of human *lcat* cDNA/cell was equivalent. Two rounds of one hour exposures with CGT_hLCATRV in the presence of 500 μg/ml of PS significantly improved the transduction efficiency compared with two rounds of overnight exposures in the presence of 8 μg/ml of PS, a concentration which was originally used for the gene transduction of h-ccdPA (Figure 1A: Kuroda *et al.*, 2011). The LCAT activity in the culture medium significantly increased in the cells with the same transduction conditions (Figure 1B).

Transplantation of m-ccdPA/*lcat* in nude mice

We transplanted the above established m-ccdPA/*lcat* subcutaneously into nude mice to examine the effect of fibrin glue as a scaffold on the secretion of hLCAT from the surviving cells without immunoreactive conditions. Blood samples collected from the mice transplanted with or without the fibrin glue were subjected to immunoprecipitation/Western (IP-Western) procedures 7 days after transplantation (Figure 2A). hLCAT was immunologically-detected clearly in the m-ccdPA/*lcat* transplanted mice, and not in the vehicle-transplanted mice (Figure 2A). The serum from the mice transplanted with the fibrin glue showed apparently increased signal intensity in comparison to those from the mice without fibrin glue (Figure 2A), indicating that the fibrin glue is effective for the cell survival after transplantation. The signal intensity analysis suggested that the concentration of the circulating hLCAT 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).

Several reports have shown that mouse (Mizuno *et al.*, 2008) and human (Cho *et al.*, 2006) preadipocytes after adipogenic induction were superior in survival potential when implanted into nude mice. We therefore examined whether adipogenic differentiation affects hLCAT delivery and the survival of m-ccdPA/*lcat* after implantation with fibrin glue. RT-PCR analysis showed that the 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 transplanted with uninduced 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 m-ccdPA/*lcat* implanted with fibrin glue survive at least 14 days after transplantation in immunosuppressive conditions.

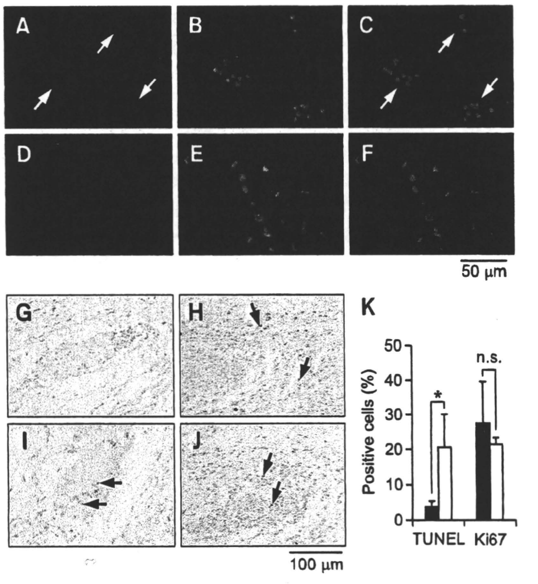

Figure 3. Immunohistochemical analysis of transplanted *lcat* gene-expressing m-ccdPA. Sections of implants from the cells with transduced (A, B, and C) or un-transduced (D, E, and F) by retroviral vector at day 28 were prepared and LCAT-immunostaining was performed. Implants were taken upon observation of PKH26 fluorescence. Immunohistochemical staining of hLCAT in fixed implants was done using rabbit anti-hLCAT monoclonal antibody as a primary antibody. Alexa Fluor 488 goat anti-rabbit IgG was used as a secondary antibody. The slides were counterstained with DAPI. Photographs of hLCAT staining (A and D), DAPI staining (B and E), and merged images (C and F) were shown. TUNEL (G and H) and Ki67 (I and J) staining of explants on 15 days after transplantation with (G and I) or without (H and J) fibrin glue were performed. Cells with positive signal were counted in four independent areas (K). *$P < 0.05$.

Effect of fibrin glue on hLCAT delivery in m-ccdPA/*lcat*-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 used as an experimental scaffold in many studies (Kitagawa and Kawaguchi, 1999; Ito *et al.*, 2005; Piasecki *et al.*, 2008). In order to consider the possibility of fibrin glue as a clinical scaffold, we analyzed the effect of fibrin glue on hLCAT delivery in comparison to Matrigel in B6 mice. The m-ccdPA/*lcat* was subcutaneously transplanted into B6 mice with fibrin glue. hLCAT immunostaining revealed that the m-ccdPA survive 14 days after transplantation and express hLCAT protein in B6 mice (Figures 3A-3F). The hLCAT expression was still detectable in the transplanted m-ccdPA 28 days after transplantation (data not shown). The TUNEL staining of transplanted sections excised on 15 days after transplantation showed that the

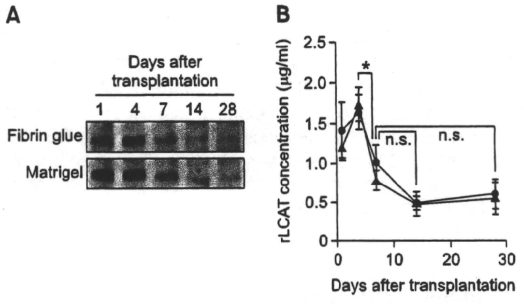

Figure 4. Effect of fibrin glue on hLCAT protein delivery. Human *lcat* gene-transduced m-ccdPA were subcutaneously transplanted in C57BL/6J mice using fibrin glue or Matrigel as scaffolds. Representative data of the experiments were shown (A), in which hLCAT delivery was monitored in a single mouse. Concentrations of hLCAT protein in cell-transplanted mice sera with Matrigel (closed circle) or fibrin glue (closed triangle) were quantified by densitometric analysis after IcP-Western experiments (B). $^*P < 0.05$.

