger RNA levels of C/EBPδ in ccdPAs were significantly higher than those in ASCs at *days 1*, *4*, and *7* (Fig. 1*A*). The expression of PPARγ2 was not detected on *days 1*, *4*, *7*, or *14* in any of the three cell lines (ccdPAs, ASCs, and BM-MSCs) (data not shown). The expression of aP2 in ccdPAs and ASCs was detected on *day 1*, and the expression levels in both ccdPAs and ASCs were decreased on *day 4*. On *days 4*, *7*, and *14*, and the aP2 expression level in the ccdPAs was significantly higher than the ASCs, but it was not significantly different from the BM-MSCs, thus indicating that the aP2 expression levels on *days 4*, *7*, and *14* in ccdPAs and ASCs are not physiologically relevant to the adipose lineage (Fig. 1*B*). The expression of leptin was not detected in ASCs and BM-MSCs at any of the time points tested. However, on *days 1*, *4*, and *7*, the expression of leptin in ccdPAs was detected and became undetectable by *day 14* (Fig. 1*C*). After 14 days of preparation, the surface marker expression profiles showed no difference between ccdPAs and SVF-derived ASCs (Fig. 1*D*). Therefore, the expression levels of adipogenic genes and surface markers were not different between ccdPAs and ASCs at 14 days after preparation.

ccdPAs show a higher adopogenic response after differentiation stimulation than ASCs derived from SVF. We evaluated the adipogenesis of ccdPA during differentiation into mature adipocytes. The ccdPAs and ASCs at 14 days after preparation

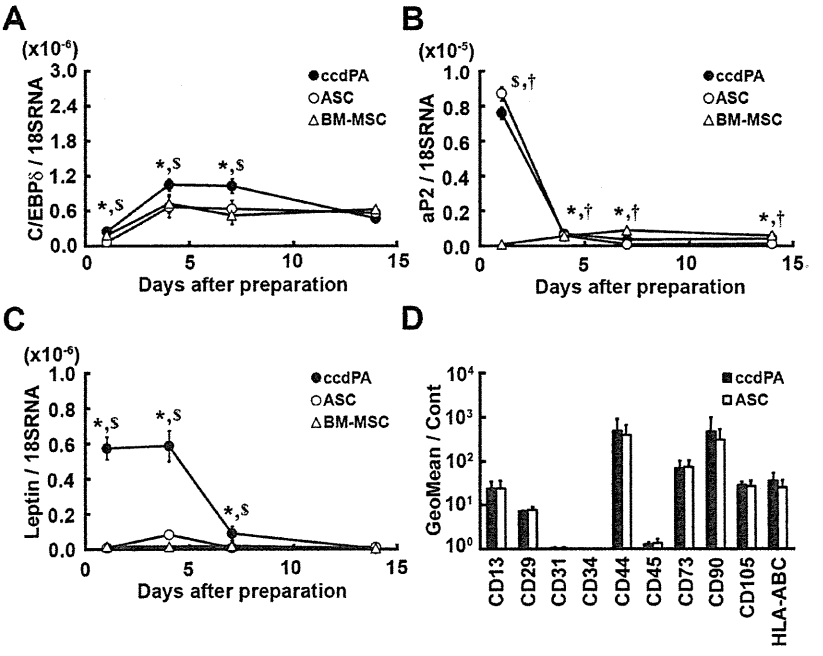

Fig. 1. Expression of adipogenic genes and cell surface markers of ceiling culture-derived proliferative adipocytes (ccdPAs) and adipose-derived stem cells (ASCs). After 7 days of primary culture with DMEM/F12-HAM supplemented with 20% fetal bovine serum (FBS), the ccdPAs and ASCs were passaged with MesenPRO medium. Bone marrow derived-mesenchymal stem cells (BM-MSCs, passage number 3 on *day 0*) were passaged in same manner. At each time point, the expression levels of mRNA for CCAAT/enhancer binding protein δ (C/EBPδ) (*A*), adipocyte protein 2 (aP2) (*B*), and leptin (*C*) were quantified by qRT-PCR. *$P < 0.05$, ccdPA vs. ASC, $$P < 0.05$, ccdPA vs. BM-MSC, †$P < 0.05$, ASC vs. BM-MSC. The expression of cell surface markers was analyzed by flow cytometry at 14 days after preparation (*D*).

were plated and grown for 3 days to confluency and then stimulated for adipogenic differentiation with medium containing insulin, dexamethasone (DEX), 3-isobutyl-1-methylxanthine (IBMX), and indomethacin (IND), and the appearance and adipogenic gene expression were analyzed for 14 days. A histological analysis suggested that the lipid droplet formation had increased in the ccdPAs compared with the ASCs (Fig. 2*A*). An adipogenesis-related gene analysis showed that the expression of PPARγ2 was detectable on *day 1* in both ccdPAs and ASCs and was gradually increased until *day 8* and then declined in both cell lines (Fig. 2*B*). The PPARγ2 expression in ccdPAs was higher than that of ASCs at all time points of stimulation (Fig. 2*B*). The aP2 expression was maximal on *day 8* or *10* (Fig. 2*C*), and its expression was also higher in ccdPAs than in ASCs at all time points (Fig. 2*C*). Therefore, ccdPAs show a higher adipogenic response during differentiation in vitro.

ccdPAs retain higher adipogenic potential than ASCs during in vitro passaging. We next examined the capability of adipogenic differentiation during passaging. Cells freshly harvested after 7 days of primary culture (designated as *day 0* in this text) and the cells that were further cultured until *day 7*, *14*, and *42* were subjected to adipogenic differentiation. During the passage period, the doubling time of ccdPAs and ASCs was not significantly different (1.60 ± 0.34 days vs. 1.57 ± 0.32 days) when they were grown in MesenPRO medium. The histological observations (Fig. 3*A*) showed that both cell lines gradually lost their capabilities for adipogenic differentiation during in vitro passage. At 14 days after stimulation, there was a clear difference in the numbers of differentiated lipid droplet-containing cells. A gene expression analysis showed that the ccdPAs expressed significantly increased levels of aP2 mRNA compared with the SVF-derived ASCs when the cells that were passaged for 0, 7, 14, and 42 days after preparation were subjected to adipogenic stimulation (Fig. 3*B*). These results show that the ccdPAs retain a higher adipogenic potential than the ASCs during in vitro passaging.

