Abstract

Fish-eye disease (FED) is an autosomal recessive disorder caused by mutations in lecithin:cholesterol acyltransferase (*lcat*) gene. We analyzed FED patient serum by non-denaturing gel electrophoresis in combination with immunodetection using apolipoproteinA-I (apoA-I) antibody, and clearly differentiated the status of apoA-I containing particles in comparison to normal sera. Based on these findings, we then evaluated the function of recombinant LCAT produced by human *lcat* gene-transduced proliferative adipocytes (ccdPA/*lcat*) which has been developed for a clinical treatment of FED as well as familial LCAT deficiency (FLD) patients by autologous cell transplantation. ccdPA/*lcat*-derived LCAT evidently converted the apoA-I containing particles to larger size on the 1D and 2D gel electrophoresis system., These results may provide the attractive bed-side evaluation system for the treatment of patients with LCAT deficiency caused not only primary but also secondary pathological conditions affecting in vivo LCAT activity in patients with not only by the deficiency.

Abbreviations: FLD, Familial LCAT deficiency; FED, fish-eye disease; apoA-I, apolipoprotein A1; UC, unesterified cholesterol; CE, cholesteryl ester; HDL-C, HDL cholesterol; 2D, two-dimensional; 1D, one-dimensional.

Introduction

Lecithin:cholesterol acyltransferase (LCAT) is responsible for the conversion of plasma unesterified cholesterol (UC) to cholesteryl ester (CE), and LCAT plays a central role in the formation and maturation of high-density lipoproteins (HDL) (1). Two classes of genetic deficiencies of LCAT have been identified: familial LCAT deficiency (FLD) and fish eye disease (FED). In FLD, plasma LCAT is either absent or exhibits no catalytic activity, and patient with partial or total LCAT deficiency often develops corneal opacity, anemia, proteinuria, and renal failure. In FED, the mutant LCAT lacks activity on HDL (α-activity) but esterifies cholesterol on low-density lipoprotein (β-activity), and causes severe corneal opacity, but not renal failure (2).

Enzyme replacement therapy by the infusion of fresh-frozen plasma improved the deranged composition of lipoproteins in FLD patients (3, 4) as also shown by *in vitro* experiments where simple incubation of the patient's plasma with purified LCAT protein from plasma of healthy subjects demonstrated significant improvement of the lipoprotein profile (5-7). However, the plasma CE diminished to the pretreatment level within two weeks probably due to short half life of the enzyme (8).

We have been developing life-long LCAT replacement therapy by transplantation of human *lcat* gene-transduced autologous adipocytes against LCAT deficient patients (Kuroda et al., submitted). To complete the development of novel cell-based therapy, the clinical and biochemical evaluation of progress in lipid disturbance in these diseases needs to be established. ApolipoproteinA-I (apoA-I) immunodetection in combination with non-denaturing two-demensional (2D) electrophoresis has recently reported to be useful to analyze HDL status and performed detailed comparison of plasma HDL profile between LCAT deficiency patients and healthy subjects (9). In this study, we for the first time present the HDL distribution of a FED patient by 2D electrophoresis and in addition, as an easy detection system, 1D electrophoresis followed by immunoblotting against apoA-I. Using these examination systems, the effect of the LCAT containing culture supernatant from human *lcat* gene-transduced adipocytes was analyzed on the change of distribution in HDL in the FED patient's serum.

Materials and methods

Analysis of patient samples

The study was approved by the Ethics Committee of Chiba University School of Medicine and informed consent was obtained from the patient. Blood samples were obtained from healthy volunteers and a FED patient in Chiba University Hospital. The serum was prepared and stored at -80 °C until use. Ultracentrifugation of lipoprotein,

electron-microscope observation, disk electrophoresis, and measurement of intima-media thickness of carotid arteries were performed as described previously (10-12). LCAT activity was measured by Anasolv LCAT kit (Sekisui Co. Tokyo, Japan).

Preparation of recombinant LCAT containing culture medium

Human *lcat* gene was transduced into human ccdPA as described (Kuroda et al., submitted). Resulted cells (ccdPA/*lcat*) were seeded and cultured with OPTI MEM I (Invitrogen, Carlsbad, CA). Culture supernatant was collected after seven days incubation, and concentrated to one-fiftieth of original volume by Amicon Ultra (MWCO=50kDa, Millipore, Billerica, MA). Amount of rLCAT in concentrated culture medium (rLCAT/ccdPA/*lcat*) was determined by immunoblotting followed by densitometric analysis. Known amount of commercially available rLCAT (Roar Biomedical, Inc., New York, NY) was used as standard. LCAT activity of concentrated medium was confirmed by the ability of cholesterol esterification using artificial apoA-I containing liposome substrate as described (Kuroda et al. submitted).