apoptotic cell death in the cells with fibrin glue was significantly decreased in comparison to those without fibrin glue (Figures 3G, 3H and 3K). On the other hand, there was no significant difference in Ki67 staining between the cells with and without fibrin glue (Figures 3I, 3J and 3K). The IP-Western analysis showed that the hLCAT protein was detected at least up to 28 days after transplantation in the serum of mice. The collected mouse sera were analyzed for hLCAT protein by IP-Western blotting and hLCAT protein was detected up to 28 days after transplantation (Figure 4A). Densitometric analysis (Figure 4B) showed that from day 4 to day 7, the density became significantly decreased, and from day 7 the concentration of hLCAT protein became relatively constant. The hLCAT levels in the serum from m-ccdPA/*lcat* transplanted with fibrin glue were comparable with those from m-ccdPA/*lcat* transplanted with the Matrigel reagent. These results showed that fibrin glue, a common clinically available material, worked as a scaffold for the *in vivo* delivery of the hLCAT protein.

Discussion

For the development of long-lasting protein replacement therapy by gene-transduced ccdPA, the use of a clinically applicable scaffold is one plausible approach for the improvement of survival and/or secretion function of transplanted cells in patients. Various types of materials have been proposed (Malafaya *et al.*, 2007; Mano *et al.*, 2007; Neuss *et al.*, 2008) as scaffolds for cell transplantation. In this study, we have chosen fibrin glue because it is already commonly used in clinics and an easy-to-use kit is commercially available. In order to evaluate the effect of fibrin glue as a scaffold in the survival and function of transplanted adipocytes, we established an autologous mouse model system using m-ccdPA/*lcat*. The results using the mice showed that fibrin glue supported human enzyme delivery from the transplanted m-ccdPA/*lcat* at a level equivalent to Matrigel, which is known as an efficient scaffold in experimental models. Thus, fibrin glue could be a candidate as a scaffold in the clinical transplantation of h-ccdPA/*lcat* in LCAT-deficient patients to prevent the development of renal insufficiency and/or corneal opacity.

Preliminary experiments showed that hLCAT protein was secreted by m-ccdPA/*lcat in vitro*, however, hLCAT was barely detectable in the transplanted mouse serum probably because of the lower capability of secretion. The integrated copy number and the LCAT activity in the culture medium could be elevated approximately three fold with the conditions suitable for m-ccdPA (Figure 1). As a result, IP-Western analysis was sensitive enough for the quantification of the serum hLCAT protein in the mice, and the analysis indicated that the delivered protein is equivalent to that of 15 μg of HDL (Figure 2A). These optimizations enabled us to establish an *in vivo* mouse model to monitor the effect of fibrin glue as a scaffold for the transplanted m-ccdPA. Adipogenic differentiation did not significantly affect the hLCAT delivery and the cell survival in this model using fibrin glue as a scaffold with m-ccdPA (Figure 2B). In this context, our results may suggest that the transplanted cells with fibrin glue were differentiated into adipocytes without adipogenic pretreatment (Cho *et al.*, 2006; Torio-Padron *et al.*, 2007a). The immunohistochemical observation did not clearly show that ccdPA would undergo adipogenic differentiation after transplantation, but the transplanted ccdPA were clearly identified as hLCAT-delivery cells in the transplanted sites of the recipient mice (Figures 3A-3F). Immunohistochemical analysis of transplanted sections suggested that action of fibrin glue was prevention of apoptotic cell death rather than proliferation stimulation of the transplanted cells after transplantation (Figures 3G-3K), and thus, caused the increase in the hLCAT-delivery into circulation after transplantation in mice. The analysis of the m-ccdPA/*lcat* with fibrin glue revealed that the serum hLCAT concentration decreased to one half in a week, and became relatively stable at 7-14 days after transplantation (Figure 4). We could therefore discern that the hLCAT-positive cells survived and functioned for at least one month using the m-ccdPA/*lcat* transplanted mouse model.

The current study showed that the implanted cells successfully supplied a therapeutic level of hLCAT into the serum, and suggested the feasibility of ccdPA-mediated gene therapy using the ccdPA. However, there are several remaining issues to be resolved before the clinical application of this therapy if we anticipate extending this cell implantation technique to various diseases other than LCAT deficiency. First, the survival period of ccdPA needs to be assessed after transplantation into the recipient. The previous model using insulin-secreting adipocytes showed that the blood glucose-reducing activity was stably observed for two months (Ito *et al.*, 2005). The stability of the ccdPA needs to be evaluated for longer periods using the mice established in this study. Second, the protein delivery by the transplanted ccdPA into the serum is unstable at the initial phase to 7 days after subcutaneous transplantation, although the delivery became constant after the 7-day phase up to a month. The characterization of the transplanted ccdPA including the interaction between the differentiation and secretion functions is in progress using this model. Before obtaining the knowledge of the multi-phase cell conditions in the recipients, the application of this cell therapy would be restricted to the enzyme deficiency in recipients without the overdose toxicity in the enzyme-mediated metabolism. In order to resolve the above remaining problems for wide clinical applications, the established autologous cell transplantation model enables us to evaluate the effects of the environmental conditions of the transplanted ccdPA on the survival and/or function of cells in detail, which is critical for successful cell-based gene therapies in humans.

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) was used as culture media except for the adipogenic induction in which PGM-2 Bullet kit (Lonza, Basel, Switzerland) was used. The m-ccdPA were prepared from 7-8 weeks male C57BL/6J mice as described (Kuroda *et al.*, 2011).

