IDL, or VLDL remnants) contain one apolipoprotein B-100 (apoB-100) molecule per particle. CM remnants contain apoB-48, but not apoB-100 [3]. CM remnants are taken up by monocyte-derived macrophages leading to the foam cell formation. Therefore, it is assumed that the measurement of serum apoB-48 concentration can help evaluate the synthesis and metabolism of CM remnants [4]. We have established an enzyme-linked immunosorbent assay (ELISA) to measure serum apoB-48 concentrations [5]. Thus, it has recently become possible to conveniently measure serum apoB-48 concentrations, thereby estimating the number of CMs and CM-remnant particles [4,5].

2. Materials and methods

2.1. Analysis equipment and reagents

We evaluated the basal performance of a recently developed CLEIA for apoB-48 measurement kit (Fujirebio Inc., Tokyo, Japan), carried out on the Lumipulse *f* fully automated immunoassay analyzer (Fujirebio). An in-house ELIS [4,5] provided by Fujirebio was used to measure serum apoB-48 concentrations and confirm the correlativity. Choletest CHO (Sekisui Medical Ltd., Tokyo, Japan) was used for the measurement of total cholesterol (T-CHO); Choletest TG (Sekisui) for triglycerides; Choletest LDL (Sekisui) for LDL-cholesterol; Choletest N HDL (Sekisui) for HDL-cholesterol; and Metabolead RemL-C (Kyowa Medex Co, Tokyo, Japan) for remnant lipoprotein cholesterol, respectively. TBA-200FRneo fully automated chemical analyzer (Toshiba Ltd., Tokyo, Japan) was used for automated measurements.

2.2. Principle of measurement of serum ApoB-48 concentrations

Serum samples were incubated with a treatment buffer solution supplemented with surfactants for separation of apoB-48 from CMs and CM remnants. The pre-treated samples were incubated with ferrite particles coupled with murine monoclonal antibody against apoB-48 in a solid phase. After incubation for 10 min at 37 °C and washing, further incubation was carried out for 10 min at 37 °C with alkaline phosphatase-conjugated anti-apoB-48 monoclonal antibody as a second antibody. After washing, AMPPD [3-(2′-spiroadamantan)-4-methoxy-4-(3″-phosphoryloxy)phenyl-1,2-dioxetane disodium salt] (Applied Biosystems, Bedford, MA) as a substrate was added to the test cartridge, and further incubation was performed for 5 min at 37 °C. Relative chemiluminescent intensity was measured and serum apoB-48 concentration was calculated by a standard curve.

2.3. Samples

We analyzed the data of 6 patients from Osaka University Hospital and 273 clinical samples from Health Care Center Osaka University and Minami-Osaka Total Health Screening Center by multiple regression analysis to examine the influence of other serum lipid values, as well as age, sex, smoking, drinking status and body mass index (BMI) on serum apoB-48 values. The experimental protocol was approved by the institutional ethics committee of the Osaka University Hospital and informed consent was obtained from all patients and healthy volunteers.

2.4. Storage of serum samples

Specimen preservation at room temperature, 4 °C, and −20 °C was investigated in sample sera from 6 control subjects. The preservation period for serum was for 1 week, 2 weeks, and 4 weeks, and collection day, respectively.

3. Methods

3.1. Precision

Within-run and between-run imprecision was evaluated with three concentrations of quality control (QC) material. Ten aliquots were analyzed in one analytical run. Briefly, three concentrations of QC material and three concentrations of human serum were prepared as individual pools. All samples were immediately stored at −80 °C. Duplicate apoB-48 analyses were performed on each pool in two separate runs per day for 10 days. Precision was evaluated as the coefficient of variation calculated from the date series mean and standard deviation.

3.2. Limit of blank and limit of detection

The limit of blank (LoB) and limit of detection (LoD) were determined in accordance with the Clinical and Laboratory Standards Institute (CLSI) EP17-A requirement [6]. The LoD represents the 95th percentile value from measurements of analyte-free samples over several independent series.

3.3. Linearity

We assessed the dilution linearity by serial dilution of 3 human serum samples spiked with recombinant apoB-48 to concentrations covering the whole measuring range. Each sample was diluted 1:4 with analyte-free human serum as diluents in 5 consecutive steps. We measured all dilutions in duplicate with various assay applications and calculated linearity separately for each assay application and instrument.

3.4. Interference studies

The CLEIA for measuring serum apoB-48 concentration was evaluated for common interferences including those due to hyperlipidemia, hemolysis and bilirubinemia by using Interference Check A Plus (Sysmex Co, Hyogo, Japan). Each interference material was evaluated by supplementation of human serum with the indicated to create a high or low interference pool followed by serial dilution with the high to the low pool to create a dilution series.

3.5. Statistical analysis

Data were analyzed by using Stat Flex software (Ver.5.0, Artec Inc., Osaka, Japan), and Mann–Whitney test and two-way ANOVA were used to evaluate the between-group differences. A $p<0.05$ was considered statistically significant.

4. Results

4.1. Imprecision

Imprecision was evaluated as the coefficient of variation (CV%). Within-run and between-run variations were examined using 3 QC materials and 3 kinds of patient pool sera with different concentrations of serum apoB-48 (Low,Middle,High). The largest within-run CV% observed was 2.7% (apoB-48 = 29.0 μg/ml). The largest between-run CV% observed was 7.3% (apoB-48 = 3.2 μg/ml). ApoB-48 imprecision for all samples are summarized in Supplementary Table 1.

4.2. Limit of blank and limit of detection

LoB and LoD for the apoB-48 assay were determined to be 0.06 μg/ml and 0.125 μg/ml, respectively. Linearity was documented by dilution up

Table 1
Multiple regression analysis between serum ApoB-48 level and other lipids or life style (drinking, smoking and BMI).

Characteristics	Regression coefficient	SE	P value
Sex	0.787	0.248	<0.01
Age	−0.008	0.016	0.63
RLP-C	0.253	0.029	<0.01
TG	0.013	0.016	0.46
T-CHO	0.009	0.009	0.11
LDL-C	−0.013	0.009	0.11
HDL-C	−0.013	0.022	0.56
Drinking	0.014	0.013	0.92
Smoking	0.306	0.122	<0.01
BMI	-0.034	0.032	0.29

Male = 1, Female = 0; Yes = 1, No = 0 for drinking and smoking.

to 30.0 μg/ml (Fig. 1). Thus, the analytical measurement range was 0.125–30.0 μg/ml.

4.3. Recovery

ApoB-48 recovery was 96.3–103.5% after 1:9 dilution steps of 3 human serum samples spiked with recombinant apoB-48 to concentrations of approximately 15.1 and 39.9 μg/ml. Identical values were obtained for all assay applications and instruments.

4.4. Interferences

Interference was defined as a>10% change in apoB-48 concentration in the presence of supplemented interference compared to the apoB-48 concentration in the absence of supplemented interference. The acceptable interference concentrations were observed at 19.4 mg/dl (bilirubin F), 20.9 mg/dl (bilirubin C), 523 mg/dl (hemoglobin). These data are demonstrated in Supplementary Fig. 2a, b and c.

4.5. Correlation with ELISA

Fig. 2(a) shows the correlation of the apoB-48 data between CLEIA and ELISA. Correlation of the test results by the ELISA (x) with those by CLEIA (y) for serum samples gave the following regression equation for apoB-48: $y = 1.02 \times -1.59$, $r = 0.953$ ($p < 0.0001$). Fig. 2 (b) shows the Bland–Altman plot which demonstrates a slight overall bias of 0.3% for the CLEIA compared with the ELISA. The 95% confidence interval was from −0.2% to 0.8%.

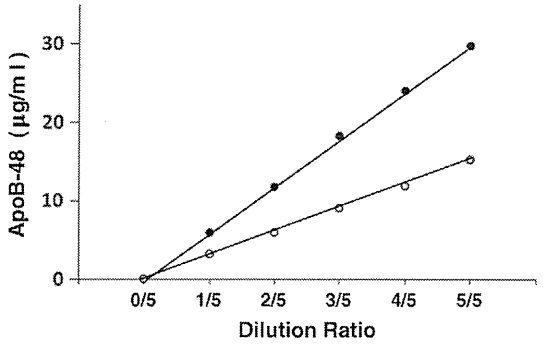

Fig. 1. Linearity of CLEIA for measuring serum ApoB-48. We used the dilution buffer as the blank. CLEIA was performed in duplicate measurements. Two serum samples with a high concentration of apoB-48 (30.0 μg/ml) and a low concentration of apoB-48 (20.6 μg/ml), respectively, were progressively diluted with a dilution buffer, and then assayed.

4.6. Storage of serum samples

Specimen preservation at room temperature, 4 °C, and −20 °C was investigated in sample sera from 6 control subjects. The preservation period for serum was for 1 week, 2 weeks, and 4 weeks, and collection day, respectively.