Sample preparation and immunoblotting

Concentrated medium containing rLCAT/ccdPA/*lcat* was mixed and incubated at 37 °C with patient serum for 24h. Inactivation of rLCAT (13) was performed by incubation at 56 °C or addition of 5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB, Sigma-Aldrich, St. Louis, MO). Non-denaturing two-dimensional (2D) gel electrophoresis was performed essentially as described previously with some modifications (9). Serum samples were first separated on the 0.7% agarose gel in 50mM barbital buffer (pH8.6) at 250V followed by the second dimensional separation on the 5-20% polyacrylamide gradient gel in running buffer (90mM Tris, 80mM boric acid, 3mM EDTA, pH8.3) at 100V for up to 20h. For non-denaturing 1D gel electrophoresis, serum was loaded on 10-20% polyacrylamide gradient gel and separated by electrophoresis in running buffer (90mM Tris, 80mM boric acid, 3mM EDTA, pH8.3) at 100V for 20h in ice-cold bath as essentially described previously (14). Separated serum proteins were transferred to PVDF membrane (Bio-Rad Laboratories Inc., Hercules, CA) and apoA-I was immunodetected by specific antibodies (Academy Bio-Medical Company, Houston, TX, or Calbiochem ,Darmstadt, Germany).

Quantification of cholesteryl ester (CE)

CE contents of samples were measured using Cholesterol Quantification kit (BioVision, Mountain View, CA) following the vendor's instructions.

Statistical analysis.

Data are presented as means ± S.D. Statistical comparison were made by ANOVA followed by the post hoc Tukey test to compare using SPSS software. In all cases, P values of less than 0.05 were considered as significant.

Results

Patient profile

The patient was a 38 years old Japanese male who had homozygous mutation in *lcat* gene which caused T123I amino acid substitution in LCAT protein confirmed by DNA sequencing. This mutation was described previously to cause FED phenotype (15). Both of his parents had heterozygous mutation. The patient showed corneal opacity (Fig. 1A) but no proteinuria (Table 1). Serum triglyceride level was higher than normal values. Free/total cholesterol ratio, as well as serum HDL-C, apoA-I, and apoA-II levels, were lower than normal values. Plasma LCAT activity was decreased to half of lower limit of normal range (Table 1). Disk electrophoresis showed a pattern of HDL, LDL and VLDL (Fig. 1B) similar to a FLD patient as reported (10). Midband lipoproteins were observed between LDL and VLDL (Fig. 1B). Obvious differences in morphologies of HDL and VLDL separated by ultracentrifugation were not observed in comparison to normal subject (Fig. 1C).

HDL profile of 2D electrophoresis

We performed 2D analysis to examine whether the differences in HDL subpopulation distribution between patient serum and normal serum were detectable. Immunodetection of apoA-I revealed that patient serum clearly showed different distribution of HDL subpopulation compared with healthy serum (Fig. 2A, B). The disturbed HDL subpopulation in the FED patient serum was rather similar to that as reported in FLD patients previously (9). Thus, the abnormal HDL subpopulation in a FED patient with T123I amino acid substitution could be identified by 2D-electrophoresis, although the morphological appearance of HDL particles was not obviously different from normal (see Fig. 1C).

Effect of recombinant LCAT produced by ccdPA/lcat on HDL particle distribution

We next analyzed the disturbed distribution of HDL subpopuration of the FED patient could be improved by the addition of rLCAT *in vitro*. The patient serum was incubated with the cultured supernatant of ccdPA/*lcat* (Kuroda et al. submitted) at the

final concentration of rLCAT (6.6 μg/ml) equivalent to healthy subject (2, 16, 17) and analyzed by electrophoresis. The apoA-I containing lipoprotein distribution in the patient serum was drastically shifted to larger molecular weight region when the cultured supernatant of ccdPA/*lcat* was added (Fig. 2C) but not ccdPA without *lcat* gene transduction (Fig2D). The effects were diminished by heat-inactivation of cultured supernatant before incubation (Fig. 2E). These results showed that the abnormal distribution of HDL subpopulation in FED patient is able to be improved by the *in vitro* incubation with rLCAT and this could be achieved by the protein produced by ccdPA/*lcat.*

Establishment of 1D electrophoresis system detecting HDL maturation profile in FED patient