ccdPAs exhibit an increased response to the partial adipogenic stimulation compared with ASCs. To further characterize the adipogenic status of the ccdPAs in terms of lineage, we employed different combinations of DEX, IBMX, and IND. After 14 days of stimulation, fine lipid-containing cells were observed in the presence of DEX alone in both the ccdPA and ASC cultures (Fig. 4*A*) but not in the presence of IBMX or IND alone (data not shown). We next omitted each reagent from the full cocktail with DEX, IBMX, and IND. Notably, ccdPAs formed relatively large lipid droplets when IBMX was omitted, whereas the ASCs formed only fine droplets (Fig. 4*A*). Moreover, it was difficult to observe any lipid droplet in the ASCs cultured without IND, whereas the ccdPAs formed lipid droplets. We therefore compared the mRNA levels of the PPARγ2 and aP2 genes in ccdPAs and ASCs (Fig. 4*B*). The ccdPAs expressed both adipogenic genes at levels approximately twofold of those in ASCs on *day 14* after incubation with the full stimulatory cocktail (Fig. 4*B*). The difference in the PPARγ2 mRNA levels of ccdPAs and ASCs was increased to 14-fold when the cells were cultured without IND (Fig. 4*C*). The difference in the aP2 mRNA levels of ccdPAs and ASCs were also obviously increased by ~90-fold under the conditions without IND (Fig. 4*D*). Therefore, the ccdPAs clearly have an increased adipogenic differentiation potential during the partial stimulation in the presence of DEX.

DISCUSSION

We have shown that gene-transduced adipocytes can supply insulin (6) and growth hormone (7) at levels sufficient to

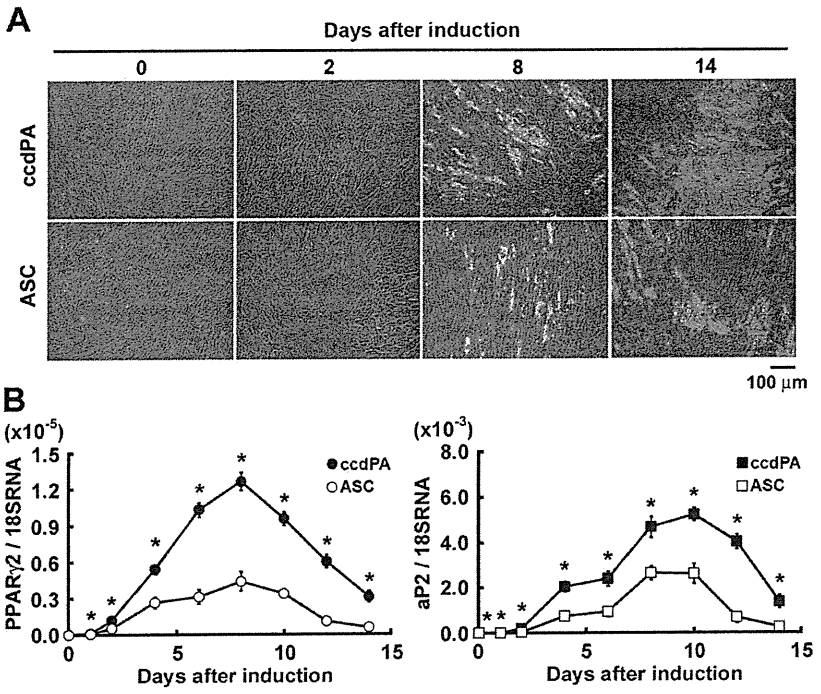

Fig. 2. Comparison of the expression of adipogenic markers in ccdPAs and ASCs during the induction of adipogenesis. *A*: adipogenic induction was performed using ccdPAs (*top*) and ASCs (*bottom*) cultured for 14 days in MesenPRO medium following 7 days of primary culture. The appearance of cells at each time point is shown. *B*: levels of peroxisome proliferator-activated receptor γ2 (PPARγ2) and aP2 gene expression were examined at each time point by qRT-PCR. $*P < 0.05$.