Optimization of gene transduction

Human *lcat* gene-expressing amphotropic retrovirus vector, CGT_hLCATRV (Kuroda *et al.*, 2011), was used for gene transduction at the concentration of 2.0×10^9 RNA copies/ml. Based on the report of Landazuri *et al.* (2007), we examined 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. Subsequently, LCAT activities secreted in culture medium were measured to examine the effect of transduction conditions using artificial liposome substrate as described (Kuroda *et al.*, 2011).

Real-time PCR and RT-PCR

Genomic DNA extractions from cultured cells and mice transplants, and quantification of transduced human *lcat* gene were performed as described (Kuroda *et al.*, 2011). Total RNA was prepared by RNeasy Plus Mini kit (QIAGEN). Single-stranded cDNA was synthesized with ReverTra Ace-α-™ kit (TOYOBO, Osaka, Japan). PPARγ2 expression was examined by RT-PCR using primers as follows; 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. The amplified products were subjected to 2% agarose gel electrophoresis and visualized with staining with GelStar® Nucleic Acid Stain reagent.

Detection of LCAT protein

Mice sera 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 rotation. Twenty microliters of TrueBlot™ anti-Rabbit Ig IP Beads (eBioscience, San Diego, CA) was added and incubated with rotation for 2 h at 4°C. Beads/proteins complex was washed with PBS-NP40, and treated by boiling in 10 μl of 2 × Laemmli's sample buffer. Samples and standards (recombinant human LCAT (Roar Biomedical, Inc., New York, NY) or human plasma HDL (Calbiochem, Merck, Darmstadt, Germany)) were subjected to western blotting using anti-LCAT rabbit polyclonal antibody (Novus Biologicals, Littleton, CO) and TrueBlot anti-Rabbit IgG HRP (1:5000) (eBioscience) as primary and secondary antibody, respectively. 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 (hLCAT) as HDL into mice serum was 101.0 ± 9.5%.

In vivo experiment

Animal experiments were carried out according to the Guidelines for Animal Research of Chiba University or

ORIENTAL YEAST Co., Ltd.. Male nude and C57BL/6J mice (Charles River Japan) were used as recipients. The cells were stained using PKH26 Red Fluorescent Cell Linker kit for General Cell Membrane Labeling (Sigma-Aldrich) to identify the transplanted cells *in vivo*.

Bolheal (The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan) was used as clinically available fibrin glue. Fibrinogen solution and thrombin solution were diluted four and two times using DMEM-HAM/F12 (Sigma) respectively before use. Cells were suspended at 1×10^7 cells/ml by diluted thrombin solution, and 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 5×10^6 cells/ml. In both cases, 5×10^6 cells were transplanted.

All mice were allowed free access to regular chow and water. Three animals were sacrificed to take serum samples at day 1, 14, 28. In C57BL/6J mice experiments, blood samples were taken from tail without sacrifice to monitor the hLCAT delivery in same animal at day 1, 4, 7, 14 and 28. Transplanted region was taken under fluorescent microscopic observation by SZX16 reflected fluorescence system (OLYMPUS corp. Tokyo, Japan).

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 anti-LCAT rabbit monoclonal antibody (250:1; EPITOMICS) and Alexa Fluor 488 goat anti-rabbit IgG (1000:1; Invitrogen) as primary and secondary antibody, respectively. The slides were counterstained with DAPI using VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, Inc., Burlingame, CA). TUNEL staining of the explanted tissues were performed using *In situ* Apoptosis Detection Kit (TaKaRa Bio Inc., Shiga, Japan). Ki67 imuunostaining was performed using anti-mouse Ki67 Rabbit polyclonal antibody (Abcam plc., Cambridge, UK), followed by biotin-conjugated anti-Rabbit Ig/HRP-conjugated streptavidin reaction. Signals were visualized by HRP reaction with DAB and the slides were counterstained with hematoxylin for TUNEL and Ki67 staining.

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.

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Ceiling Culture-Derived Proliferative Adipocytes are A Possible Delivery Vehicle for Enzyme Replacement Therapy in Lecithin: Cholesterol Acyltransferase Deficiency

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Abstract: Human proliferative adipocytes propagated *via* ceiling culture technique from subcutaneous fat tissue (designated as ccdPA) were herein evaluated for their potential as a recipient for retroviral vector-mediated gene transduction of a therapeutic protein delivery. Exposure to the ZsGreen-expressing vector supernatant using a cell preparation generated by a 7-day ceiling culture induced a 40-50% transduction efficiency, with less than two integrated copies of viral genome per cell on average. The *lcat* gene-transduced human ccdPA secreted functional LCAT protein, correlating with the integrated copy number of vector genome. The gene-transduced cells could be expanded up to nearly 10^{12} cells from 1 g of fat tissue within one month after fat tissue preparation. The cells also maintained the potential to differentiate into adipocytes *in vitro*. The presence of human LCAT protein in serum was immunologically identified upon transplantation of *lcat*-expressing ccdPA into the adipose tissue of immune-deficient mice. These results indicated that human ccdPA has a novel therapeutic potential for LCAT-deficient patients. The clinical application in combination with cell transplantation shed a light on a development of a life-long protein replacement therapy for LCAT-deficient patients.

Keywords: Protein replacement therapy, lecithin:cholesterol acyltransferase, adipocyte, ceiling culture, gene therapy.

INTRODUCTION

The intriguing biology of pluripotent stem or progenitor cells has suggested the sustained production of therapeutic proteins to be a treatment for patients with serum protein deficiencies [1, 2]. The ability of cells to self-renew at a high proliferation rate has led to the expectations that these cells are ideal targets for retroviral vector-mediated transgene delivery. Studies examining this concept have described the treatment of various diseases in animal models [3-10].