Based on the above 2D analysis, we assumed that 1D non-denaturing acrylamide gel in combination with immunoblotting with anti apoA-I antibody could detect the change of size distribution in FED patient serum (Fig. 3A). The obvious difference of immunodetectable apoA-I containing lipoprotein distribution was appeared between the patient (Fig. 3A, lane 1) and normal subject (Fig. 3A, lane 2). We next assessed the effect of rLCAT by 1D analysis. ApoA-I containing HDL particles were shifted to large size by incubation with cultured supernatant of ccdPA/*lcat* dose-dependently (Fig. 3B, lanes 5-7) as well as the incubation with recombinant LCAT (Fig. 3B, lane 12). The incubation with cultured supernatant of ccdPA (without transduced *lcat* gene, lane4) or PBS (11) did not cause any change from the original serum pattern of the patient. Pre-heat-treatment of cultured addition of DTNB (lane 8) or supernatant (lane 9) diminished the effects on HDL particle shifting of the incubation with the cultured supernatant of ccdPA/*lcat.* To confirm the involvement of LCAT activity produced by ccdPA/*lcat* on the conversion of HDL particles, we measured the CE content in the HDL fractions after removal of LDL/VLDL from the above reaction mixture by precipitation. Addition of cultured supernatant of ccdPA/*lcat* significantly elevated the CE level in HDL fractions (lane 7), as observed in the addition of rLCAT (lane 12), in agreement with the shift observed in 1D gel (Fig. 2B). Taken together with the result in Fig. 2, 1D electrophoresis in combination with immunoblotting analysis is enough sensitive for the detection of abnormality in HDL subpopulation distribution in FED, and the effect of *in vitro* incubation with cultured supernatant of ccdPA/*lcat* could be detected using the 1D system.

Discussion

In this study, we for the first time reported the disturbed HDL profile of FED patient who had T123I mutation in 2D and 1D electrophoresis in combination with immunoblot analysis using apoA-I antibody, and the possible LCAT replacement treatment of the patient's abnormal HDL by the cell transplantation of ccdPA/*lcat*. Using the apoA-I immunoblotting system for the evaluation of the enzyme effect *in vivo*, 1D, in addition to 2D, non-denaturing gel electrophoresis could detect the difference of apoA-I status between serum of FED patient and normal subject, and the gel system clearly demonstrated the disturbed distribution of apoA-I-containing HDL particles are able to be improved by the incubation with recombinant LCAT protein produced by ccdPA/*lcat* (Kuroda et al., submitted).

Asztalos et al. have reported the status of HDL subpopulation of LCAT deficiency by 2D analysis in combination with immunoblot analysis using antibodies against apolipoproteins, and clearly demonstrated that the lipoprotein distribution of FLD was distinguishable in contrast to normal subjects (9). These results showed that LCAT was essential for development of large, spherical and apoA-I containing HDL and subsequent maturation of HDL. Nakamura et al. have analyzed the metabolism of pre-β-HDL by the mobility shift of apoA-I in 1D gel in normal plasma, and showed that LCAT was involved in the metabolism of pre-β-HDL (14). Holmquist et al. have incubated the mixture of enriched HDL fraction and autologous plasma from FED patient with purified normal LCAT, and showed LCAT-dependent increase in apparent molecular weight of HDL using 1D gel after density gradient fractionation without immunodetection method (18). Conversion of HDL3 to HDL2-like particles by LCAT was shown by *in vitro* incubation assays using ultracentrifugation (19-23). Thus, LCAT is known to be important for the production and maturation of HDL, and the complete deficiency causes obvious shift in HDL particles in the 1D and 2D gel. However, the HDL profile in FED, not complete deficiency of LCAT, has not been yet demonstrated by 2D gel electrophoresis followed by immunoblotting of apoA-I. Particularly, the development of easy-handling and sensitive system is expected to evaluate the pathological conditions in FED patient and to develop the consistent replacement therapy for these patients. Thus, we utilized 1D and 2D analysis in combination with immunoanalysis to examine the effect of rLCAT produced by ccdPA/*lcat*.

The efficacy of LCAT replacement therapy has been reported by infusion of normal plasma (4). However, the effects were temporal and therefore, life-long treatment has been expected to be established. We have developed an *lcat* gene transduced-adipocytes for LCAT replacement therapy against LCAT deficient patient via auto-transplantation of adipose tissues of patient (Kuroda et al., submitted). The application of clinical study

using the adipocyte transplantation is now in progress in Japan as a first trial of *lcat* gene transduced-adipocyte transplantation. The sensitive and easy-handling clinical examination of the HDL profile in the treated patients largely contribute to the evaluation of novel cell therapy.