As shown in Fig. 3a, the apoB-48 concentrations of sample sera kept at room temperature for 2 weeks decreased gradually. The stability of samples was kept for at least 2 weeks at 4 °C (Fig. 3b). The apoB-48 concentration of one sample had decreased at 4 weeks from the beginning. The samples frozen at −20 °C remained stable for at least 4 weeks (Fig. 3c). Thus, it was preferable to keep the samples cold after blood drawing and to freeze them below −20 °C for prolonged storage.

4.7. Healthy reference value of serum ApoB-48

As shown in Fig. 4, the healthy reference value was confirmed for 273 clinical samples from Health Care Center Osaka University and Minami-Osaka Total Health Screening Center. Serum apoB48 concentration distributed log-normally, and the reference values were set at the 95th percentile of the distribution of serum apoB-48. The reference interval of apoB-48 was 0.8–9.7 μg/ml (median: 2.8 μg/ml) in the total subjects. In addition, the reference interval was 1.0–10.3 μg/ml (median: 3.3 μg/ml) for males and 0.7–5.6 μg/ml (median: 2.2 μg/ml) for females, respectively. Therefore, a significant gender difference was observed in the serum apoB-48 concentrations ($P < 0.01$).

4.8. Correlations of lipid values and lifestyle factor

We analyzed the data of 273 clinical samples from Health Care Center Osaka University and Minami-Osaka Total Health Screening Center by multiple regression analysis to examine the influence of other serum lipid values or Life Style (drinking, smoking and BMI). Serum apoB-48 concentrations were correlated with gender and RemL-C concentrations ($P < 0.01$). Moreover, the correlativity was admitted by smoking in the lifestyle as show in Table 1. We compared the serum apoB-48 concentrations according to smoking status and gender. In males, the concentrations of serum apoB-48 of smoker subjects was 1.0–10.8 μg/ml (median: 3.8 μg/ml), that of ex-smokers was 1.0–15.6 μg/ml (median: 2.8 μg/ml), and that of nonsmokers was 0.9–7.1 μg/ml (median: 2.8 μg/ml). The P value of difference by Mann–Whitney test between smokers and ex-smokers, smokers and nonsmokers, ex-smokers and nonsmokers was 0.006, 0.14, and 0.95, respectively (Fig. 5). In contrast, in female subjects, the concentrations of serum apoB-48 were 1.6–6.6 μg/ml (median: 2.3 μg/ml) for smokers, 0.8–4.6 μg/ml (median: 2.1 μg/ml) for ex-smokers, and 0.6–4.6 μg/ml (median: 2.1 μg/ml) for nonsmokers, respectively. The P value of difference by Mann–Whitney test between smokers and ex-smokers, smokers and nonsmokers, ex-smokers and nonsmokers was 0.11, 0.29, and 0.43, respectively. No significant differences in serum apoB-48 concentrations were observed in females according to the smoking status.

4.9. Serum ApoB-48 concentrations in patients with metabolic syndrome

In Japan, the patients with the metabolic syndrome (Mets) can be identified as increased waist circumference at the umbilicus concentration (≥85 cm in men and ≥90 cm in women, respectively), and the presence of 2 or more of the following components: 1) systolic blood pressure ≥130 mm Hg and/or diastolic blood pressure ≥85 mm Hg, 2) fasting serum TG concentrations ≥150 mg/dl and/or serum HDL-C< 40 mg/dl, 3) fasting glucose ≥110 mg/dl [7]. Pre-Mets subjects are identified as having increased waist circumference and one Mets component. Among 211 patients who underwent medical examinations for Mets, 62 were classified as having Mets or pre-Mets. The apoB-48 concentration of these 62 Mets and pre-Mets subjects was higher than

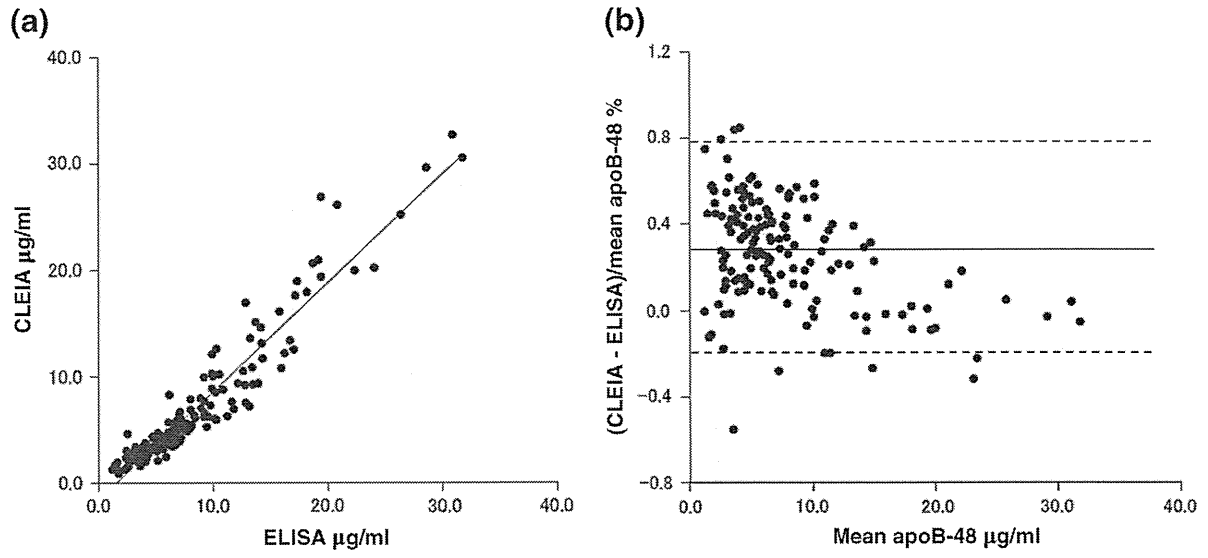

Fig. 2. Correlation of serum ApoB-48 levels determined by ELISA and CLEIA. (a) The serum apoB-48 levels determined by either ELISA or CLEIA in 159 serum samples were well correlated with a correlation coefficient $r = 0.953$ ($y = 1.02x - 1.59$). (b) Bland-Altman analysis showing overall bias of 0.3% for the CLEIA compared with the ELISA. The dashed lines represent 95% limit of agreement.

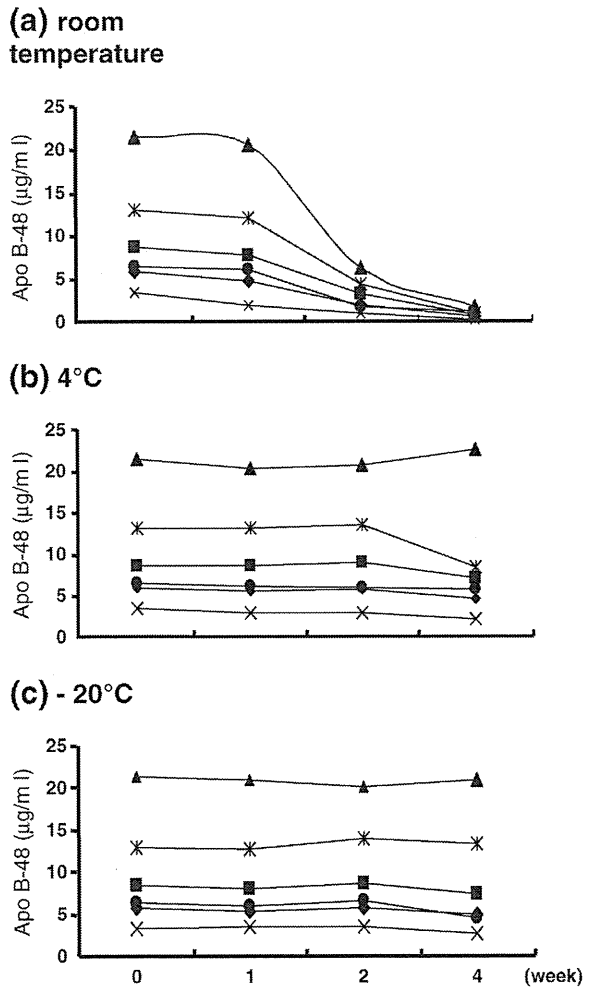

Fig. 3. Effects of storage conditions on serum ApoB-48 concentrations. (a) Samples were stored for indicated number of weeks at room temperature until analysis. (b) Samples were stored for indicated number of weeks in a refrigerator at 4 °C until analysis. (c) Samples were stored for indicated number of weeks in freezer at −20 °C until analysis.

that of non-Mets subjects. The apoB-48 values of Mets and pre-Mets subjects were 1.2–10.2 μg/ml (median: 3.1) while those of non-Mets subjects were 0.8–8.2 μg/ml (median: 2.8), thereby revealing a significant difference ($P = 0.01$) (Fig. 6).