Lecithin:cholesterol acyltransferase (LCAT) is a plasma protein responsible for the conversion of plasma unesterified cholesterol into cholesteryl ester, and plays a central role in the formation and maturation of high-density lipoproteins (HDL), which are involved in reverse cholesterol transport. Genetic LCAT deficiencies have been identified, and more than forty different mutations have been identified to date (refer to HGMD: http://www.hgmd.cf.ac.uk/ac/index.php). Plasma LCAT is either absent or exhibits no catalytic activity in patients with a familial LCAT deficiency. Cholesteryl ester levels are markedly reduced in lipoproteins, abnormal cholesterol deposition is observed in the tissues of these patients, and patients often develop corneal opacity, anemia, proteinuria, and renal failure [11]. The efficacy of LCAT replacement therapy was shown by infusion of normal plasma [12, 13], but the effects were transient. In addition, replacement therapy with recombinant LCAT protein has not been established mainly because this is a rare condition, and due to the associated expenses for production of the recombinant protein. Therefore, life-long treatment with autologous cell-based therapy may contribute to the continuous replacement of enzymes.

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Recently, much attention has been paid to adipose tissue as a source of proliferative cells for cell-based gene therapy [14] and for regenerative therapy [15, 16]. Two types of preparations have been reported to be sources of adipose-tissue derived proliferative cells. One is stromal-vascular fractions (SVFs), which can be obtained as sediment by the centrifugation of collagenase-digested fat tissue [17, 18]. The obtained cells are pluripotent and can differentiate to yield various cell types, including cardiomyocytes, chondrocytes, and osteoblasts, in addition to adipocytes [19]. The other cell preparation is obtained from the floating mature fat cell fraction of the centrifugation, followed by a ceiling culture [20]. The cultured cells maintain the ability to differentiate into mature adipocytes at a high frequency [10, 20, 21], and are presumably more committed to the adipocytes lineage.

In the present study, a target cell population was prepared from adipose tissue using the ceiling culture technique to develop a cell-based gene therapy of LCAT-deficient patients, and we designated the target cells as ceiling-culture derived proliferative adipocytes (ccdPA). The current study established this production procedure, and optimized the gene transduction conditions of human ccdPAs as therapeutic gene recipient cells. In addition, we assessed the capability and the safety of the *lcat* gene-transduced ccdPA as a LCAT-secreting device for protein replacement therapy. Therefore, we developed stable protein-producing human ccdPAs with self-renewing and high expansion capacities.

MATERIALS AND METHODS

Construction of pCGThLCAT, A Retroviral Vector Plasmid Encoding the Human *Lcat* Gene

The pDON-AI, Moloney Murine Leukemia virus (MoMLV) vector plasmid (TaKaRa Bio Inc., Shiga, Japan) was used as a recipient for the human *lcat* cDNA. The *lcat* cDNA was derived from total RNA prepared from HepG2 cells. The resulting cDNA was amplified by PCR using the following primer pair: 5'-ATCGGATCCAGGGCTGGAAATGGGGCCGCCC-3' (forward) and 5'-ATCGGATCCGTCGACGGAAGGTCTTTATTCAGGAGGCGGGGG-3' (reverse). The forward primer contained a *Bam*HI restriction site (underlined) and a Kozak sequence, and the reverse primer contained a *Sal*I restriction site (underlined). The reverse primer also eliminated the polyA signal from the original *lcat* cDNA. The amplified PCR products were digested by *Bam*HI and *Sal*I and cloned into the corresponding sites of the pDON-AI plasmid. Thereafter, the neomycin resistant gene was removed by *Sal*I and *Xho*I digestion and subsequent self-ligation, yielding the pCGThLCAT plasmid.

Production of the Amphotropic Retroviral Vector

The GMP grade retroviral vector CGT_hLCATRV was produced by TaKaRa Bio Inc. In brief, the pCGThLCAT vector was transfected into the ecotropic packaging cell line GP+E86 (ATCC#: CRL-9642), and the supernatant was collected. The supernatant was used to infect the amphotropic packaging cell line GP+envAM-12 (ATCC#: CRL-9641) to produce a master cell bank (MCB) for vector production. CGT_hLCATRV was prepared from culture supernatant of the MCB. The vector solution was aliquoted and stored at -80 °C until use. The vector titer was quantified by TaKaRa Bio Inc. using the One Step SYBR PrimeScript RT-PCR Kit with primer pairs from Retrovirus Titer Set (TaKaRa Bio Inc.). The *ZsGreen*-gene expressing retrovirus vector was similarly propagated.

Cell Culture and Medium

Dulbecco's modified Eagle's medium [DMEM]/F12-HAM (Sigma-Aldrich, St. Louis, MO) and MesenPRO medium (Invitrogen, Carlsbad, CA) were used to maintain cultured cell lines. Fetal bovine serum (FBS) was purchased from SAFC Biosciences (Lenexa, KS). Cell passaging was performed twice a week.