Distribution of apoA-I containing HDL profile in 2D analysis showed that rLCAT evidently changed the abnormal HDL size populations in FED patient toward the pattern in normal subject. This change in HDL particles was also detected in 1D electrophoresis with rLCAT-dependent formation of CE in HDL. Thus, incubation with rLCAT derived from ccdPA/*lcat* stimulated CE formation and subsequent maturation in HDL subpopulations of FED patient serum. This *in vitro* system could serve as an evaluation system of the LCAT-dependent conversion in lipoprotein profiles of FED patient not only by cell transplantation but also by natural course of disease itself. In addition, rather easier handled 1D analysis, as a clinical examination, is expected to reflect the rLCAT dose-dependent conversion of HDL size based on mobility shift of apoA-I.

In conclusion, the disturbed HDL particle distribution in FED, a partial deficiency of LCAT, as well as FLD previously reported by others (9), could be identified by 2D and 1D electrophoresis followed by apoA-I immuno blotting. The system could detect the improvement of HDL pattern in the patient by novel replacement therapy using ccdPA/*lcat*. This detection system may provide an attractive bed-side evaluation system for the *in vivo* LCAT activity in patients with partial deficiency as well as total deficiency of LCAT, caused by not only primary but also secondary pathological conditions affecting *in vivo* LCAT activity.

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Table 1. Clinical and lipid profiles of the patient and his parents

	Normal values	Patient	Father	Mother
Age, y		38	68	65
Total cholesterol, mg/dL	150-220	124	201	180
Free cholesterol, mg/dL	30-60	50	53	45
Free / total cholesterol	0.23-0.27	0.4	0.26	0.25
Triglyceride, mg/dL	50-150	190	124	80
HDL-cholesterol, mg/dL	40-96	3	30	39
Apo A-I, mg/dL	119-165	34	102	110
Apo A-II, mg/dL	24-36	2.8	21.2	31.9
Apo B, mg/dL	66-109	90	130	96
Apo C-II, mg/dL	1.5-4.6	3.2	3.9	4.2
Apo C-III, mg/dL	5.4-10.0	5.2	8.7	8.1
Apo E, mg/dL	2.7-4.6	4.8	4.8	3.1
LCAT activity[a] (nmol/mL/h)	53-108	24.8	61.9	79.2
Apo E phenotype		3/3		
Corneal opacity		+	−	−
Proteinuria		−	−	−
Intima-media thickness	<1.0	0.7		

[a]see materials and methods analysis of patient samples.

Figure legends

Figure 1. Clinical observations of the patient. (A) Corneal opacity. (B) Scanned lipoprotein profile separated by disk gel electrophoresis. Arrow, midband. (C) Electron micrographs of HDL (left panels) and VLDL (right panels) in patient (upper panels) and normal subject (lower panels). Bar=0.1 μm.

Figure 2. Distribution of apoA-I-containing particles. Serum samples of normal subjects (A) and FED patient (B, C, D and E) were analyzed by 2D gel electrophoresis followed by immunoblotting against apoA-I. The patient serum without incubation (B). The

patient serum was incubated at 37°C for 24 hours with cultured supernatant derived from lcat gene-transduced ccdPA (C) or cultured supernatant from ccdPA (D), and same as sapmle C but with pretreatment of heat-inactivation of cultured supernatant (E).

Figure 3. 1D analysis of mobility changes in apoA-I containing particles by in vitro incubation with rLCAT. A, The pattern of apoA-I signals of serum samples from FED patient (lane1) and normal subject (lane2) after 1D gel electrophoresis followed by immunodetection of apoA-I. **B,** Culture medium (lanes 1 and 2), cultured supernatant of un-transduced (lanes 3 and 4) or human *lcat* gene-transduced (lanes 5 to 9) ccdPA, phosphate-buffered saline (PBS, lanes 10 and 11), and recombinant LCAT 60 μg/mL (Roar company) (lane 12) were added to patient serum and incubated at 37°C for 24 hours (lanes 2, 4-9, 11, and 12). Samples without incubation (lane 1, 3, and 10) were included as controls. Heat-inactivated cultured supernatant of human *lcat* gene-transduced ccdPA was used in lane 9. DTNB was included in the reaction mixture for lane 8. Concentrations of ccdPA-derived LCAT in the reaction mixtures were 0.7 (lane 5), 2.2 (lane 6), and 6.6 (lane 7 to 9) μg/ml, respectively. HDL-CE was quantified in the reaction mixtures of those samples analyzed and shown in the bar graph at the bottom. Quantification of HDL-CE for lane 8 was not performed due to interference of DTNB with the enzymatic determination of cholesterol (24). *p<0.05.