5. Discussion

We previously established an ELISA to measure serum apoB-48 concentrations, using a microplate assay [4,5]. By using this ELISA, we have

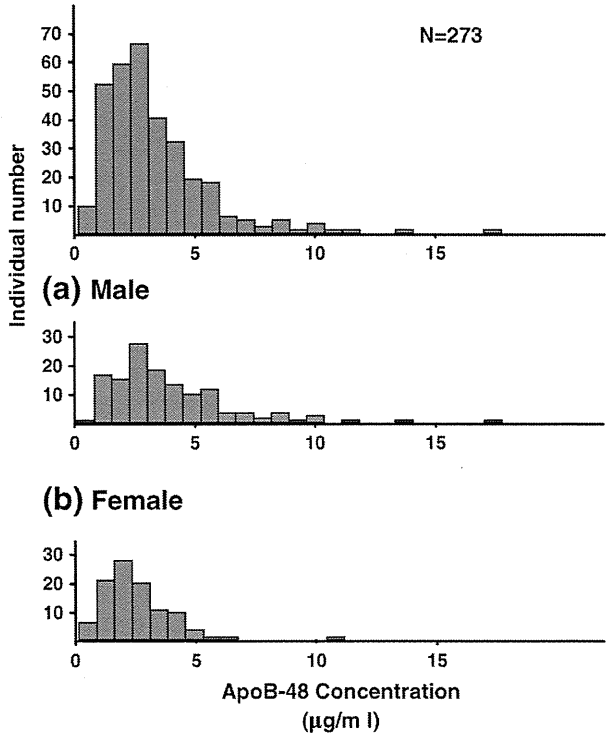

Fig. 4. Histogram of serum ApoB-48 concentration in males and females. (a) Histogram of serum apoB-48 concentration in males ($n = 115$). (b) Histogram of serum apoB-48 concentration in females ($n = 96$).

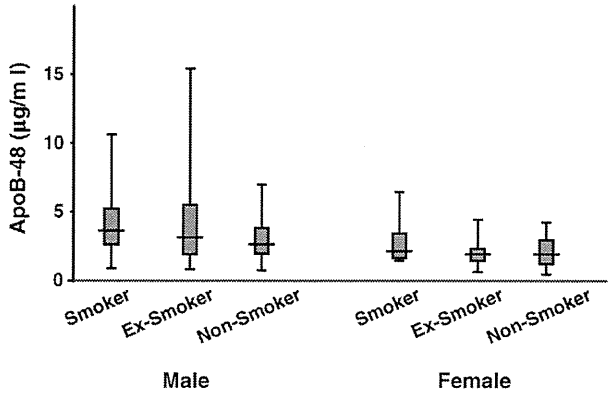

Fig. 5. Interrelationship between serum ApoB-48 levels and smoking status in males and females, respectively. Boxes represent interquartile ranges (IQRs); bold horizontal lines are median; whiskers are lowest and highest values within smokers, ex-smokers and non-smokers. Two-way ANOVA was performed to test the effect of smoking and gender status on apoB-48. Significantly different from baseline, $p < 0.05$.

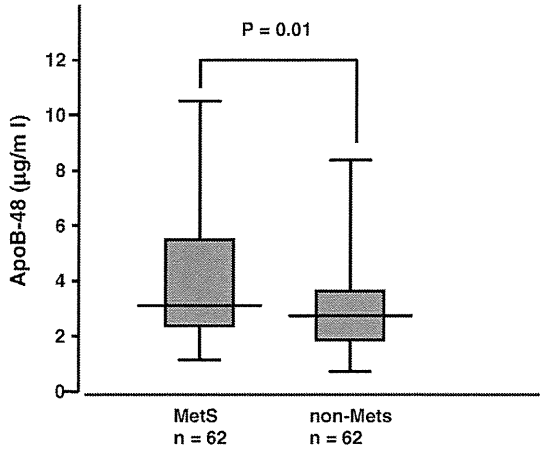

Fig. 6. Comparison of serum ApoB-48 levels between patients with the metabolic syndrome including those with only one risk component and normal subjects. Boxes represent interquartile ranges (IQRs); bold horizontal lines are median; whiskers are lowest and highest values within patients with metabolic syndrome and normal subjects.

reported the apoB-48 concentrations in a variety of patients with lipid disorders [8–11]. ApoB-48 is contained in CMs which are synthesized and secreted from the small intestines as well as in CM remnants. Abnormalities in CM remnant metabolism are related to coronary artery disease [12] and postprandial hyperlipidemia [13,14], and apoB-48 concentration is the one of the indices of abnormality in CM-remnant metabolism. In the current study, we have evaluated the basal performance of a recently developed CLEIA for measuring serum apoB-48, using the Lumipulse *f* automated immunoassay analyzer. The results reported here confirmed the reproducibility, analysis sensitivity, and linearity of the CLEIA. The measurement of apoB-48 by CLEIA was performed easily and conveniently with enough precision, exactness, and correlativity. Our data indicated that samples need to be kept refrigerated at 4 °C for short-term storage and that freezing at −20 °C is preferable in case of prolonged storage.

We analyzed the correlations between serum apoB-48 concentrations and serum lipid values as well as lifestyle-related factors. Serum apoB-48 concentration was correlated with RemL-C concentration, because RemL-C includes cholesterol in the lipoproteins containing apoB-48 (CM remnants) and in those containing apoB-100 (VLDL remnants or IDL). Mets is known to be a strong risk factor for cardiovascular diseases. Serum apoB-48 concentration was higher in male subjects than in female subjects as reported previously [5]. In the current study, we have demonstrated for the first time that serum apoB-48 value was higher in subjects who smoked. Regarding the effect of smoking and gender on serum apoB-48 concentrations, it was speculated that the significant difference between smokers and non-smokers was not due to the smoking habit itself, but to gender. Therefore, the necessity to set a standard value of serum apoB-48 in each sex was suggested. We also found that serum apoB-48 concentrations are increased in subjects with the metabolic syndrome.

It has been shown that Mets facilitates the progression of atherosclerosis [15–17]. Mets and pre-Mets subjects have higher concentrations of apoB-48 than healthy subjects. Therefore, it was suggested that a high apoB-48 value suggests the accumulation of CMs and CM-R in serum, and may be linked to atherosclerosis including cardiovascular diseases.

The mechanisms by which atherosclerosis is enhanced by CM remnants have been investigated in vitro [18]. The particle size of CM remnants is smaller than CMs and these remnants can infiltrate into the vascular sub-endothelial space. Moreover, CM remnants are taken up by macrophages without being oxidized unlike LDL. This causes the formation of foamy macrophages. Furthermore, CM remnants enhance the expression of plasminogen activator inhibitor type I (PAI-1), apoptosis and dysfunction of endothelial cells. CM remnants also accelerate the proliferation of vascular smooth muscle cells and inflammation of vessels. The injury of vascular endothelial cell is also enhanced by an increase in small dense LDL-C and a decrease in HDL-C [19], which are accompanied in patients with postprandial hyperlipidemia.

In patients with atherosclerotic cardiovascular diseases, the increment of serum TG value after meals can be amplified by a delayed catabolism of CM remnants or an increased synthesis of CMs even if fasting serum TG value is normal. A correlation between postprandial hyperlipidemia and cardiovascular diseases has been suggested [1]. Therefore, it is important to determine the number of CM remnants at fasting and after meal for prevention of atherosclerosis.

The presence or absence of postprandial hyperlipidemia cannot be diagnosed easily by the concentrations of fasting TG. Furthermore, the amount of fat in the oral fat tolerance test varies among researchers. A method measuring the concentration of remnants by immune-affinity chromatography technique has been used, and recently a direct method for measuring CM remnants and VLDL remnants using surfactants has been developed and widely used [20]. We were able to quantitatively measure RLP-C [21]. Although, RLP-C measures CM remnants and VLDL remnants, RLP fraction contains large CMs, VLDL and HDL with apoE. Therefore, it has been difficult to distinguish between CM remnants and VLDL remnant by previous techniques [22]. In the current study, we have established and evaluated the CLEIA of apoB-48, which is included in CMs and CM- remnants, each having one apoB-48 molecule per particle.

Taken together, the apoB-48 CLEIA may allow us to evaluate the synthesis and catabolism of exogenous lipoproteins such as CMs and their remnants. A clinical study has demonstrated that the concentration of fasting apoB-48 concentration is closely correlated with the presence of postprandial hyperlipidemia [23]. Therefore, it is necessary to examine and establish the importance of measuring the concentration of fasting serum apoB-48 as a convenient tool for diagnosis and treatment of postprandial hyperlipidemia.

6. Conclusion

We performed the basal examination of apoB-48 by Lumipulse *f* automated immunoassay analyzer. The results were satisfactory enough to develop a clinical application. The concentration of fasting serum apoB-48 is a good marker of exogenous lipoproteins (CMs and CM remnants), and may contribute to the susceptibility of atherosclerotic cardiovascular diseases.

Supplementary materials related to this article can be found online at doi:10.1016/j.cca.2011.09.013.

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Original Article

Early weight changes after birth and serum high-molecular-weight adiponectin level in preterm infants

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Abstract ***Background***: Extra-uterine growth retardation (EUGR) is associated with an increased risk for cardiometabolic diseases later in life. The aim of the present study was to examine the relationship between early weight change after birth in preterm infants and adiponectin (adn) multimeric complexes.

Methods: Subjects included 28 preterm infants born between weeks 24 and 33 of gestation. Serum adn multimeric complexes and the anthropometric parameters were measured in preterm infants at birth and at corrected term.