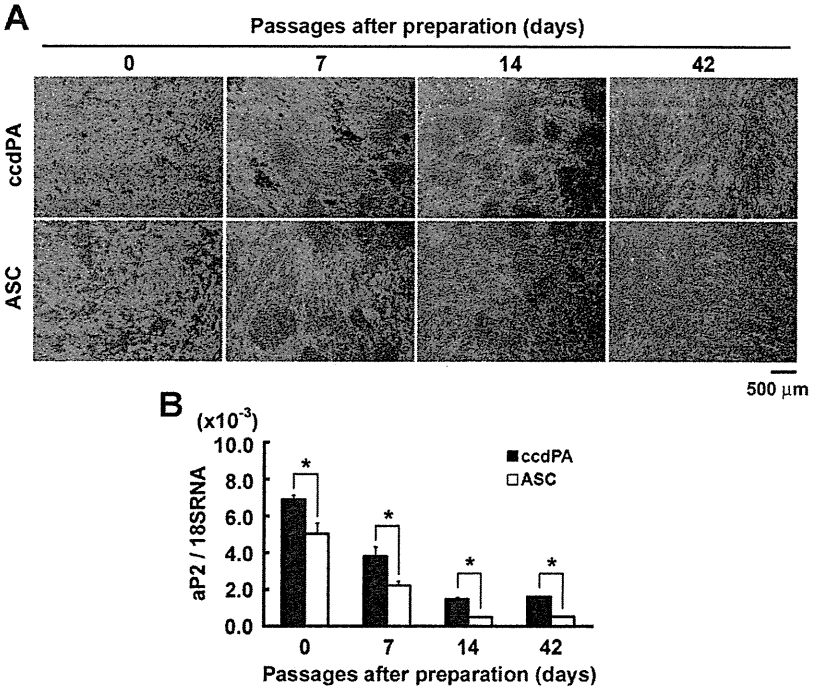

Fig. 3. The effects of consecutive in vitro passaging on the adipogenic potential of ccdPAs and ASCs. The ccdPA and stromal vascular fraction (SVF)-derived cells were obtained after a 7-day ceiling culture and were further cultured in MesenPRO medium for 7, 14, or 42 days. Cells were seeded and incubated for 3 days to confluency, and the medium was replaced by adipogenic induction medium. On *day 14*, the differentiation of the cells was evaluated by the appearance of lipid droplet formation (*A*) and by the expression of the aP2 gene as determined by qRT-PCR (*B*). $*P < 0.05$.

provide improvement of systemic disturbances in animal models. During the development of adipocyte-based protein replacement therapy, the transplanted cells are required to exhibit stable and controllable characteristics of gene transduction efficiency, maintenance of the transduced gene, proliferation, and survival after transplantation, in addition to posing a minimal risk for unexpected phenotypic changes. Considering the successful outcomes for these applications, the properties required for the transplanted adipocytes are different from those for typical regenerative medicine, i.e., homogeneity to

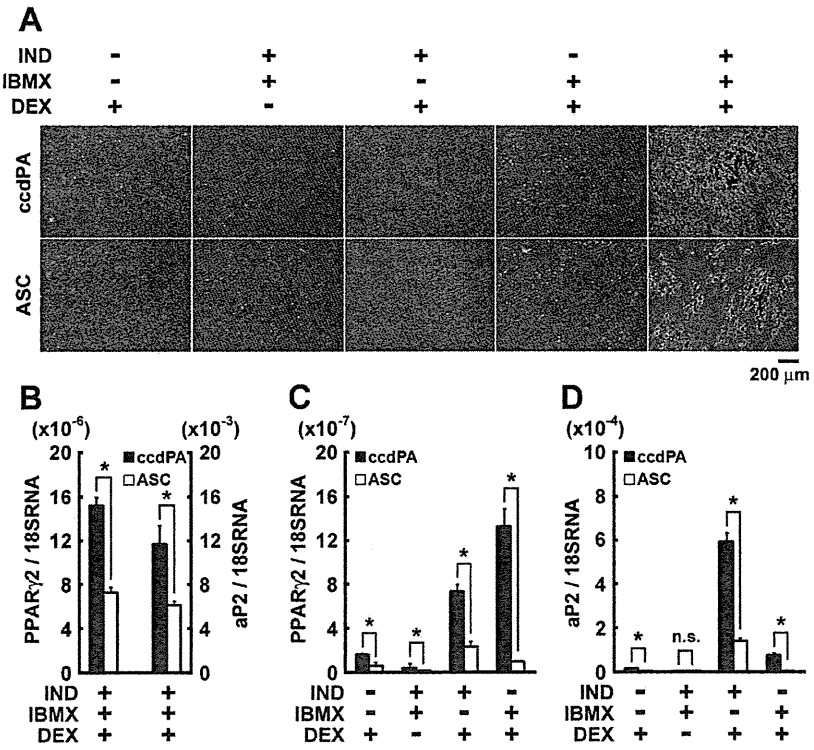

Fig. 4. Effects of differentiation-inducing agents on the adipogenecity and the gene expression levels in ccdPAs. *A*: cells were cultured for 2 wk in growth medium before induction. The appearances of the ccdPAs and ASCs on *day 14* after adipogenic induction with medium containing combinations of the indicated agents are shown. Insulin was included in all medium for the adipogenic induction. The accumulated lipids were stained with Oil Red O. The expression levels of the PPARγ2 and aP2 genes in the cells induced by the full cocktail (*B*) and different combinations (*C*, *D*) of the reagents were quantified on *day 14*. IND, indomethacin; IBMX, 3-isobutyl-1-methylxanthine; DEX, dexamethasone. $*P < 0.05$.

maintain cell stability, but not heterogeneity to keep the multipotentiality.

We have previously utilized the ceiling culture technique to obtain proliferative cells for retrovirus-mediated gene transduction and designated these cells as ccdPAs (8). We identified the optimal primary culture period to be 7 days for high transduction efficiency with minimal integrated copies of therapeutic gene per cell. The obtained gene-transduced ccdPAs stably maintain the exogenously introduced gene during their subsequent culture in vitro. In the present study, we further addressed their adipogenic potential to clarify the suitability of ccdPAs as transplantation cells for use in long-term protein replacement therapy.

The ccdPAs showed increased expression levels of mRNA for the aP2 and leptin genes on *day 1* after 7 days of ceiling culture (see Fig. 1, *C* and *D*). These expression levels of late genes for adipogenic markers had declined to baseline within 7 days of the following culture. At 14 days after preparation, these cells showed no significant difference in their morphological appearance and surface antigen profiles compared with ASCs. However, they exhibited clearly different responsiveness to adipogenic stimuli (see Fig. 2). Even after consecutive in vitro passages, the ccdPAs still had a higher adipogenic potential than the ASCs (see Fig. 3). This higher adipogenic potential was reflected by the observation that ccdPAs expressed increased levels of PPARγ2 and aP2 mRNAs compared with the SVF-derived ASCs (see Figs. 2 and 3). The differences between ccdPAs and ASCs in terms of the mRNA levels for the PPARγ2 and aP2 genes were even more pronounced when the cells were cultured without IND (see Fig. 4). These results suggest that ccdPAs can be easily differentiated into mature adipocytes and/or that ccdPAs are highly homogeneous preadipocytes, most of which retain an adipogenic potential higher than that of ASCs. On the other hand, these results imply that the ccdPAs are less suitable for applications as regenerative medicine in which the cells are intended to differentiate into other cell lineages. In the present study, we used MesenPRO medium as the regular culture medium for ccdPAs, since the medium has greater advantages for expansion capability (8) and the chromosomal stability. It is possible that different culture conditions may be required to be developed for these regenerative medicine purposes. The implication of these findings for the therapeutic strategies based on adipocyte engineered protein delivery includes many metabolic diseases in addition to congenital circulating enzyme deficiencies. The high adipogenic potential of ccdPAs suggests the possible use of ccdPA for improving the cosmetic and metabolic abnormalities observed in lipodystrophy (3, 12, 14). The expandability of the transplanted ccdPA with the secretion properties of leptin and other cytokines should therefore be further studied in future studies.