Isolation of ccdPAs from Human Fat Tissue

Subcutaneous adipose tissues were obtained from 16 healthy volunteers (C001-C016) with ages ranging from 19 to 42 years after informed consent was obtained with the approval and guidelines of the ethical committee at Chiba University School of Medicine, according to the Declaration of Helsinki. Ceiling culture techniques [20] were employed and optimized using C001-C012 fat tissues to isolate human ccdPAs as follows. Fat tissue was weighed, and each 1.0 g was digested with gentle agitation for 1 hr at 37 °C in 3 ml of Hank's balanced salt solution (HBSS) containing 2 mg/ml collagenase (Collagenase NB 6 GMP Grade, SERVA, Heidelberg, Germany) and 40 μg/ml gentamicin (GENTACIN, Schering-Plough Co., Kenilworth, NJ). Thereafter, the solution was diluted with 10 ml of DMEM/F12-HAM containing 20% FBS and 40 μg/ml gentamicin (DMEM/FBS), mixed, and centrifuged at 400 x g for 1 min. The pellet was removed as an SVF. The dilution steps were repeated 4 times to collect the floating cell fraction. The floating fraction was filtered with a 500-μm mesh (Netwell Insert, Corning Inc., Corning, NY) and seeded into flasks, which were filled with DMEM/FBS. After 7 days ceiling culture, cells that grew at the ceiling surfaces were harvested and seeded into flasks for the subsequent steps.

Gene Transduction

In preliminary experiments, the acceptability of the MoMLV vector for human ccdPA propagated in the course of ceiling culture revealed that longer culture times resulted in a higher resistance to retroviral vector transduction (data not shown). Therefore, the cells obtained by 7 day-ceiling culture were evaluated as a potential recipient for retroviral vector-mediated gene transduction. Human ccdPAs were seeded and incubated in DMEM/FBS at 37 °C for 24 hrs. Protamine sulfate (PS, Novo-Protamine Sulfate, 100 mg for I.V. Injection, Mochida Pharm. Co. Tokyo, Japan) was used to optimize the transduction conditions (0.5-16 μg/ml). Gene transduction was performed in the presence of 20% FBS and 8 μg/ml PS at 37 °C for 24 hrs. The viral vector concentration used for transduction was 2.0×10^9 RNA copies/ml, unless otherwise specified. After transduction, the medium was replaced with growth medium.

Flow Cytometry

Cells were suspended in phosphate buffered saline containing 2% FBS (PBS/FBS). Fluorescein isocyanate (FITC) or phycoerythrin (PE)-conjugated antibodies were purchased from BD Farmingen (San Diego, CA) or Beckman Coulter (Fullerton, CA), or Ancell Corporation (Bayport, MN). Aliquots of cell suspensions (4.5 x 10^4 cells) were mixed with primary antibody in a total volume of 90 µl and were incubated for 30 min at RT. The cell suspension was washed twice with PBS/FBS, and the cells were fixed in 200 µl of PBS/FBS containing 1% paraformaldehyde. Five thousand events were acquired for each antibody on a FACS Calibur apparatus using the CELLQuest acquisition software program (Becton, Dickinson and Company, Flanklin Lakes, NJ). *ZsGreen* expression was also examined in human ccdPAs. Non-transduced cells were used as a negative control.

Quantification of Transduced Gene

Genomic DNA was extracted from cultured cells and mouse adipose sections with the DNeasy Blood & Tissue kit and the Gentra Puregene kit (QIAGEN, Hilden, Germany), respectively. The integrated vector copy number was quantified with the SYBR *Premix Ex Taq* (Perfect Real Time) kit (TaKaRa Bio Inc.). A known amount of pCGThLCAT DNA was used as a standard. The primer pairs were from the Retrovirus Titer Set (TaKaRa Bio Inc.). The DNA content in a human normal cell (6 pg/cell) [22] was used for calculating the average integrated copy number. Existence of transduced gene in transplanted adipose tissue was quantified with a TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA) using *lcat*-cDNA specific primers and probes designed by the Probe Finder Software program (Roche Diagnostics, Mannheim, Germany). All the real-time PCR reactions were performed using the ABI7500 Real-time PCR system (Applied Biosystems).

Detection of LCAT Protein

Culture medium and mice sera were diluted to a volume of 500 µl with ice-cold phosphate buffered saline containing 0.2% Nonidet P-40 (PBS-NP40) and were incubated with 2.5 µl of anti-LCAT rabbit monoclonal antibody (EPITOMICS, Burlingame, CA) for 18 hrs at 4 °C with gentle rotation. Twenty micro-liters of TrueBlot 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 with PBS-NP40, and eluted by boiling in 10 µl of 2X Laemmli's sample buffer. Immunoprecipitated samples were subjected to immunoblotting. Purified human LCAT (Roar Biomedical, Inc., New York, NY) or human plasma HDL (Calbiochem, Merck, Darmstadt, Germany) was used as a standard. An anti-LCAT rabbit polyclonal antibody (Novus Biologicals, Littleton, CO) and TrueBlot anti-Rabbit IgG HRP (1:5000) (eBioscience) were used as primary and secondary antibodies, respectively. The signals were detected with the SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific Inc.) and the LAS1000 apparatus (FUJI film, Tokyo, Japan).

Measurement of LCAT Activity

The procedure described by Ishii *et al.* [23] was modified to prepare the liposome substrate for the LCAT analyses. Two hundred microliters of [^{3}H]-cholesterol (American Radiolabeled Chemicals, Inc., St. Louis, MO) were evaporated to dryness by flushing N_2 gas, and 5 ml of the substrate mixture of Anasolv LCAT kit (SEKISUI MEDICAL Co. Tokyo, Japan) was added. The solution was sonicated with a Digital Sonifier Model 250 (BRANSON, Danbury, CT) at an amplitude of 40% and 0.5 second pulse cycles for 1 min a total of six times in an ice bath. The sonicated mixture was centrifuged at 3,000 rpm and stored at 4 °C until use. The reaction mixture contained 100 µl of labeled substrate, 4.5 mM β-mercaptoethanol, 36 µg of apolipoprotein A1 (Athens Research & Technology, Athens, GA), and 100 µl of culture medium in a total volume of 220 µl. The reaction was performed at 37 °C for 1 hr, and was terminated by the addition of 1.6 ml of chloroform/methanol (2:1). One hundred microliters of water was added, and the organic phase was obtained by centrifugation. Fifty microliters of the organic phase was spotted onto Whatman flexible thin layer chromatography (TLC) plates (Whatman plc, Kent, UK). Sample-spotted plates were developed with standards of cholesterol and cholesterol oleate in a glass tank using a solvent mixture of hexane/ethyl ether/acetic acid (146:50:4) by TLC. Developed TLC plates were air-dried and stained with iodine (Wako Pure Chemicals, Osaka, Japan). Cholesteryl ester spots were excised, and the radioactivity was determined by liquid scintillation spectrometry.