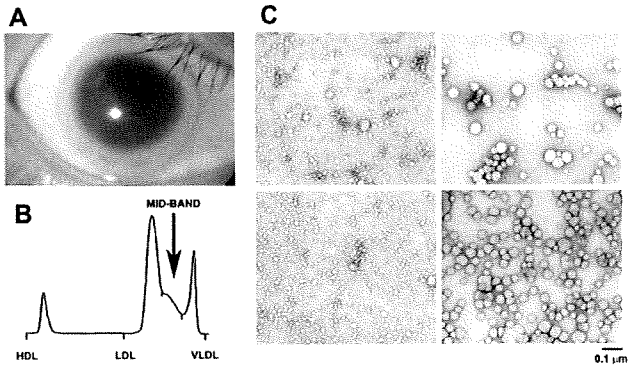

Figure 1..

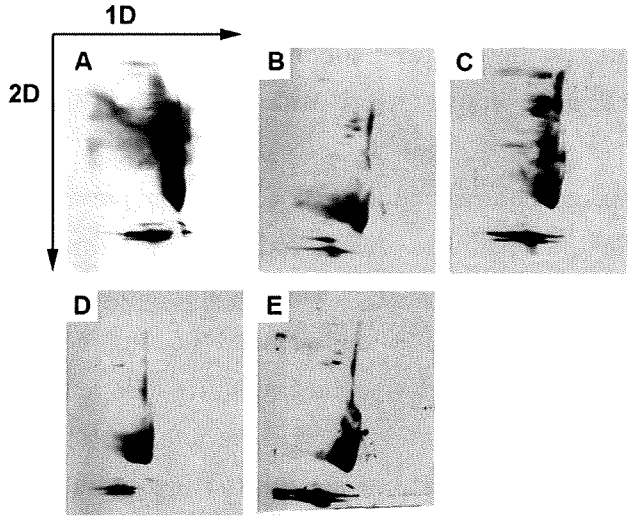

Figure 2.

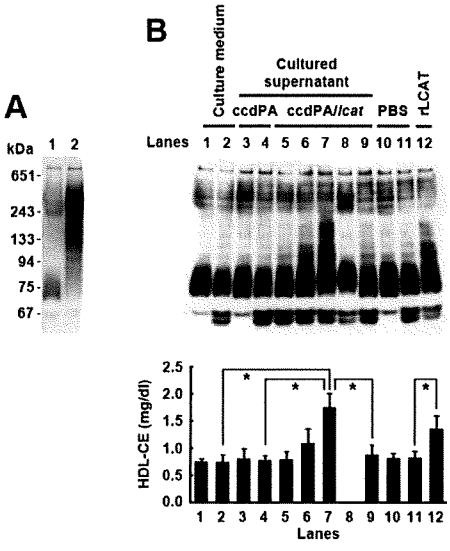

Figure 3.

Fibrin glue increases the cell survival and the transduced gene product secretion of the ceiling culture-derived adipocytes transplanted in mice

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Short title: Fibrin glue and ceiling culture-derived transplanted adipocytes

Abstract

Fat tissue is a common material for autologous transplantation in plastic and reconstructive surgery. To establish a novel protein replacement therapy using multi-potential preadipocytes in aspirated fat with fibrin glue as clinically applicable scaffold, we have developed a mice model system to monitor the delivery of the transgene product into the blood and the survival of transduced cells after transplantation. Human lecithin-cholesterol acyltransferase (LCAT) gene was tranduced into mouse ceiling culture-derived proliferative adipocytes (m-ccdPA) by retrovirus vector and subcutaneously transplanted into mice combined with fibrin glue to compare with Matrigel, an established experimental scaffold. By means of sensitive immunoprecipitation/western blotting procedure, we could detect the gene expression comparable levels of recombinant human LCAT for 28 days in the blood stream of the mice delivered by the implanted gene-transduced m-ccdPA with fibrin glue as those with Matrigel. This *in vivo* system would serve as a platform to study the scaffold conditions for the stable and long-lasting cell-based replacement of defective proteins in patients with the deficiency.

Introduction

Aspirated fat is a common source of autologous tissue transplantation for the correction of tissue defects in plastic and reconstructive surgery (Billings and May, 1989; Pinski and Roenigk, 1992; Patrick, 2000; Coleman, 2001; Patrick, 2001; Yoshimura et al., 2009). Recent development in regenerative cell biology has shown that the preadipocytes existing in aspirated fat are multipotential rather than just differentiated adipocytes (Stashower et al., 1999; Zuk et al., 2001; Zuk et al., 2002; De Ugarte et al., 2003; Dragoo et al., 2003; Gimble et al., 2007). One of such potentials is known as the high capability for exogenous gene transduction and secretion of the incorporated gene products (Ito et al., 2005). We have recently identified human ceiling culture-derived proliferative adipocytes (h-ccdPA) existing in subcutaneous adipose tissue, and proposed the application of gene-transduced h-ccdPA to the long-time replacement therapy for variety of inherited or acquired gene defective diseases (Kuroda et al., submitted).