In summary, ccdPAs retain their capability for adipogenic differentiation longer than ASCs, although the basal levels of the adipogenic differentiation markers examined are undistinguishable between the two cell lines. More precise investigations of ccdPAs using SVF-derived ASCs as reference cells will be helpful not only to distinguish ccdPAs from ASCs but also to provide a better understanding of the mechanism of adipogenesis and the physiology of adipose tissue.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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Amelioration of circulating lipoprotein profile and proteinuria in a patient with LCAT deficiency due to a novel mutation (Cys74Tyr) in the lid region of LCAT under a fat-restricted diet and ARB treatment

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ABSTRACT

Familial lecithin-cholesterol acyltransferase (LCAT) deficiency is a hereditary disease characterized by an abnormal lipid profile, corneal opacity, anemia and progressive renal disease. We report a patient with complete loss of LCAT activity due to a novel *lcat* gene mutation of Cys74Tyr in the lid region of LCAT protein. Esterification of cholesterol in this patient was disturbed by disruption of a substrate binding loop of Cys50-Cys74 in LCAT protein. She had progressive renal dysfunction, proteinuria, corneal opacity, anemia and an abnormal lipid profile. Her serum lipids showed a significant increase in abnormal lipoproteins at the original position in agarose gel electrophoresis and VLDL-cholesterol, and a severe decrease in serum HDL-cholesterol. Lipoprotein analyzes also revealed the presence of an abnormal midband lipoprotein, and a maturation disturbance of HDL particles. Renal function and proteinuria improved following the adoption of a fat-restricted diet and administration of an angiotensin II receptor blocker. The abnormal lipoproteins also decreased after this treatment.

1. Introduction

Lecithin-cholesterol acyltransferase (LCAT) deficiency is an uncommon autosomal recessive disorder which results from a gene mutation of LCAT. Since the first identification of LCAT as a unique plasma enzyme [1], 86 mutations in the LCAT gene have been described. Patients with LCAT deficiency show an abnormal circulating lipoprotein profile as a result of the disturbed esterification of free cholesterol incorporated into high-density lipoproteins. Increased plasma concentrations of unesterified cholesterol, triglyceride (TG) and phosphatidylcholine result in lipid deposition in the tissue. LCAT deficiency develops as two clinically distinct syndromes, familial LCAT deficiency (FLD) and fish eye disease (FED). Patients with FLD show corneal opacities, hemolytic anemia, and progressive renal disease [2]. Renal disease occurs as a result of the loss of enzyme activity against β-lipoproteins rather than against α-lipoproteins. Meanwhile, FED patients develop corneal opacities as a result of a partial deficiency in LCAT activity.

A number of approaches to the treatment of LCAT deficiency have been proposed. LCAT replacement therapy by plasma transfusion produced a marked improvement in the deranged composition of TG-rich lipoproteins and Apo-E concentrations [3,4]. Recent advances in gene therapy have allowed the transplantation of *ex vivo* lcat gene-transduced adipocytes and subsequent production of human LCAT protein in circulating plasma [5,6]. Further, a clinical trial of synthetic LCAT in patients with coronary arterial disease is also currently underway at NIH (NCT01554800). In contrast, a fat-restriction diet improves the hypertriglyceridemia in these patients by reducing TG-rich lipoproteins. The lipid-lowering drugs nicotinic acid and fenofibrate have been shown to ameliorate renal function and proteinuria [7], and corticosteroids and renin–angiotensin–aldosteron (RAA) system blockers such as ACE inhibitors and angiotensin II receptor blockers (ARB) also decreased proteinuria [8–10].

Here, we report a novel LCAT gene mutation in a patient which resulted in disruption of the disulfide bridges essential to the