Results: Bodyweight (BW) decreased during the first week of life, with birthweight restored at approximately 19 days after birth. Nineteen of the subjects had EUGR at corrected term. Total (T)-adn, high-molecular-weight (H)-adn, and the ratio of H-adn to T-adn (H/T-adn) were significantly elevated at corrected term than at birth. Postmenstrual age, birthweight, birth length and lowest BW after birth were positively correlated with H-adn and H/T-adn. Weight reduction after birth was negatively correlated with H-adn. Age to restore birthweight was negatively correlated with T-adn, H-adn and H/T-adn. Stepwise multiple regression analysis indicated age to restore birthweight as the major predictor of T-adn and H-adn.

Discussion: Early weight changes after birth may alter serum adn level in preterm infants at corrected term. The appropriate nutritional support in the early postnatal period could reduce the prevalence of EUGR and the future risk for cardiometabolic diseases.

Key words adiponectin, cardiovascular risk factors, extra-uterine growth retardation, postnatal growth, preterm infant.

Epidemiological studies on humans and experimental studies on animals have shown that small-for-gestational-age (SGA) term infants might have increased risk for cardiovascular disease and metabolic syndrome later in life.[1] To date, most appropriate-for-gestational-age (AGA) preterm infants, especially those born at <34 weeks of gestation, are smaller than AGA term infants at corrected term. In other words, most preterm infants have extra-uterine growth retardation (EUGR).[2] The initial period of weight loss and the delay before birthweight is restored may lead to EUGR in preterm infants.[3] This finding suggests that current early nutritional support for preterm infants in neonatal care may be insufficient for normal growth.[4,5] Recent reports indicate that EUGR children also have a reduction in insulin sensitivity of similar magnitude to that in SGA term children, thereby suggesting that EUGR children have increased risk for cardiometabolic diseases such as diabetes mellitus, dyslipidemia, and coronary heart disease.[6,7] The underlying mechanism that links EUGR with these future risks, however, has yet to be fully clarified.

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In the present study, to clarify the mechanism that links EUGR and cardiometabolic risk, we focused on the relationship between early weight change after birth in preterm infants and adiponectin (adn), one of the adipocytokines secreted by adipocytes, which has several physiological functions. Serum adn consists of three multimer species: high-, middle- and low-molecular-weight adns (H-adn, M-adn, and L-adn, respectively), with H-adn considered to be an active form of this protein.[8,9] Furthermore, the ratio of H-adn to total adn (T-adn; H/T-adn) is more significantly associated with insulin resistance than T-adn alone, thereby suggesting the usefulness of H/T-adn in diagnosing insulin resistance.[8] In adults, serum levels of adn are inversely associated with adiposity-related parameters, insulin resistance, inflammatory markers, and risk factors of cardiometabolic disease.[10–12] In contrast, serum levels of adn in neonates are positively associated with birthweight, with the levels being much higher than those in adults and schoolchildren.[13,14] In preterm infants, serum levels of adn are positively associated with bodyweight (BW) until corrected term. In addition, we demonstrated that the ratio of H-adn to T-adn is lower in preterm infants at corrected term than in term infants.[15] In the present study, we investigated the relationship between early weight changes after birth and serum levels of adn multimeric complexes at corrected term in preterm infants.

Table 1 Early bodyweight changes after birth and clinical variables in preterm infants

Variable	
No. subjects (F/M)	28 (12/16)
Postmenstrual age at birth (weeks)	30.8 ± 2.8 (24–33)
Birthweight (g)	1644 ± 436 (726–2226)
Birth height (cm)	40.6 ± 3.9 (32.0–47.0)
Lowest bodyweight after birth (g)	1431 ± 416 (538–1916)
Weight reduction after birth (%)	13.8 ± 5.2 (6.6–26.1)
Age to lowest bodyweight (days)	6.9 ± 2.5 (3–13)
Age to restore birthweight (days)	19.4 ± 4.9 (13–32)
Energy intake (kJ/kg per day)	
At age to restore birthweight	403.5 ± 58.7 (301.7–548.9)
At corrected term	504.9 ± 80.0 (383.4–712.3)
Bodyweight at corrected term (g)	2291 ± 277 (1724–2928)
Age at corrected term (day)	43.7 ± 24.4 (14–108)
Bodyweight gain ratio (g/day)	13.7 ± 7.7 (5–26.6)

Methods

Subjects

The subjects consisted of 28 preterm infants born between 2005 and 2007. Infants were born between weeks 24 and 33 of gestation to mothers with an uncomplicated pregnancy who had been admitted to the Ryukyu University Hospital, Japan. All infants were born AGA (between 10th and 90th percentile according to Japanese intra-uterine growth grids).[16] Breast milk was started within the first day of life. If breast milk was unavailable, parenteral amino acids (0.5–1.0 g/kg per day) or small-volume formula was started within the third day of life while awaiting breast milk supply. When enteral feeding volumes reached 100–120 mL/kg per day, venous fluid support and parenteral amino acids were stopped. Written informed consent was obtained from the infants' parents. The Ethics Committee of Ryukyu University approved the study protocol.

Clinical and growth-associated factors

Gestational age was confirmed on ultrasound before week 20 of gestation. Birthweight and birth length were measured without delay after birth. The subjects' medical charts were reviewed retrospectively, with data of daily recordings of BW and energy intake (kJ/kg per day) each calculated as early BW-change-related variables (Table 1). Weight reduction after birth (%) and BW gain ratio (g/day) were calculated as (birthweight [g] – lowest BW after birth [g])/birthweight [g])×100 and (BW at corrected term [g] – birthweight [g])/age at corrected term [days], respectively.

Laboratory measurements

Umbilical vein serum samples and serum samples at corrected term, before the discharge from hospital, were stored at –40°C until assay. Serum adn concentrations were measured using sandwich ELISA kits (Daiichi Pure Chemicals, Tokyo, Japan) with a dynamic range of 0.075–4.8 ng/mL. Intra-assay variations (CV) were 5.3% (T-adn), 4.1% [M-adn + H-adn], and 3.3% (H-adn), as described previously.[9]

Statistical evaluation

Differences between series of data were determined using Wilcoxon's rank–sum test. Pearson correlation coefficient was computed to assess the associations between adn and parameters. Because the distributions of T-adn, H-adn, and H/T-adn were markedly skewed, these parameters were normalized using log transformation. Stepwise multiple regression analysis was performed by entering the independent variables with the highest partial correlation coefficient at each step until no variable remained with $F \geq 4$. Data are reported as mean ± SD. Significance was set at $P < 0.05$. All analyses were performed with JMP 5.1 (SAS Institute, Cary, NC, USA).

Results

Clinical characteristics and early BW change indexes are listed in Table 1. Mean weight reduction after birth was 13.8%. Mean age to restore birthweight was 19.4 days. Energy intakes of the infants at age to restore birthweight and at corrected term were 403.5 kJ/kg per day and 504.9 kJ/kg per day, respectively. Nineteen of the infants (19/28; 67.9%) were EUGR at corrected term (defined as BW ≤ 10th percentile according to Japanese intra-uterine growth grids).[16] Mean BW gain ratio from birth to corrected term was 13.7 g/day. As shown in Table 2, T-adn, H-adn, and H/T-adn were significantly higher at corrected term than at birth. Table 3 lists the univariate correlations between early BW change indexes and log T-adn, log H-adn, and log H/T-adn at corrected term. Postmenstrual age, birthweight, birth height, and lowest BW after birth were positively correlated with log H-adn and log H/T-adn. Percent weight reduction after birth was negatively correlated with log H-adn. Age to lowest BW was not correlated with log T-adn, log H-adn, or log H/T-adn. In contrast, age to restore birthweight was negatively correlated with log T-adn, log H-adn, and log H/T-adn. Energy intake and BW gain ratio had no influence on the serum concentration levels of adn multimer complexes. Because each of these parameters can potentially contribute directly to adn multimer complexes, we performed stepwise multiple regression analysis with adn multimers as the dependent variables and the other parameters as independent variables. As shown in Table 4, the major predictor of log T-adn and log H-adn was age to restore birthweight. Age at corrected term was the only predictor of log H/T-adn.

Discussion

The present results show that serum concentrations of T-adn and H-adn at corrected term were inversely correlated with age to restore birthweight in preterm infants. BW at corrected term and BW gain ratio were not selected as significant parameters for

Table 2 Adiponectin multimer complexes vs time in preterm infants

	At birth	At corrected term	*P*
T-adn (μg/mL)	5.9 ± 3.1	21.5 ± 15.0	<0.001
H-adn (μg/mL)	2.4 ± 1.9	10.0 ± 5.7	<0.001
H/T-adn	0.37 ± 0.18	0.49 ± 0.12	0.004

H-adn, high-molecular-weight adiponectin; T-adn, total adiponectin.