Adipogenic Differentiation Assay

Human ccdPA (3.5 x 10^4 cells) were seeded into BioCoat Collagen I 48-well Multiwell Plates (BD Biosciences) and grown to confluency over 3 days. Differentiation was induced with the PGM Bullet Kit (Lonza, Basel, Switzerland), and the cells were incubated for 2 weeks. The cells were fixed in 4% paraformaldehyde, washed twice with PBS, incubated with 60% isopropanol for 1 min, and stained with Oil Red O solution (Chemicon International, Inc. Temecula, CA) for 20 min. The accumulation of triglycerides was examined to confirm adipogenic differentiation using the Triglyceride E-test kit (Wako Pure Chemicals) according to the manufacturer's instructions. The protein content of the lysate was also determined with the Quick Start Bradford Dye Reagent (Bio-Rad Laboratories Inc.).

Clonality Analysis by Southern Blotting

Abnormal amplification of specific cell clones resulting from the integration of the retroviral vector genomic sequence was examined by Southern blotting according to the DIG (Digoxigenin) protocol (Roche Diagnostics). Genomic DNA extracted with the Gentra Pure Gene kit (QIAGEN) was digested with *Hin*dIII (Roche Diagnostics). Digested DNA (6 µg) was subjected to agarose gel electrophoresis, followed by capillary transfer to a positively-charged nylon membrane (Roche Diagnostics). Human *lcat* cDNA was used as a template to synthesize probes by the PCR DIG Probe Synthesis kit (Roche Diagnostics). Hybridization was performed at 50 °C overnight. The membrane was washed and reacted with Anti-digoxigenin-AP, Fab fragments (Roche Diagnostics). The signals were detected using CDP-Star with the LAS1000 apparatus (FUJI film). As positive control, 293 (European Collection of Cell Cultures) cells were transduced with a neomycin-resistant gene-containing version of the *lcat*-expressing retroviral vector, and typical single copy-integrated clones were selected.

Colony Formation Assay by Soft-Agar Containing Medium

Anchorage-independent colony formation was examined by the soft-agar assay using the CytoSelect 96-well Cell Transformation Assay kit (Cell Biolabs, Inc., San Diego, CA). Ten thousand gene-transduced human ccdPAs were seeded into 96-well plates in triplicates along with 100, 1000, or 10,000 HeLa cells (European Collection of Cell Cultures) as a positive control.

Monitoring Human LCAT Secretion in Mouse Model

Animal experiments were performed in the Central Institute for Experimental Animals (CIEA, Kanagawa, Japan) according to the Ethical Guidelines for Animal Experimentation from CIEA to examine the delivery of LCAT protein *in vivo*. To identify the transplanted cells, cells were stained using the PKH26 Red Fluorescent Cell Linker kit for General Cell Membrane Labeling (Sigma-Aldrich) one passage prior to transplantation. Expanded cells were harvested, washed with Ringer solution containing 0.5% human serum albumin (HSA, Benesis Corp. Osaka, Japan) four times, and re-suspended to a final cell concentration of 3×10^7 cells/ml. The cell suspension (50 μl) was injected into the adipose tissue between the shoulder-blades of NOD/Shi-*scid* IL-$2R\gamma^{null}$ (NOG) mice [24]. Buffer alone was injected as a control. All mice were bred in a vinyl-isolator and six animals were sacrificed to collect serum samples at each time point (Day 1, and at 1, 3, and 6 months). Six and three animals were used for the transplanted and control groups, respectively, for each time point. The transplanted region was taken using fluorescent microscopy on a SZX16 reflected fluorescence system (OLYMPUS corp. Tokyo, Japan), and sections were frozen at -80 °C until use.

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 Dunnett or Tukey test using the SPSS software program. The integrated copy number, positive rate, and LCAT activity 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. In all cases, P-values of less than 0.05 were considered to be statistically significant.

RESULTS

Preparation of Gene-Transduced Human ccdPAs

The optimization of cell-processing steps was carried out with fat tissues obtained from 16 healthy volunteers (C001-C016). Adipose tissue-derived proliferative cells were assessed for their suitability in ceiling culture, gene transduction, and cell expansion, using two culture media, DMEM/F12-HAM supplemented with 20% FBS (DMEM/FBS) and MesenPRO medium, respectively. The ceiling culture was performed in DMEM/FBS in comparison to MesenPRO medium. The cell yield of C012 after the ceiling culture from 1 g adipose tissue was $7.1 \times 10^5 \pm 1.0 \times 10^5$ and $2.1 \times 10^5 \pm 0.2 \times 10^5$ cells in DMEM/FBS and MesenPRO medium, respectively, showing that a higher cell yield was obtained in DMEM/FBS than in MesenPRO medium ($p<0.05$). The flow cytometric analyses showed that cells in DMEM/FBS tended to be homogeneous in shape and size, in comparison to those grown in MesenPRO medium (Fig. **1a**). The gene transduction of the cells after the ceiling culture was next assessed using the two medium types. The above cells which were frozen after ceiling culture (C010) in DMEM/FBS were recovered, incubated for 4 days, and seeded for gene transduction in MesenPRO medium or DMEM/FBS medium. After transduction with the *lcat*-expressing retroviral vector, the cells were passaged several times in the respective medium, and cell samples were subjected to copy number quantification 12 days after transduction. DMEM/FBS was more effective than MesenPRO medium for the gene transduction of human ccdPAs when a retroviral vector was employed under the appropriate conditions (0.94 ± 0.10 copies/cell vs. 0.36 ± 0.09 copies/cell, $p<0.05$). Finally, the effects of the