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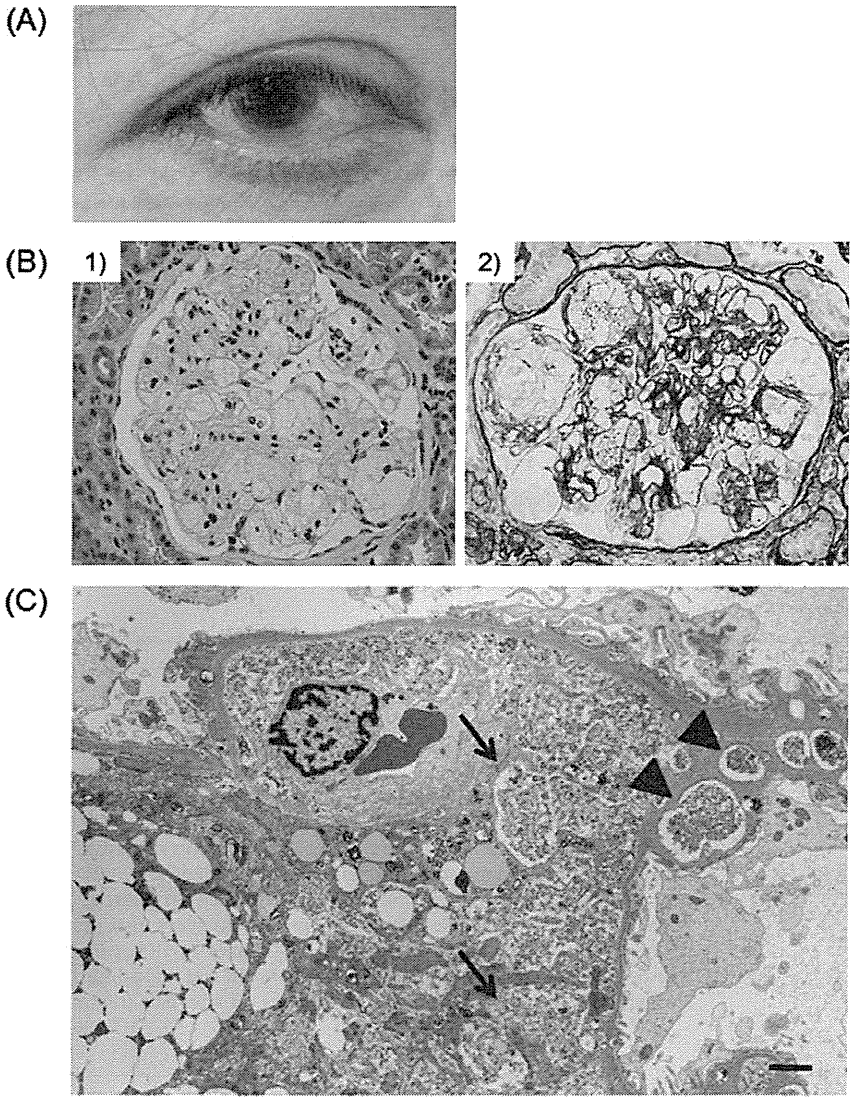

Fig. 1. Ocular and renal pathology (A) Corneal opacity in the patient. (B)-1) PAS staining of a renal biopsy specimen. 400×. An increase in cell number and matrix expansion are seen in the mesangial area of the glomerulus. Foam cell infiltration into capillary loops and the mesangial area are seen in the glomerulus. (B)-2) PAM staining shows irregular thickening and double contours in the glomerular basement membrane (GBM). Foam cells within granular structures are present in the capillary lumen. (C) Electron micrograph of the glomerulus shows the presence of clear vacuoles containing granular structures in the mesangium (↓) and within the GBM (▼). 3000×. Bar = 2 μm.

enzyme lid region. This patient had a complete deficiency in LCAT activity and renal insufficiency. We also investigated the effects of a fat-restriction diet and administration of an ARB on plasma lipoprotein profiles and proteinuria in this patient.

2. Materials and methods

2.1. Biochemical and genetic analysis

Biochemical and urine samples were analyzed by enzymatic methods using a chemical autoanalyzer (Hitachi Co., Tokyo, Japan). Esterified cholesterol concentrations were calculated as the difference between total and free cholesterol. LDL-cholesterol was determined a Determiner-L LDL-C (Kyowa Medex, Tokyo, Japan). LCAT activity in serum was measured using a colorimetric method for analyzing cholesterol esterification rate (CER) with synthetic dipalmitoyl lecithin sol [11]. Alpha-LCAT activity was also measured using Anasolb LCAT® (Sekisui Medical, Tokyo, Japan). Genomic DNA was purified from plasma with a QIAamp DNA kit (QIAGEN, Hilden, Germany). Genomic fragments were amplified by PCR, followed by agarose gel purification and direct sequencing. The entire sequence of the *lcat* gene locus thereby obtained was compared with a reference sequence (NM_000229) to identify nucleotide substitution. The study was approved by the Ethics Committee of Chiba University School of Medicine, and informed consent was obtained from the patient. Both parents were deceased, and informed consent for genetic analysis was not obtained from her younger sister, who shows corneal opacity.

2.2. Lipoprotein analysis

The serum lipoprotein profile was determined by polyacrylamide gel disc electrophoresis [12]. Two-dimensional electrophoresis was performed as described previously [13]. Lipoproteins were also evaluated by agarose gel electrophoresis using the rapid electrophoresis system [14–17]. After electrophoresis, cholesterol and triglycerides in the plates were separately stained and analyzed with a Cho/Trig COMBO kit according to the

manufacturer's instructions (Helena Laboratories, Saitama, Japan). Using the serum total cholesterol and TG concentrations in the blood samples, concentrations of cholesterol and TG in each fraction were determined using the detected ratio of cholesterol and TG after automatic densitometric analysis.

3. Results

3.1. Patient

A 61-year-old Japanese woman was transferred to the Department of Nephrology, Kitasato University Hospital. She complained of dyspnea on walking for the preceding five months, and eyelid and pretibial edema for one month. Family history showed her married parents were cousins, and that her younger sister had corneal opacity and mental retardation. She had anemia (Hemoglobin 9.5 g/dl) bilateral corneal opacities (Fig. 1A) and pitting edema. Urinalysis revealed proteinuria at 2 g/day, 1 + microscopic hematuria, and an N-acetyl-β-D-glucosaminidase (NAG) level of 29.1 unit/L (normal range: 1–4.2). Blood chemistry showed total protein 6.4 g/dL, albumin 3.4 g/dL, total cholesterol 235 mg/dL, TG 235 mg/dL, HDL-cholesterol 22 mg/dL, LDL-cholesterol 39 mg/dL, urea nitrogen 40 mg/dL, creatinine 1.83 mg/dL, and uric acid 8.2 mg/dL. CER of normal sera without heat inactivation was 96.3 ± 10.4 nmol/ml/h ($n = 3$), indicating normal LCAT activity. In contrast, CER of patient sera without heat-inactivation was 16.3 nmol/ml/h, which was below the CER of heat-inactivated normal sera (21.3 ± 1.8 nmol/ml/h, $n = 3$), indicating the total loss of LCAT activities in the patients. The 84 units of alpha-LCAT activity in patient serum measured by Anasorb LCAT® was also markedly low as compared with a standard level of 382–512 units. No abnormalities on chest X-ray, electrocardiography or echography findings were detected in either kidney. The renal biopsy specimen showed glomerular mesangial expansion, and foam cell infiltrates into glomerular tufts and mesangium. PAM staining revealed irregular thickening, double contouring, and vacuolation of the glomerular basement membrane (GBM) (Fig. 1B). Electron microscopic findings revealed numerous small vacuoles and granular structures within the vacuoles in the GBM and mesangial matrix (Fig. 1C). Immunofluorescence revealed negative staining for immunoglobulins of IgG, IgA and IgM, and for complement components of C1q, C4 and C3. These findings are consistent with the findings of LCAT deficiency.