A key factor for the protein delivery system via auto-transplantation of gene-transduced various kinds of cells is the regulation of survival and secretory function of gene transduced cells at the transplanted space. We have shown that the nutritional condition of the recipient is one of the important factors for the survival and gene expressions of adipocytes in the fat graft after a subcutaneous transplantation in mice (Matsumoto et al., 2002). As well, a bioactive molecule, VEGF, secreted from vascular system constructed around the transplanted graft in recipients is also important for their long cell survival (Yamaguchi et al., 2005). Particularly, recent studies have highlighted the importance of the various cytokines for the regulation of cell functions and the surrounding matrix conditions (Kuramochi et al., 2008; Cho et al., 2006; Tsuji et al., 2009; Kimura et al., 2003; Torio-Padron et al., 2007; Kawaguchi et al., 1998; Ning et al., 2009; Tabata et al., 2000). Together with the consideration of cytokine delivery for the transplanted cells, the development scaffold transplanted with adipocytes contribute to the earlier construction of surrounding matrix conditions around the transplanted site. The insulin gene transduced cells transplanted with Matrigel reagent as a scaffold have shown to be survived as insulin-secreting functioning adipocytes for three months after transplantation (Ito et al., 2005). Thus, it is critical to set up an appropriate clinically applicable scaffold for the adipocyte transplantation into patients, which allows not only longer survival of the implanted cells but also guarantees longer-lasting secretion of the therapeutic gene product into blood stream.

In this study, we have optimized the gene transduction condition for the most

effective retrovirus vector-mediated gene transduction using ceiling culture derived-proliferative adipocytes from mouse adipose tissue (m-ccdPA). We established a mouse model for the transplantation with expanded human enzyme gene-transduced m-ccdPA for the evaluation of protein delivery in the serum of the mice. Using the *in vivo* model, we analyzed the effect of fibrin glue (Neuss et al., 2008; Malafaya et al., 2007; Mano et al., 2007) as a clinically applicable scaffold on the efficacy of the circulating enzyme delivery.

Materials and Methods

Cell culture

Dulbecco's modified Eagle's medium/F12-HAM (Sigma-Aldrich, St. Louis, MO) supplemented with 20% fetal bovine serum (FBS, SAFC Biosciences, Lenexa, KS) and 40 μg/ml gentamicin (GENTACIN, Schering-Plough Co., Kenilworth, NJ) (DMEM/FBS) was used as culture media except for the adipogenic induction in which PGM-2 Bullet kit (Lonza, Basel, Switzerland) was used. Cells were detached by TrypZean (Sigma-Aldrich) treatment, harvested by centrifugation at 300g at 4°C, re-suspended, seeded in fresh growth medium. Expansion culture was performed in CellSTACK-1, 2, 5, and 10 Chamber (Corning Inc.) flasks. Passage was performed essentially twice a week.

Isolation of ccdPA from fat tissue

Adipose tissues were obtained from 7-8 weeks male C57BL/6J mice and proliferative adipocytes population was propagated by ceiling culture technique as described (Sugihara et al., 1986; Sugihara et al., 1987, Kuroda et al., submitted). After 7 days ceiling culture, cells that grew at the ceiling surface were detached with TrypZean (Sigma-Aldrich) treatment, harvested and seeded into flasks for next step.

Gene transduction

Human *lcat* gene-expressing amphotropic retrovirus vector, CGT_hLCATRV, which was described elsewhere (Kuroda et al., submitted), was used for gene transduction. The cells obtained by ceiling culture were used as a recipient of retrovirus vector mediated gene transduction. Based on the report of Landazuri et al., we examined relatively high concentration (100-500 μg/ml) of protamine sulfate (PS, Novo-Protamine Sulfate, 100 mg for I.V. Injection, Mochida Pharm. Co. Tokyo, Japan) to enhance transduction efficiency in comparison to 8 μg/ml. Gene transduction was performed at 37°C in the presence of 20% FBS and PS. Virus vector concentration used for transduction was 2.0×10^9 RNA copies/ml. After transduction, the medium was changed

to growth medium.

Quantification of transduced gene

Genomic DNA of the cultured cells and mice transplants was extracted with DNeasy Blood & Tissue kit (QIAGEN, Hilden, Germany) and Gentra Puregene kit (QIAGEN), respectively. Integrated vector copy number of the cells was quantified by SYBR® Premix Ex Taq™ (Perfect Real Time) kit (TaKaRa Bio Inc., Shiga, Japan) using Value of DNA content of normal cell (6 pg/cell) (Rogachev et al.) was used for calculation for averaged integrated copy number. Existence of *lcat* cDNA in transplanted implants was quantified by TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA). All the real-time PCRs were performed using ABI7500 Real-time PCR system (Applied Biosystems) using pCGThLCAT DNA was used as a standard.