Table 3 Adiponectin multimer complexes at corrected term vs clinical variables of preterm infants: univariate correlations ($n = 28$)

	Log T-and		Log H-adn		Log H/T-adn	
	r	*P*	*r*	*P*	*r*	*P*
Postmenstrual age at birth (weeks)	0.22	n.s.	0.45	0.016	0.48	0.010
Birthweight (g)	0.29	n.s.	0.51	0.004	0.47	0.012
Birth length (cm)	0.23	n.s.	0.45	0.016	0.46	0.015
Lowest bodyweight after birth (g)	0.32	n.s.	0.54	0.003	0.47	0.011
Weight reduction after birth (%)	−0.36	n.s.	−0.53	0.004	−0.36	n.s.
Age to lowest bodyweight (days)	−0.04	n.s.	−0.11	n.s.	−0.15	n.s.
Age to restore birthweight (days)	−0.41	0.028	−0.60	<0.001	−0.39	0.042
Energy intake (kJ/kg per day)						
At age to restore birthweight	0.01	n.s.	0.01	n.s.	−0.01	n.s.
At corrected term	−0.05	n.s.	−0.06	n.s.	−0.02	n.s.
Bodyweight at corrected term (g)	0.36	n.s.	0.40	0.036	0.09	n.s.
Age at corrected term (days)	−0.23	n.s.	−0.47	0.011	−0.51	0.006
Bodyweight gain ratio (g/day)	0.24	n.s.	0.17	n.s.	−0.16	n.s.

H-adn, high-molecular-weight adiponectin; T-adn, total adiponectin.

predicting serum concentrations of T-adn and H-adn at corrected term, thereby suggesting that age to restore birthweight in preterm infants might be an important factor in attaining adn multimer status similar to that in AGA term infants.

In AGA term infants, BW increases rapidly during the third trimester of gestation, suggesting that fetal adipose tissue is developed during this period. In contrast, preterm infants appear to be chronically undernourished during the first few weeks of life.[4] Thus, EUGR is generally found in preterm infants regardless of intra-uterine growth status at birth.[4,5] In the present study, 67.9% of AGA preterm infants had EUGR at corrected term. In AGA term infants, serum concentrations of adn complexes were positively associated with birthweight.[15] In the case of AGA preterm infants, serum concentrations of adn complexes are positively associated with BW at corrected term, with concentrations much higher than those in adults and schoolchildren.[13,15] H/T-adn in preterm infants at corrected term, however, is lower than that in AGA term infants.[15] In the present study, serum concentrations of H-adn had a strong association with age to restore birthweight. This finding suggests that insufficient early weight gain after birth in AGA preterm infants may affect the development of adipose tissue quantitatively and qualitatively.

Ibáñez *et al.* recently reported that SGA term children tend to be viscerally adipose and hypoadiponectinemic, even if they are not overweight.[17] Furthermore, recent reports have shown that the risks for developing insulin resistance in intra-uterine growth retardation children are increased if postnatal catch-up growth is evident.[18] Kim *et al.* reported that serum levels of adn are a starvation signal from growing adipose tissue, and that normal-sized adipocytes do not induce insulin resistance, even in the obese condition.[19] Wang *et al.* showed that ectopic fat accumulation occurred even in non-obese mice that had hyperinsulinemia, insulin resistance, and hypertrophy of adipocytes because of subnormal adipocyte storage capacity,[20] while Ruderman *et al.* reported metabolically obese, normal-weight patients.[21] The results of these previous studies, combined with those of the Kim *et al.* study, strongly suggest that in neonates, normal development of adipose tissue is important to prevent future development of cardiometabolic diseases such as diabetes, hypertension, and dyslipidemia. Subnormal development of adipose tissue in neonates may reduce the number of adipocytes. Serum concentrations of adn in preterm infants may be a marker of the development of adipose tissue. If our hypothesis is valid, the limited number of adipocytes in preterm infants with EUGR may induce hypertrophy of each adipocyte more readily than occurs in term infants with AGA. As a result, preterm infants with EUGR may be at high risk for cardiometabolic diseases in the future, even if normal weight is maintained. Taken together, these data suggest that the period of weight gain during the third trimester of gestation may be crucial for the development of cardiometabolic diseases later in life.

Table 4 Stepwise multiple regression for prediction of adn multimers in preterm infants at corrected term

	Selected independent parameters	r^2	*P*
Log T-adn	Age to restore birthweight (days)	0.17	0.029
Log H-adn	Age to restore birthweight (days)	0.36	<0.001
Log H/T-adn	Age at corrected term (days)	0.26	0.006

H-adn, high-molecular-weight adiponectin; T-adn, total adiponectin.

The present results show that the active form of adn multimer complexes increased in the conditions of less weight reduction after birth and shorter time taken for birthweight to be restored. Early weight change after birth may affect the development of adipose tissues. It is of interest that the positive correlation between serum concentrations of adn and BW disappeared at 1 month of age in AGA term infants.[22] The so-called physiological weight reduction after birth may be linked to this phenomenon.

In Japan, the current feeding protocol in neonatal intensive care units is total parenteral nutrition with early lowest enteral feeding, to reduce the risk of infection, necrotizing enterocolitis, and nutritional deficit.[23] Weight reduction, however, is commonly found in preterm infants in the first few weeks. Thus, additional early aggressive nutritional support may be required to minimize early weight reduction after birth.[5] Although further studies are needed, we consider that appropriate nutritional support in the

early postnatal period could reduce the prevalence of preterm infants with EUGR and their future risk for cardiometabolic diseases.

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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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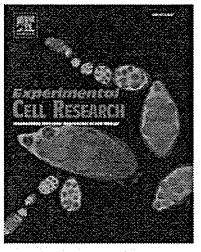

Research Article

Fibrin glue is a candidate scaffold for long-term therapeutic protein expression in spontaneously differentiated adipocytes *in vitro*

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ABSTRACT

Adipose tissue is expected to provide a source of cells for protein replacement therapies via autotransplantation. However, the conditioning of the environment surrounding the transplanted adipocytes for their long-term survival and protein secretion properties has not been established. We have recently developed a preparation procedure for preadipocytes, ceiling culture-derived proliferative adipocytes (ccdPAs), as a therapeutic gene vehicle suitable for stable gene product secretion. We herein report the results of our evaluation of using fibrin glue as a scaffold for the transplanted ccdPAs for the expression of a transduced gene in a three-dimensional culture system. The ccdPAs secreted the functional protein translated from an exogenously transduced gene, as well as physiological adipocyte proteins, and the long viability of ccdPAs (up to 84 days) was dependent on the fibrinogen concentrations. The ccdPAs spontaneously accumulated lipid droplets, and their expression levels of the transduced exogenous gene with its product were maintained for at least 56 days. The fibrinogen concentration modified the adipogenic differentiation of ccdPAs and their exogenous gene expression levels, and the levels of exogenously transduced gene expression at the different fibrinogen concentrations were dependent on the extent of adipogenic differentiation in the gel. These results indicate that fibrin glue helps to maintain the high adipogenic potential of cultured adipocytes after passaging in a 3D culture system, and suggests that once they are successfully implanted at the transplantation site, the cells exhibit increased expression of the transduced gene with adipogenic differentiation.

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Introduction

Much attention has been paid to adipose tissue as a source of transplanted cells for cell-based gene therapy [1] and regenerative therapy [2,3]. The adipose tissue-derived preadipocytes, which can be harvested from aspirated fat tissue, proliferate rapidly and differentiate into mature adipocytes both *in vitro* and *in vivo*. Preadipocytes have been focused on as one of cell systems used to deliver therapeutic genes, since fully differentiated adipocytes are currently utilized in clinical transplantation for the correction of tissue defects in plastic and reconstructive surgery [4–6]. Therefore, we postulated that adipocyte-based protein replacement therapy via subcutaneous transplantation of gene-transduced ceiling culture-derived proliferative preadipocytes (ccdPAs) could be utilized for patients with gene deficiencies such as lecithin: cholesterol acyltransferase (LCAT) deficiency, hemophilia, lysosomal diseases, and diabetes [7–10]. The identified cells have previously been shown to differentiate into mature adipocytes in plate culture upon stimulation, and their differentiation was not affected by the exogenous gene transduction [8,11]. In the clinical application of this strategy for gene-deficient patients, it is assumed that the transplanted cells will reside stably in the subcutaneous adipose space, differentiate into adipocytes, and finally reconstruct the adipose tissue.

For successful treatment in such cell transplantation-based therapies, it is important to select suitable scaffolds for the transplanted preadipocytes, adapting the transplantation site to optimize their survival, differentiation and protein expression. In this context, fibrin glue (FG) is capable of supporting the secretion of the exogenously transduced-gene product *in vivo* [12]. In order to investigate the cell fate and protein secreting function, and also to develop alternative therapeutic applications, the establishment of an *in vitro* long-term evaluation system is required. Various synthetic and naturally-derived materials have so far been investigated as biocompatible scaffolds for adipose tissue 3D models [13–18].

In this study, we employed an *in vitro* long-term 3D culture system using FG as a therapeutic cell transplantation scaffold, and examined the cell survival, differentiation, and the expression of the transduced gene, of the ccdPAs cultured under these conditions.