3.2. Gene analysis

Direct sequencing of the *lcat* gene and comparison with a reference sequence (NM_000229) showed that the proband had a novel homozygous G to A nucleotide substitution in exon 2 resulting in Cys74Tyr [c.293 G > A (p.Cys74Tyr)]. The amino acid substitution was a novel mutation in the lid region of the LCAT protein. The substituted cysteine was one of four cysteine amino acid residues which formed the disulfide bonds in construction of the enzyme lid structure [23].

3.3. Lipid and lipoprotein profiles

Serum TG and free cholesterol values were higher than normal, while cholesterol ester, LDL-cholesterol and HDL-cholesterol values appeared lower (Table 1). Densitometric analysis for lipid staining of lipoproteins on disc polyacrylamide gel electrophoresis of serum showed a significant decrease in α and preβ-β positions, and a tiny abnormal "midband" localized on preβ-β position (Fig. 2A). Two-dimensional gel electrophoresis followed by immunodetection for apoprotein showed that distribution of apoprotein was shifted to the smaller HDL particles, indicating that the maturation of HDL particles was impaired (Fig. 2B). The production of LDL particles was thus severely disturbed, as evidenced by the presence of an abnormal "midband" lipoprotein and the disturbed maturation of HDL particles resulting in a severe decrease in HDL lipoprotein.

Table 1
Lipid profiles in a patient with LCAT deficiency following a fat-restriction diet and administration of losartan for 8 months. Lipid profiles at admission and after adoption of a fat-restriction diet consisting of 10 g fat, 45 g protein and 1570 kcal energy per day and administration of losartan 50 mg for 8 months on lipid profile in a patient with LCAT deficiency.

Lipid fraction		Normal value	At admission	At 8 months of treatment
Total cholesterol	(mg/dl)	120–220	235	80
Triglyceride	(mg/dl)	30–150	235	142
LDL-cholesterol	(mg/dL)	70–139	39	33
HDL-cholesterol	(mg/dL)	40–96	22	19
Free cholesterol	(mg/dL)	30–65	205	71
Cholesterol ester	(mg/dL)	90–200	30	9
Free/total cholesterol	(%)	70–80	87	88

Cholesterol and TG staining for lipoproteins which migrate to the α-position on agarose gel electrophoresis could not be seen in the serum at admission (Fig. 3A). Lipoproteins which migrate to the preβ-position were also decreased on both cholesterol and TG staining at admission (Fig. 3A), whereas lipoproteins which migrate to the β-position showed broad bands in both cholesterol and TG staining. The amount of cholesterol and TG in each lipoprotein fraction of serum at admission is shown in Table 2. Apolipoproteins AI and AII, which are predominantly contained in HDL-lipoprotein,

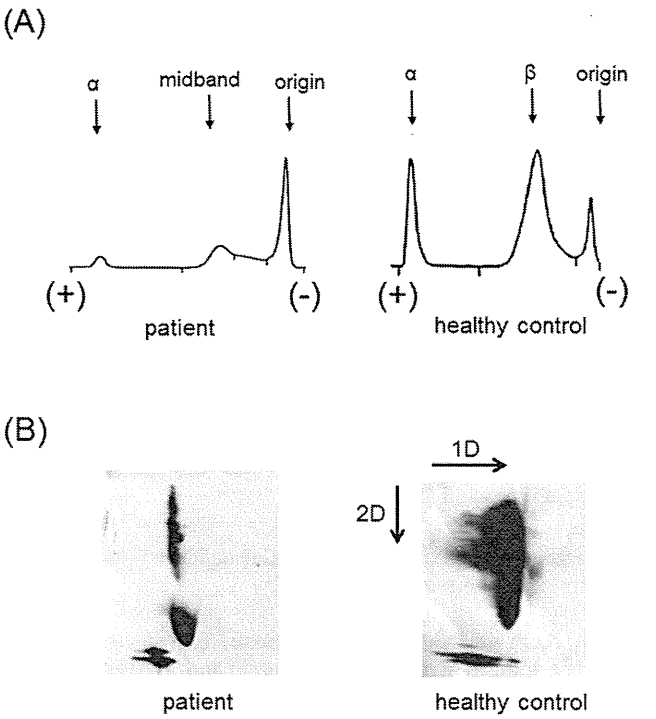

Fig. 2. Densitometric analysis of lipoproteins on disc polyacrylamide gel electrophoresis in the patient and a healthy control. (A) Serum at admission shows an increase in original position lipoproteins, a decrease in α-position lipoproteins, and the appearance of midband lipoproteins instead of β-position lipoproteins. (B) Two-dimensional disk electrophoresis consisting of charge separation for the first dimension and molecular weight separation for the second dimension followed by immunostaining for apolipoprotein. Apolipoprotein distribution was shifted towards the smaller HDL particles, indicating that the maturation of HDL particles in this patient was impaired.