RT-PCR

Total RNA was propagated by RNeasy Plus Mini kit (QIAGEN). Single-stranded cDNA was synthesized with ReverTra Ace-α-™ kit (TOYOBO, Osaka, Japan) according to manufacturer's instruction. The sequences of the mouse PPARγ2 primers were: PPARγ2-F (5'-GGTGAAACTCTGGGAGATTC-3') and PPARγ2-R (5'-CAACCATTGGGTCAGCTCTTG-3'). The amplification was performed with TITANIUM Taq DNA polymerase (TaKaRa Bio Inc.) under the following condition: 94 °C for 5 min/94 °C for 30 s, 58 °C for 30 s, and 72 °C for 90 s (28 cycles)/72 °C for 7 min. As control, G3PDH was amplified using control primers included in the ReverTra Ace-α-™ kit. The amplified products were subjected to 2% agarose gel electrophoresis and visualized with staining with GelStar® Nucleic Acid Stain reagent.

Measurement of LCAT activity

To assess LCAT activities in culture medium, we used artificial liposomes containing lecithin-cholesterol as substrate, as described elsewhere (Kuroda et al., Submitted).

Detection of human LCAT protein

For detection of human LCAT (hLCAT) in culture medium and mice sera, test samples were diluted up to 500 μl with ice-cold phosphate buffered saline containing 0.2% Nonidet P-40 (PBS-NP40) and incubated with 2.5 μl of anti-LCAT rabbit monoclonal antibody (EPITOMICS, Burlingame, CA) overnight at 4°C with gentle rotation. Twenty microliters of TrueBlotTM anti-Rabbit Ig IP Beads (eBioscience, San Diego, CA) was added and incubated with rotation for 2 hrs at 4°C. Bound proteins were

pelleted by centrifugation, washed five times with 1 ml of PBS-NP40 buffer, and eluted by boiling in 10 μl of 2 x Laemmli's sample buffer. Immunoprecipitated samples and standards (purified hLCAT (Roar Biomedical, Inc., New York, NY) or human plasma HDL (Calbiochem, Merck, Darmstadt, Germany)) were separated by 10% acrylamide gel and transferred to nitrocellulose membrane. The membrane was blocked for 1 hour in SuperBlock T20 (TBS) (Thermo Fisher Scientific Inc., Rockford, IL) and incubated overnight at 4°C with the anti-LCAT rabbit polyclonal antibody (Novus Biologicals, Littleton, CO). After washing the membrane filter with Tris-buffered saline containing 0.1% Tween 20 was incubated with TrueBlot anti-Rabbit IgG HRP (1:5000) (eBioscience) for 1 hour at room temprature. The signals were detected by SuperSignal® West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific Inc.) with LAS1000 apparatus (FUJI film, Tokyo, Japan). Preliminary experiments demonstrated that the efficiency of recovery of input human LCAT as HDL into mice serum was 101.0 ± 9.5%.

In vivo experiment

To examine the ability to deliver hLCAT protein *in vivo*, mice experiments were performed. Recipient mice were obtained from Charles River Japan. Animal experiments were carried out according to the Guidelines for Animal Research of Chiba University. To identify the transplanted cells *in vivo*, the cells were stained using PKH26 Red Fluorescent Cell Linker kit for General Cell Membrane Labeling (Sigma-Aldrich) one passage before transplantation with slight modification of manufacturer's instructions.

Bolheal (The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan) was used as clinically available fibrin glue. The fibrin glue product consists of two components. Fibrinogen solution (containing fibrinogen, plasma fibronectin, factor XIII, plasminogen, and aprotinin) and thrombin solution (containing thrombin and calcium chloride) were diluted four and two times before injection using DMEM-HAM/F12 (Sigma) respectively. After expansion, cells were detached by TrypZean treatment and harvested, washed with DMEM-HAM/F12 containing 1% mouse serum (T. K. Craft Co., Ltd. Gunma, Japan). The harvested cells were re-suspended at 1 x 10^7 cells/ml by diluted thrombin solution, and were injected subcutaneously into the mouse with same volume of diluted fibrinogen solution using injection apparatus included in Bolheal kit. We also transplanted the cells suspended in Matrigel (BD Biosciences, Bedford, MA) at 5 x 10^6 cells/ml. In both cases, 5 x 10^6 cells were transplanted.