Materials and methods

Preparation lcat gene transduced ceiling culture-derived proliferative adipocytes (ccdPA/lcat)

Subcutaneous adipose tissues were obtained from healthy donors after informed consent was obtained with approval from the ethics committee of Chiba University School of Medicine, and all studies were performed according to the guidelines of the Declaration of Helsinki. The preparation of ccdPAs, subsequent *lcat* gene transduction, and quantification of the copy number of transduced *lcat* genes were performed as described previously [8]. The cells utilized in this study had an average *lcat* gene copy number of 1.01 ± 0.03 copies/cell. LCAT secretion into the culture supernatant was examined by immunoprecipitation/western blot analysis and measuring the esterifying activity, as described previously [8].

Culture in the fibrin scaffold

Benesis (Benesis, Osaka, Japan) or Bolheal (The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan) was used as a clinically available material for the fibrin gel. Fibrinogen and thrombin solutions were diluted with Ringer's Solution (Fuso Pharmaceutical Industries, Osaka, Japan) containing 0.5% human serum albumin (Mitsubishi Tanabe Pharma., Tokyo, Japan). The ccdPA/*lcat* cells were suspended at 1 and 3×10^7 cells/ml in the diluted fibrinogen and thrombin solution. Because of the substantial gel shrinkage observed on Day 84 in the cultures with 3×10^7 cells/ml, the experiments were performed at a concentration of 1×10^7 cells/ml for long-term evaluation of the cultured cells. The final concentration of fibrinogen was 4, 8, or 40 mg/ml and the thrombin solution was used at 1 U/ml. To form fibrin clots, 50 μl of the cell-fibrinogen suspension was added to each cell culture insert (Falcon 3104; Becton Dickinson, Franklin Lakes, NJ), then shortly thereafter, 50 μl of the cell-thrombin suspension was added into the insert, mixed by pipetting, and incubated at room temperature for 2 h. The inserts with fibrin clots were placed in 12-well culture plates, and 1 ml and 2 ml of MesenPRO medium (Invitrogen, Carlsbad, CA), which had been shown to have the potential to significantly increase the proliferation and stability of ccdPAs and mesenchymal stem cells in comparison to conventional medium [8,19–21], were added to the inserts and wells, respectively. The plates were incubated at 37 °C in a 5% CO_2 incubator. At each time point, culture media were collected and stored at −30 °C until analysis. For collection of the culture supernatant, the culture medium was changed to fresh medium 3 days prior to the harvest.

Measurement of leptin and plasminogen activator inhibitor-1 (PAI-1)

The leptin and PAI-1secretion into the culture supernatant were examined using ELISA kits obtained from MILLIPORE (Billerica, MA) and R&D Systems, Inc. (Minneapolis, MN), respectively.

Cell viability analysis

The cell viability in the 3D gels was examined by the formation of water-soluble formazan dye using the Cell Counting Kit-8 (DOJINDO, Tokyo, Japan) according to manufacturer's instructions, with slight modifications. The FG/ccdPA/*lcat* 3D gel was prepared at a final cell concentration of 1×10^7 cells/ml with 4, 8, or 40 mg/ml of fibrinogen, and cells were subsequently cultured as described in the above section. To examine the cell viability, 100 μl or 200 μl of WST-8 solution was added to the inserts and wells, respectively, and the cells were incubated at 37 °C in a 5% CO_2 incubator for 30 min. The culture supernatants of the inserts and wells were mixed, and the absorbance was measured at 450 nm. The value without cells (negative control) at 450 nm was subtracted from each value.

Histological and immunohistochemical analyses

Photographs of the gels were taken at each time point, and the areas of each gel were calculated using the WinROOF software program (Mitani Corporation, Tokyo, Japan) and used to evaluate the degree of gel shrinkage. All samples of ccdPA/*lcat* cultured in

the fibrin scaffold were washed with PBS (Sigma-Aldrich, St. Louis, MO) and embedded in OCT compound (Sakura Finetek Japan, Tokyo, Japan). These samples were stored at −80 °C until they were analyzed. Sections (5 μm thick) were fixed in 10% formaldehyde and stained with hematoxylin and eosin (HE). Adipogenic differentiation was identified by Oil Red O (Sigma-Aldrich) staining for 15 min at 37 °C, and the specimens were counterstained with hematoxylin. The cell numbers were counted, and the area positive for the Oil Red O signal was measured in four independent areas using the WinROOF software program (Mitani Corporation). We defined the lipid droplet accumulation rate (lipid area/cell number) as the lipid accumulating index.

LCAT production was investigated by immunohistochemistry using an anti-human LCAT antibody. Endogenous peroxidase was inactivated by incubating the samples in 0.3% H_2O_2 in methanol for 10–20 min. Nonspecific binding was blocked by treatment with 5% skim milk and 2% bovine serum albumin in PBS for 30 min. Specimens were then incubated with an anti-LCAT rabbit monoclonal antibody (Epitomics, Burlingame, CA) at a dilution of 1:250 in a humidified chamber at 4 °C overnight. The primary antibody reactions of LCAT were enhanced using the Envision+kit (DAKO, Glostrup, Denmark). The immunoreaction was visualized with 0.05% DAB (Sigma-Aldrich) solution for 30 s to 2 min at room temperature. After washing in distilled water, the specimens were counterstained with hematoxylin, dehydrated and mounted.

RNA extraction and quantitative real-time RT-PCR

RNA was extracted from ccdPAs on the fibrin scaffold by using the TRIZOL reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Quantitative real-time RT-PCR was performed to investigate the expression level of PPARγ2, aP2, leptin, *lcat*, and 18S rRNA as an internal control. Five hundred nanograms of total RNA was used for the synthesis of cDNA, by using a ReverTraAce qPCR RT Kit (Toyobo, Osaka, Japan). Quantitative real-time PCR was carried out on a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA), using TaqMan Gene Expression Assays (Applied Biosystems). In all, 1–2 μl of cDNA solution corresponding to 50–100 ng of total RNA was subjected to 40 PCR cycles of 15 s at 95 °C, then 60 s at 60 °C in a 20 μl mixture containing 10 μl TaqMan Gene Expression Master Mix (Applied Biosystems), 1 μl of TaqMan Gene Expression Assays (target gene) and 1 μl of TaqMan Gene Expression Assays (18S rRNA). The abundance of the mRNA expression of the target genes was normalized to that of 18S rRNA, and the ΔCT was produced by subtracting the mean CT of controls from the CT of each target gene.

Statistical analyses

The data are presented as the means ± S.D. Statistical comparisons were made by Student's *t*-test or by ANOVA followed by the post hoc Tukey test using the SPSS software program. The gel shrinkage, cell viability, lipid accumulating index, and mRNA levels for the *lcat*, PPARγ2, aP2, and leptin genes were analyzed to determine whether there was a linear correlation between these variables. For this analysis, we calculated a linear correlation coefficient (Pearson r value) and the corresponding P-value (two tailed) based on these assumptions. P values <0.05 were considered to be significant.

Results

ccdPAs secrete the functional protein translated from an exogenously-transduced gene, as well as physiological adipocyte proteins, in the 3D fibrin gel culture system

The suitability of fibrin gel as a scaffold in the 3D culture system for the secretion of protein produced in stable exogenous gene-expressing preadipocytes was studied using ccdPA/*lcat*, the ceiling culture-derived proliferative adipocytes transduced with the human *lcat* gene [8]. The FG/ccdPA/*lcat* 3D gel was prepared at a final cell concentration of 1×10^7 cells/ml, and cultured in MesenPRO medium (see Materials and methods). The secreted LCAT protein, the exogenously transduced-gene product, was detected by immunoprecipitation/western blot analysis in the culture supernatant (Fig. 1A). The supernatant of the ccdPA/*lcat* in 3D gel showed significantly increased cholesterol esterifying activity in comparison to that of ccdPA cultures (without *lcat* gene transduction) in the gel (Fig. 1B). The amount of leptin and PAI-1, an active molecule important for the regulation of lipid and glucose metabolism, and a regulator in plasminogen activator/plasmin system [22–24], respectively, were increased in the course of 3D culture (Fig. 1C). Thus, the functional transduced gene product was secreted, in addition to the physiologically produced adipocyte-derived proteins, in the media of the FG/ccdPA/*lcat* 3D gels.

The ccdPAs survive for at least 84 days in 3D gels in a fibrinogen concentration-dependent manner

We analyzed the viability of preadipocytes cultured in the FG/3D gel for longer periods, up to 12 weeks. Obvious gel shrinkage was observed time-dependently in the wells cultured with the 4 or 8 mg/ml concentrations of fibrinogen at a cell concentration of 1×10^7 cells/ml (Fig. 2A). The WST-8 assay showed that the cell viabilities in the gels cultured with 4 or 8 mg/ml fibrinogen were significantly lower than that of the cells treated with 40 mg/ml, throughout the culture period (Fig. 2B). The cell viabilities were significantly and positively correlated with the extents of gel shrinkage among the analyzed points with the three different concentrations of fibrinogen ($p<0.05$, $r=0.934$) (Fig. 2C). Thus, the long-term viability of ccdPAs (for up to 84 days) was regulated by the fibrinogen concentrations in the 3D gel culture system.