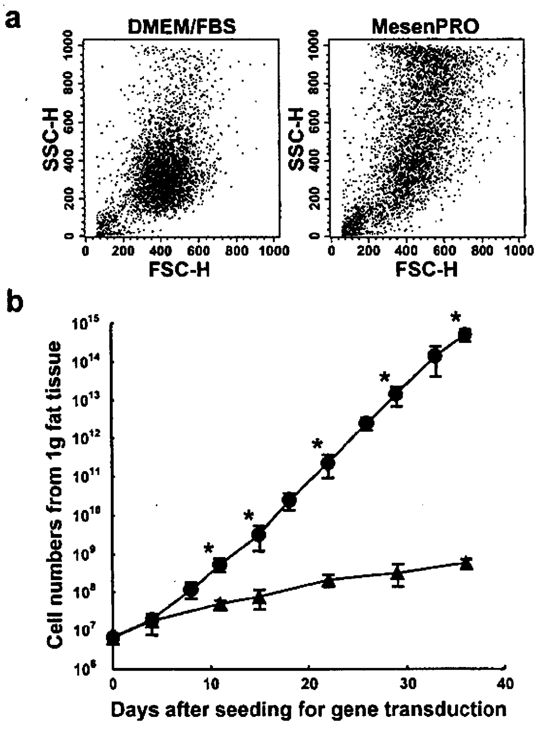

Fig. (1). Comparison of DMEM/FBS and MesenPRO media for the preparation of human ccdPAs. (**a**) The cells (C012) prepared by ceiling culture in DMEM/FBS (left panel) or MesenPRO medium (right panel) were subjected to a FACS analysis. The dot-plot (forward-scattered vs. side-scattered) of both cell populations are shown. A representative plot is shown for each medium. (**b**) The cells derived from C013 were used for expansion. Cell numbers were counted during proliferation for 35 days in DMEM/FBS (closed triangle) or MesenPRO medium (closed circle) after gene transduction in DMEM/FBS. Cell numbers are presented from 1 g of fat tissue. Data are presented as the mean ± SD (n=3). *$p<0.05$ vs. MesenPRO medium at each day after seeding.

incubation media on the gene-transduced cell expansion were examined in C013 cells. The doubling times of the cells in the MesenPRO medium were significantly shorter than those in DMEM/FBS (31.7 ± 4.8 hours vs. 119.4 ± 29.6 hours, $p<0.05$). The transduced cell number expanded to more than 3 x 10^4 fold of the original number in a month when grown in MesenPRO medium (Fig. **1b**). Therefore, DMEM/FBS was chosen for the ceiling culture and gene transduction, and the MesenPRO medium for cell expansion of ccdPA, respectively, in subsequent experiments.

Characterization of Human ccdPAs

The cell surface antigen profile was analyzed by FACS for human ccdPAs (Fig. **2a**). The populations of $CD31^-$/$CD45^-$ cells were significantly increased in the ccdPA preparation, in comparison to SVF-derived cells (99.1 ± 0.3% vs. 95.6 ± 0.1%, $p<0.05$), indicating that ceiling culture technique excludes CD31-positive and/or CD45-positive cell populations in comparison with cells prepared from SVF. The ccdPAs were positive for CD9, CD10, CD13, CD29, CD44, CD59, CD90, CD105, CD146, and HLA-ABC, and