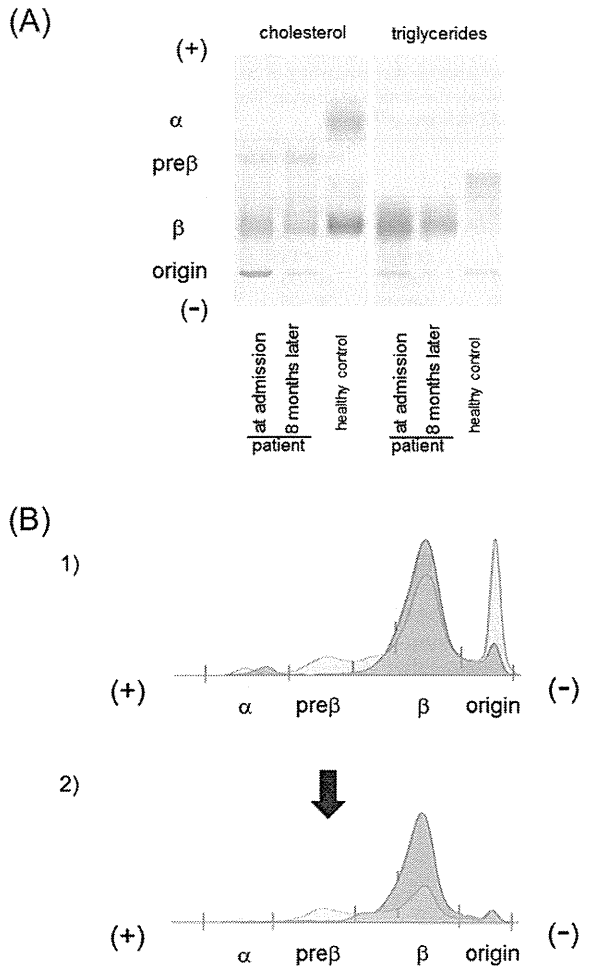

Fig. 3. Staining patterns for cholesterol and triglycerides in lipoproteins on agarose gel electrophoresis. (A) In cholesterol staining, the lipoproteins at original position increased, while the lipoproteins at α- and β-position decreased in the patient at admission compared with those of a healthy control. After 8 months on a fat-restriction diet and administration of losartan 50 mg, a decrease in original position lipoproteins is seen. In triglyceride staining, the serum sample at admission shows a decrease in preβ-position lipoproteins and increase in β-position lipoproteins compared with levels of a healthy control. (B) Densitometric analyzes for staining pattern. Cholesterol staining is shown as a red area, and triglycerides staining as a blue area. (B)-1) Staining patterns for lipoproteins in the patient's serum at admission. (B)-2) Staining patterns for lipoproteins in the patient's serum at 8 months after a fat-restriction diet and administration of losartan 50 mg. Original and β-position lipoproteins stained for cholesterol at admission have decreased after treatment for 8 months.

were decreased at admission (Table 2), whereas apolipoprotein CII and E were increased (Table 2).

3.4. Effects of a fat-restriction diet on LCAT deficiency-induced lipid profile and kidney disease

The patient was prescribed a fat-restricted diet consisting of meals containing 10 g of fat, 45 g of protein and 1570 kcal of energy during admission, and treatment with losartan, an ARB, was started by single daily administration at 50 mg from 14 days after admission. This treatment was continued following discharge, and follow-up at 8 months showed that compliance with both the diet and medication was good. Her body weight decreased from 64 to 52 kg at 6 month after the start of the fat-restricted diet, and was thereafter maintained. At one year of treatment, her proteinuria had decreased from 2.04 g/g·creatinine (Cr) to 0.62 g/g·Cr, and her serum Cr level had decreased from 1.83 mg/dl to 1.10 mg/dl.

Table 2
Apolipoprotein profile in a patient with LCAT deficiency before and after treatment with a fat-restriction diet and administration of losartan for 8 months. Apolipoprotein profile at admission, and effect of a fat-restriction diet consisting of 10 g fat, 45 g protein, and 1570 kcal energy per day for 8 months on apolipoproteins in a patient with LCAT deficiency.

Apolipoprotein fraction		Normal values	At admission	After 8 months of treatment
apoA-I	(mg/dL)	126–165	39	34
apoA-II	(mg/dL)	24–33.3	5.1	3.3
apoB	(mg/dL)	66–101	63	55
apoC-II	(mg/dL)	1.5–3.8	5.6	1.3
apoC-III	(mg/dL)	5.4–9.0	8.6	3.5
apoE	(mg/dL)	2.8–4.6	10.8	5.4

Changes in lipid and apolipoprotein fractions in sera samples collected at admission and 8 months of treatment are shown in Tables 1 and 2, respectively. Surprisingly, adoption of the fat-restriction diet resulted in a decrease in cholesterol, while TG contents and abnormal lipoproteins migrated to their original position (Table 2, Fig. 3A&B). The cholesterol content of lipoprotein at the β-position was also substantially decreased (Table 2, Fig. 3A&B). These data showed that the decrease in abnormal lipoproteins at the original position and change in lipid content in β-lipoproteins were the result of the fat-restriction diet and administration of an ARB.

4. Discussion

In this study, we report a patient with LCAT deficiency who experienced progressive renal dysfunction, proteinuria, anemia and corneal opacity. Her renal biopsy specimen showed many foam cell infiltrates into glomerular capillary tufts and mesangium, and numerous clear vacuoles containing granular structures within the GBM and mesangial matrix. Glomerular foam cells infiltrates are a characteristic feature of LCAT deficiency [18–20]. The numerous clear vacuoles within the GBM and mesangial matrix are also consistent with the findings of a previous study of LCAT deficiency [21]. However, the findings in our patient were not consistent with the structures of odd-shaped electron-dense materials with a membranous profile within clear vacuoles described in that study [21]. Differences in structure in clear vacuoles may be related to patient age or lipid composition in the vacuoles. It is conceivable that the glomerular lipid deposits are fully or partially composed of LpX, a cationized lipoprotein [22]. The glomerular charge barrier is composed of negatively-changed proteins and may be influenced by deposited cationized lipoproteins, resulting in the exacerbation of proteinuria. Indeed, agarose gel electrophoresis of serum from our patient at admission revealed a substantial amount of abnormal cationized lipoproteins suggestive of LpX at the original position (Fig. 3B-1). Proteinuria in this patient might have been induced by the deposition of this abnormal cationized lipoprotein into glomeruli.