All mice were allowed free access to regular chow and water. Three animals were

sacrificed to take serum samples at Day1, 14, 28. In CB57BL/6J mice experiments, blood samples were taken from tail without sacrifice to follow the LCAT delivery in same animal at day1, 4, 7, 14 and 28. Transplanted region was taken under fluorescent microscopic observation by SZX16 reflected fluorescence system (OLYMPUS corp. Tokyo, Japan) and sections were frozen at -80°C until use.

Histological staining

The explanted tissues were fixed in 4% paraformaldehyde following replaced 30% gum-saccarose and embedded in Tissue–Tek O.C.T. Compound (Sakura Finetechnical Co., Ltd, Tokyo, Japan). Immunohistochemical staining was performed using monoclonal rabbit anti-human LCAT antibody (250:1; EPITOMICS) as primary antibody and Alexa Fluor 488 goat anti-rabbit IgG (1000:1; Invitrogen) as secondary antibody. The slides were counterstained with DAPI using VECTASHIELD Mounting Medium with DAPI (Vector Laboratories).

Statistical analysis

Data are presented as means ± S.D. Statistical comparison were made by Student's t-test or by ANOVA followed by the post hoc Tukey test to compare using SPSS software. In all cases, P values of less than 0.05 were considered as significant.

Results

Establishment of human lcat gene transduced m-ccdPA

We have recently established a ceiling culture-derived human proliferative adipocytes (h-ccdPA) which secret functionally active LCAT, a key circulating enzyme for serum cholesterol esterification, and proposed that subcutaneous transplantation of the cells for the long-lasting replacement of the protein in the patients with LCAT deficiency (Kuroda et al. submitted). In order to establish the mouse model suitable for the evaluation of the effect of scaffold on the survival and function of transplanted adipocytes, we first prepared m-ccdPA for the *lcat* gene transduction as donor cells for the recipient mice. The biochemical characterizations showed that the prepared m-ccdPA have morphological feature and surface antigen expression pattern similar to those of h-ccdPA (unpublished data). The transduced m-ccdPA secreted much less amount of hLCAT than h-ccdPA when the average copy number of human *lcat* cDNA/cell was equivalent. Two rounds of one hour exposure with CGT_hLCATRV in the presence of 500 μg/ml of PS significantly improved the transduction efficiency compared with two rounds of overnight exposure in the presence of 8 μg/ml of PS, a concentration

which was originally used for the gene transduction for h-ccdPA (Figure 1A: ref. Kuroda et al. submitted). The LCAT activity in the culture medium was significantly increased in the cells with same transduction condition (Figure 1B).

Transplantation of lcat gene transduced m-ccdPA in nude mice

We transplanted the above established human *lcat* gene-transduced m-ccdPA subcutaneously into nude mice to examine the effect of fibrin glue as a scaffold on the secretion of LCAT from the survived cells without immuno-reactive conditions. Blood samples collected from the mice transplanted with fibrin gel were subjected to IP-western procedure 14 days after transplantation (Figure 2A). Human LCAT was clearly immunologically detected in the *lcat* gene-transduced m-ccdPA transplanted mice, and not in vehicle transplanted mice (Figure 2A). The immunodetected signal suggested that the concentration of circulating LCAT protein is over or equivalent to those of the 15 μg of human HDL, which is a major distribution site of LCAT (Fielding and Fielding, 1995; Jonas, 2000).

Several reports have shown that mouse (Mizuno et al., 2008) and human (Cho et al., 2006) preadipocytes after adipogenic induction were superior in survival when implanted into nude mouse. We therefore examined whether adipogenic differentiation affects LCAT delivery and survival of *lcat* gene-transduced m-ccdPA after implantation with fibrin glue. RT-PCR showed that PPARγ2 expression level was significantly induced in cells cultured in adipogenesis inducing medium for three days prior to transplantation (data not shown). The serum hLCAT concentration was not significantly different between mice transplanted with differentiation induced cells and those with un-induced cells (Figure 2B). The *lcat* gene recovery analysis suggested that the adipogenesis inducing pretreatment did not affect the cell survival rate (Figure 2B). These results indicate that the transplanted *lcat* transduced m-ccdPA with fibrin glue survive at least 14 days after transplantation in immunosuppressive conditions.

Effect of fibrin glue on human LCAT delivery in lcat gene transduced m-ccdPA transplanted B6 mice

We and others have already shown that exogenous gene transduced adipocytes survive more than 28 days when subcutaneously transplanted with Matrigel, which is known as experimental scaffold in many studies (Ito et al., 2005; Kawaguchi et al., 1998; Piasecki et al., 2008; Kitagawa et al., 1999; Tabata et al., 2000; Planat-Benard et al., 2004). In order to consider the possibility of fibrin glue as a clinical scaffold, we analyzed the effect of fibrin glue on human LCAT delivery in comparison to Matrigel in