The ccdPAs accumulate lipid droplets spontaneously and in a fibrinogen concentration-dependent manner in 3D gels

We next analyzed the lipid accumulation in ccdPAs to determine their ability to differentiate into mature adipocytes in the 3D gel culture system. Oil red O staining of embedded cells in sections suggested that the cells spontaneously started to accumulate lipid droplets around Day 28, and then the droplets became larger and more prevalent during the reamining period (Fig. 3A, at 4 mg/ml fibrinogen). The lipid accumulation analysis clearly showed that the cells cultured with 4 or 8 mg/ml of fibrinogen started to accumulate intracellular lipids after 56 days, and the cells cultured in the 40 mg/ml concentration started the accumulation after 84 days in culture (Fig. 3B). The ratios of lipid accumulation/cell number on Days 56 and 84 were significantly higher in the cells cultured with 4 mg/ml of fibrinogen than those cultured

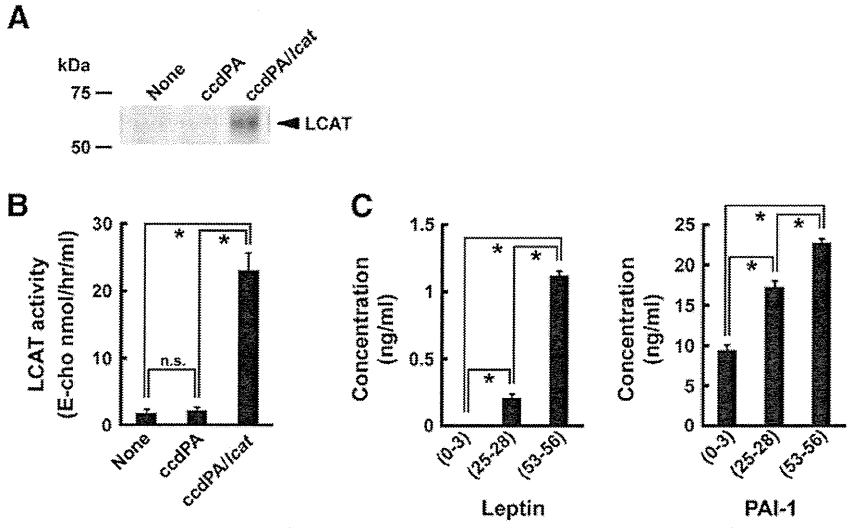

Fig. 1 – 3D fibrin gel culture of *lcat*-expressing ccdPAs. 3D fibrin gels were prepared without cells (None), or with ccdPA with or without *lcat* gene transduction, and were subsequently cultured for up to 84 days. The LCAT secretion was examined by immunoprecipitation/western blot analysis (A) and by measuring the cholesterol-esterifying activity (B) in the culture supernatant from Day 4 to Day 7. *$p < 0.05$. (C) Leptin and PAI-1 were quantified by an ELISA in the three day culture supernatant of the FG/ccdPAs/*lcat* culture collected from Day 0 to Day 3 (0–3), from Day 25 to Day 28 (25–28), and from Day 53 to Day 56 (53–56). *$p < 0.05$. Leptin in the supernatant from Day 0 to Day 3 was below detection limit.

in 8 or 40 mg/ml of fibrinogen, and were also higher in those cultured with 8 mg/ml of fibrinogen than in those cultured with 40 mg/ml of fibrinogen (Fig. 3C), suggesting that the lower concentrations of fibrinogen induce lipid accumulation in ccdPAs earlier and to a greater extent in the gel. The observation that the cell numbers in a single field apparently increased during the culture period in Fig. 3A, together with the identified gel shrinking (see Fig. 2), prompted us to analyze the cell density in the gel as a regulator of lipid accumulation at different fibrinogen concentrations. The quantitation of cell numbers in sections showed that cell densities were gradually increased during the culture from 7 days to 84 days, and reached numbers on Day 84 that were about 3 to 4-fold those on Day 7 days at all of the above concentrations of fibrinogen (Fig. 3D). This suggests that the lipid accumulation starts at a cell density of 200/field in the gel. Therefore, ccdPAs spontaneously accumulate lipid droplets, and the accumulation can be regulated by the fibrinogen concentration, possibly through the effects of the cell density in the 3D gel.

The effects of cell density and the fibrinogen concentration on the expression of the exogenously introduced gene in ccdPAs

An exogenously transduced gene has previously been shown to be stably expressed for 3 months in plate culture without any modification of the proliferative activity of ccdPAs [8]. We herein examined the transduced gene expression in cells cultured in 3D-fibrin gel (3D/FG). The *lcat* expression levels were not significantly different between the cells that were seeded at a density

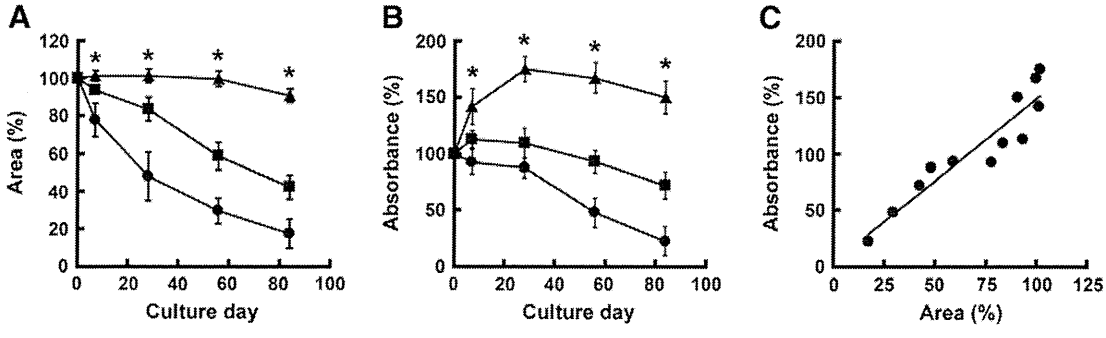

Fig. 2 – The status of 3D gel shrinkage and the survival of ccdPAs/*lcat* in the gel. The 3D fibrin gels were prepared with ccdPAs/*lcat* using 4 (closed circle), 8 (closed square), or 40 mg/ml (closed triangle) of fibrinogen, and were subsequently cultured for up to 84 days. (A) The status of gel shrinkage was expressed by the area (%) using the area of the original gel as 100%. (B) The viability of the cells in the gels was also examined, and expressed using the absorbance values with original gels as 100%. The data are presented as the means ± SD ($n = 4$). Asterisks (*) depict that the differences were significant among the three concentrations of fibrinogen ($p < 0.05$). (C) The correlation of the cell viability with the degree of gel shrinkage was evaluated in ccdPAs cultured with the three different concentrations of fibrinogen in the 3D gel ($p < 0.05$).

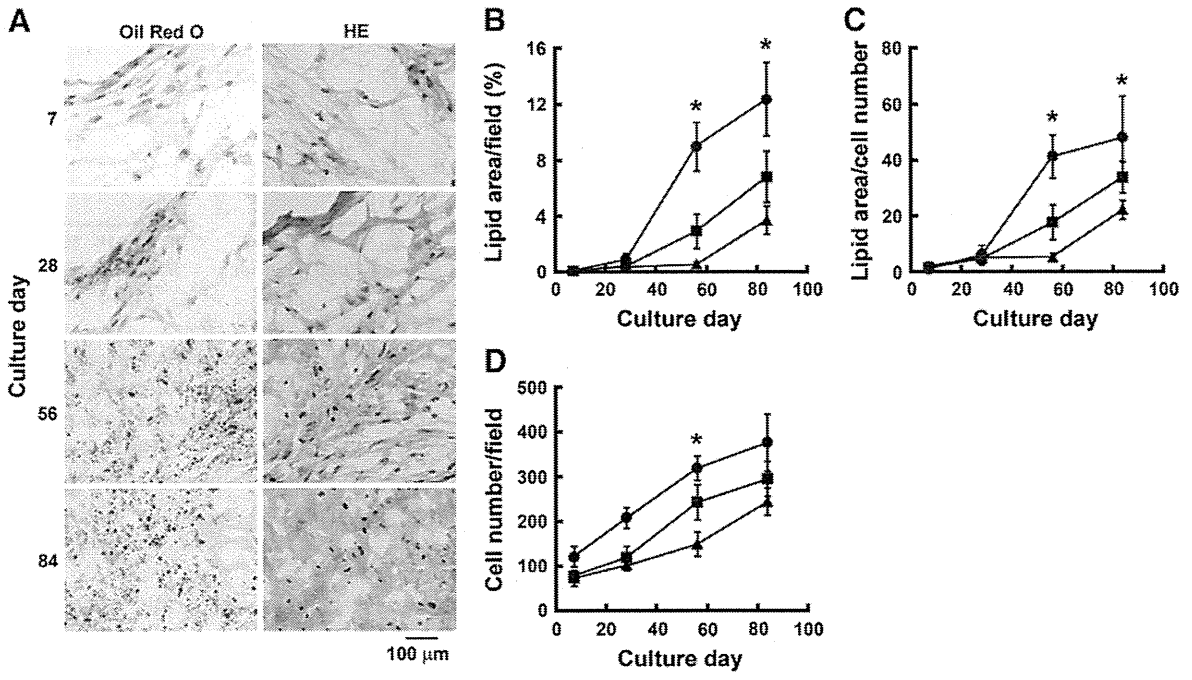

Fig. 3 – The effects of the fibrinogen concentration on the lipid accumulation of ccdPAs cultured on fibrin scaffolds.
(A) Representative data from the histological analyses of cross-sections with 4 mg/ml fibrinogen are shown. Lipid accumulation was observed by Oil Red O staining (left panel). The sections were examined by staining with hematoxylin and eosin (HE) (right panel). (B–D) The cross-sections of the 3D fibrin gels with 4 (closed circle), 8 (closed square), and 40 mg/ml (closed triangle) fibrinogen were examined. (B) Lipid droplets were quantified by evaluating the red colored area using Oil Red O stained specimens. (C) The lipid accumulation rates were evaluated based on the lipid accumulating index. (D) The cell number in the fibrin gels was evaluated by counting the number of nuclei. The data are presented as the means ± SD ($n=4$). Asterisks (*) depict that the differences were significant among the three concentrations of fibrinogen ($p<0.05$).