Our patient had a novel mutation of Cys74Tyr in the lid region of LCAT protein. LCAT protein contains two functional disulfide bridges, Cys50-Cys74 and Cys313-Cys356 [23]. It appears likely that the Cys50-Cys74 bond was disrupted in our patient. In previous study, disruption of the Cys313-Cys356 bond by an amino acid substitution was shown to result in LCAT deficiency and early onset renal disease [10,24]. The former loop region in the LCAT protein, consisting of Tyr51-Asp73, has binding capacity for HDL- and LDL-cholesterol [25], and truncation of Lys53-Gly71 or Asp56-Leu68 from LCAT protein abolished the ability of LCAT protein to bind HDL- and LDL-cholesterol *in vitro* [26,27]. Our patient also showed a complete loss of LCAT activity, indicating that the former loop

region spanned by Cys50-Cys74 is essential for substrate recognition of LCAT in the esterification process [28]. Further *in vitro* and *in vivo* investigation of the effect of partial transformation of the Tyr51-Asp73 loop region on the cholesterol esterification process may contribute to our understanding of the biochemistry of enzyme–lipid interactions as well as the pathophysiology of LCAT deficiency.

Although sequential ultracentrifugation is a standard method in lipoprotein analysis, we applied agarose gel electrophoresis to detect abnormal LpX and LDL-like lipoproteins and for visualization of lipoproteins. Our patient had a significant increase in abnormal lipoproteins at the original position and a severe decrease in HDL-lipoprotein. Her serum also showed an abnormal midband lipoprotein and a disturbance in the maturation of HDL particles. We suggest that these lipoprotein abnormalities were due to the disturbance in esterification resulting from the loss of LCAT activity.

We also evaluated the effect of a fat-restriction diet and administration of an ARB on lipid profile, proteinuria and renal function in this patient. This treatment decreased proteinuria and resulted in a delay in the deterioration of renal function. A fat restriction diet obviously decreased her serum total-cholesterol and TG, except HDL-cholesterol and LDL-cholesterol at 8 months of treatment. The abnormal cationized lipoproteins at original positions, suggestive of LpX, disappeared after treatment. This disappearance of abnormal cationized lipoproteins in her serum after treatment may have induced a decreased in the amount of deposited cationized lipoproteins within the GBM, thereby resulting in restoration of the charge barrier in the GBM and decrease in proteinuria. The lipid content of lipoproteins at the β-position and the cholesterol content of the midband lipoprotein decreased also after treatment. Lipoproteins accumulating in the kidney are thought to be abnormal apoprotein E-rich lipoproteins which have migrated from the β-position [29], suggesting that the decrease in serum apoprotein E after treatment may be associated with the decrease in accumulated lipoproteins in the kidney. A fat-restriction diet in combination with ARB treatment may contribute to decrease in proteinuria and result in a delay in the deterioration of renal function in patients with LCAT deficiency.

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Original Article

Small Dense Low-Density Lipoproteins Cholesterol can Predict Incident Cardiovascular Disease in an Urban Japanese Cohort: The Suita Study

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Aim: Several lines of evidence indicate that small dense low-density lipoproteins (sd-LDL) are more atherogenic than large buoyant LDL; however, few prospective studies have addressed the role of sd-LDL in cardiovascular disease (CVD). We therefore examined the association between sd-LDL cholesterol (sd-LDL-C) and CVD in a Japanese cohort.
Methods: An 11.7-year prospective study was performed using a general population aged 30-79 without a history of cardiovascular disease. Direct LDL-C and sd-LDL-C were measured in samples from 2034 participants (968 men and 1066 women).
Results: During the follow-up period, there were 116 incident cases of CVD. The multivariable-adjusted hazard ratios (HRs) of sd-LDL-C for CVD were calculated using a proportional hazards regression model after adjusting for age, hypertension, diabetes, use of lipid-lowering drugs, body mass index, and current smoking and alcohol drinking, and found that increasing quartiles of sd-LDL-C were associated with increased risk of CVD. We also determined that age and sex-adjusted HRs per 10 mg/dL of sd-LDL-C and HRs for CVD, stroke, cerebral infarction, and coronary artery disease were 1.21 (95% CI: 1.12-1.31), 1.17 (95% CI: 1.05-1.30), 1.15 (95% CI: 1.00-1.33), and 1.29 (95% CI: 1.14-1.45), respectively.
Conclusions: It was demonstrated that sd-LDL-C was significantly associated with CVD in a Japanese population, providing evidence of sd-LDL-C as an important biomarker to predict CVD.

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Key words; Cardiovascular disease, Lipoproteins, Lipids, Risk factors, Epidemiology

Introduction

The causal relationship between high levels of serum low-density lipoprotein cholesterol (LDL-C) and cardiovascular disease (CVD) has been well established in previous cohort studies[1-5]. Recent clinical trials have also indicated significant event reduction by statins in the primary and secondary prevention of CVD[6-8]; therefore, LDL-C is one of the most important risk factors of CVD and many guidelines, including ours, recommend certain target LDL-C goals for risk management to prevent the development of CVD[5].

Although we use LDL-C as the primary target for cholesterol-lowering therapy, LDL particles are heterogeneous with respect to size and density. Compared to large, buoyant LDL, small dense LDL (sd-LDL) particles exhibit a prolonged plasma residence time, increased penetration into the arterial wall, lower affinity for the LDL receptor, and increased sus-

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