of 1×10^7 cells/ml or 3×10^7 cells/ml after 56 days in culture (Fig. 4A). We observed that the *lcat* gene expression levels in the cells after 56 days of culture were dose-dependently decreased by the fibrinogen concentration (Fig. 4B). When the fibrinogen concentration was 40 mg/ml, there was a 55% decrease in *lcat* expression in comparison to that at 4 mg/ml. Immunostaining showed that the translated *lcat* gene product could be clearly observed in ccdPA cultures on Day 56 (Fig. 4C). Furthermore, the immunodetected signal was intensely detected in the lipid accumulation area, rather than the area without lipid accumulation. Therefore, the expression levels of the transduced exogenous gene with its product in ccdPAs were maintained at 56 days in 3D/FG, and affected by the fibrinogen concentration.

The effects of the fibrinogen concentration on the adipogenic differentiation of ccdPAs in 3D gel

The above results suggested that the fibrinogen concentration caused decreased exogenous gene expression in ccdPAs, possibly by inhibiting the differentiation of adipocytes in the 3D/FG. Therefore, we analyzed the effect of the fibrinogen concentration in the gel on the expression levels of genes important for adipocyte differentiation in the cultured ccdPAs. The increased fibrinogen concentration resulted in a decrease in the mRNA level of PPARγ2, a differentiation-related transcription factor (Fig. 5A). The expression of aP2, another adipogenesis-related gene, was also significantly decreased by the 40 mg/ml fibrinogen concentration (Fig. 5B). In contrast, the expression of leptin, an adipocyte-secreting hormone, increased due to the increased fibrinogen concentration (Fig. 5C). These results indicated that the fibrinogen concentration modifies the adipogenic differentiation of ccdPAs and their exogenous gene expression levels, in the 3D gels.

The relationship between adipogenic differentiation and exogenous gene expression of ccdPAs in 3D gel

Finally, we analyzed the relationship between the exogenous gene expression and the adipogenic differentiation in 3D/FG with different concentrations of fibrinogen. The *lcat* gene expression levels significantly and positively correlated with the amount of lipid accumulation in the ccdPAs ($p<0.05$, $r=0.967$) (Fig. 6A). The *lcat* gene expression level was also significantly correlated with the PPARγ2 mRNA expression in the ccdPAs ($p<0.05$, $r=0.852$) (Fig. 6B). Therefore, the levels of exogenously transduced gene expression in the cultures with different fibrinogen concentrations were dependent on the extent of adipogenic differentiation in the 3D gels.

Discussion

For long-lasting enzyme replacement therapy, the transplanted cells need to stably and functionally survive at the transplanted site. To achieve this general requirement, the candidate cell types

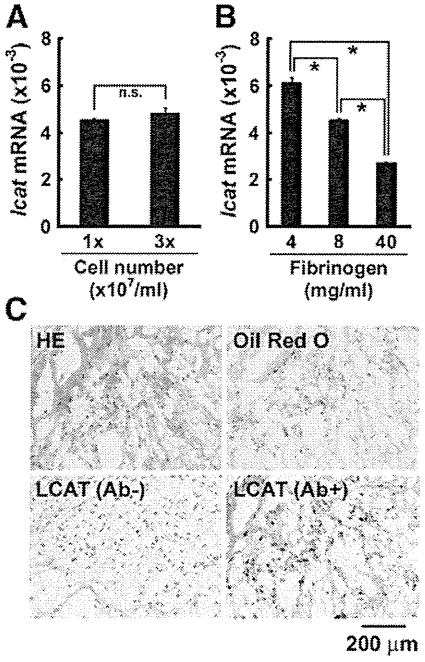

Fig. 4 – The expression of the exogenously-introduced *lcat* gene. The expression of the introduced *lcat* mRNA was compared between cultures originally generated using two different cell densities (A) and for the cultures using the three different fibrinogen densities (B) at Day 56 by quantitative RT-PCR. The mRNA expression of the treated group relative to the control was calculated using the ΔCT method. All PCR experiments were performed in triplicate. The data are presented as the means ± SD (n = 3). (C) The histological and immunostaining analyses of ccdPA/*lcat* HE in cross sections of 3D/FG on day 56 (*p<0.05).

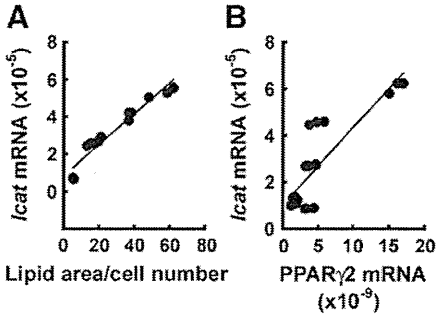

Fig. 6 – The correlations between the *lcat* expression level and lipid accumulation/cell number and PPARγ2 expression. The correlation of the expression of *lcat* with lipid accumulation/cell number (A) or PPARγ2 expression (B) was evaluated in ccdPAs cultured with different concentrations of fibrinogen in the 3D gel. *p<0.05.

are adipocytes and their progenitor cells (preadipocytes). In fact, aspirated adipocytes are widely available and currently used for cell transplantation in plastic surgery. ccdPAs are homogeneous cells identified from heterogenous preadipocytes with high adipogenic potential in long-term plate culture [11]. The homogeneity of the ccdPAs seems to be suitable for stable gene transduction in comparison to using the conventional ASCs (adipose tissue-derived stem cells) as a gene transfer vehicle. The exogenously transduced gene expression did not affect the adipogenic differentiation in a plate culture system [8]. Based on these previous findings, we evaluated the transduced gene expression characteristics of the ccdPAs using an *in vitro* 3D culture system in order to determine whether the homogenous preadipocytes could be applied for long-term protein supplementation of the exogenously transduced gene products. The ccdPAs differentiated into adipocytes which were characterized by lipid droplet accumulation and the expression of adipogenesis-specific genes in fibrin gel 3D culture, without the need for any artificial stimulation. The expression levels of the exogenously transduced gene were associated with the lipid accumulation properties of the preadipocytes in the gel. Importantly, the adipogenic potential was modified by the fibrinogen concentrations in the 3D gel, possibly as a result of the regulation of gel shrinking, and therefore the cell density, throughout the long-term culture. Thus, we propose that the 3D culture system is a candidate system suitable for evaluating gene-transduced preadipocytes prior to their subsequent physiological application.

The ccdPAs secreted the functional protein produced by the exogenously transduced gene, as well as endogenous gene products, in 3D culture (see Fig. 1). Using the exogenously transduced product-secreting preadipocytes, we investigated the cell properties using 1×10^7 cells/ml with three different concentrations of fibrinogen for 84 days in culture. The histological observations clearly showed that the fibrinogen concentration regulated the

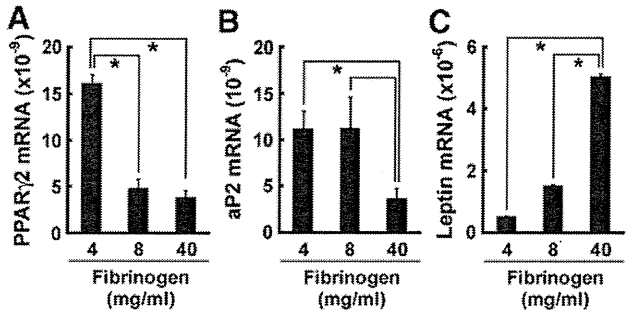

Fig. 5 – The effects of the fibrinogen concentration on adipocyte differentiation. The gene expression of adipose-specific PPARγ2 (A), aP2 (B), and leptin (C), on Day 56 were compared among the cultures grown with different fibrinogen concentrations by quantitative RT-PCR. The data are presented as the means ± SD (n = 3). *p<0.05.