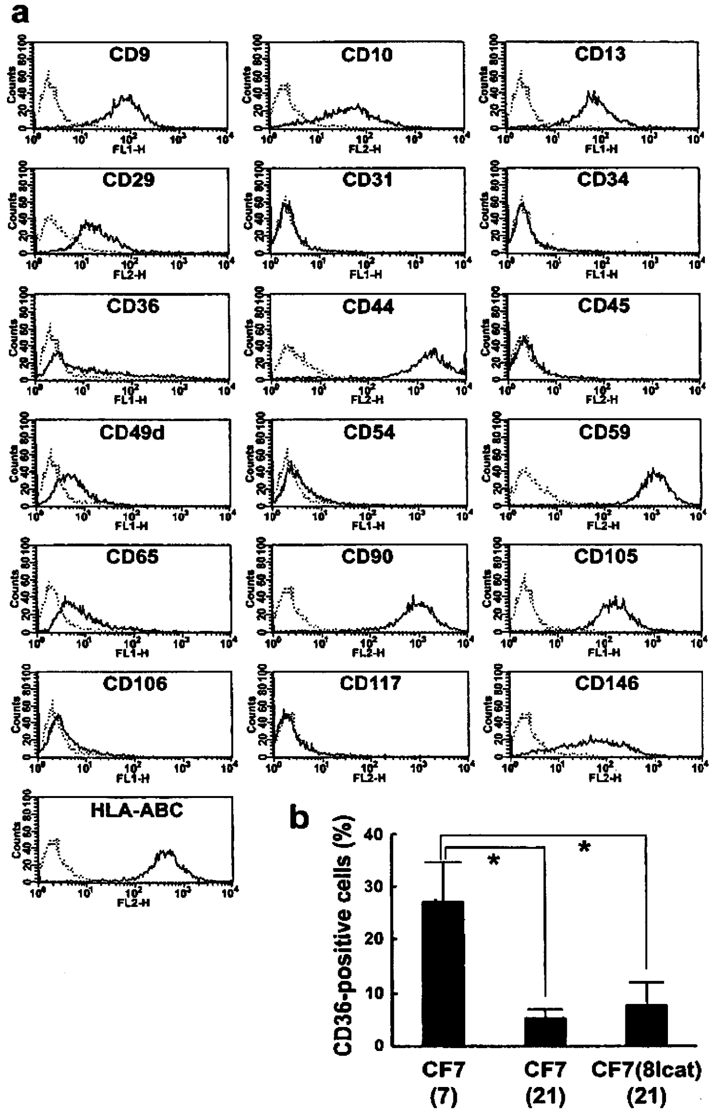

Fig. (2). Cell surface antigen profiles of isolated human ccdPAs by ceiling culture. (**a**) The cells were harvested at 7 days after ceiling culture, and were immuno-stained with the corresponding antibodies (solid line) or an isotype control (dotted line), and were subjected to a FACS analysis. Histograms for each antibody are presented. (**b**) CD36-positive cells was examined in the cells harvested from the ceiling culture (CF7(7)), the cells expanded after *lcat*-gene transduction (CF7(8lcat)(21)), and the cells expanded without gene transduction (CF7(21)). The ratio of CD36-positive cells in the prepared cells is presented as the positive cell rate (%). Data are presented as the mean ± SD (n=3). *$p<0.05$.

negative for CD31, CD34, CD45, CD54, and CD106. They were moderately positive for CD49d and CD65, and a substantial number of cells were positive for CD36, a marker for adipocytes [25]. The populations of CD36-positive cells after a 14-day *in vitro* culture of ccdPAs were significantly lower than those at 7 days ($p<0.05$, Fig. **2b**).

Retroviral Vector-Mediated Gene Transduction and Transduced Gene-Derived Protein Secretion in Human ccdPA

Human ccdPAs were evaluated as a recipient of MoMLV-based gene transduction using various concentrations of the vector and PS with single round of transduction using a ZsGreen-expressing vector. Two types of cells were analyzed, one cell type just after harvesting from the ceiling culture (CF7(7)), while another type was further cultured in the normal manner for an additional week (CF7(14)) in DMEM/FBS. The integrated copy number could be increased to approximately 1.7 and 2.5 copies/cell in CF7(7) and CF7(14) cells, respectively, and a good linear correlation was observed between the integrated copy number and the transduction efficiency (percentage of ZsGreen-positive cells) (Fig. **3a**). The transduction efficiency and the integrated copy number were significantly different between the cells of same batch at Days 1 and 2 of gene transduction (Fig. **3b**). These results showed that the cells with a higher transduction efficiency of the transduced gene and a lower integrated copy number were obtained by transduction for cells which were seeded and incubated overnight following a 7-day ceiling culture (CF7(8)). The CF7(8) cells were examined as a potential recipient for the human *lcat* gene. The transduction analyses using the *ZsGreen* vector showed that a vector concentration of 2.0×10^9 RNA copies/ml resulted in a good correlation between the integrated copy number and ZsGreen-positive cells in two different cell batches (Fig. **3a**). The use of the maximum achievable concentration (3.1×10^9 RNA copies/ml) of CGT_hLCATRV was compared with that using a concentration of 2.0×10^9 RNA copies/ml. Transduction of CF7(8) cells with 3.1×10^9 or 2.0×10^9 RNA copies/ml of the vector resulted in no difference in the integrated copy number (1.65 ± 0.12 vs. 1.56 ± 0.23 copies/cell). The LCAT protein produced by the *lcat* gene-

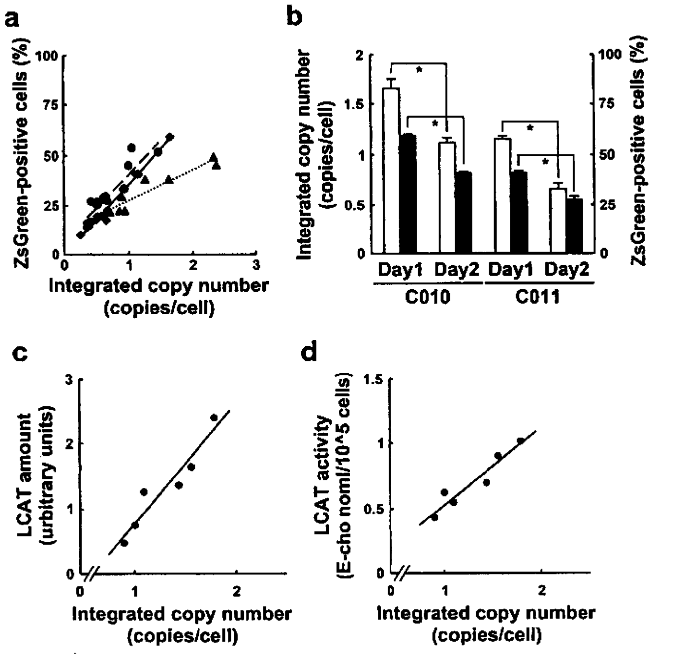

Fig. (3). ***In vitro* evaluation of human ccdPAs as recipients of MoMLV-based retroviral vector-mediated gene transduction and a vehicle for the secretion of functional LCAT protein.** (**a**) Integrated copy number (copies/cell) and ZsGreen-positive cells (%) were plotted for C010 CF7(7) (closed rhombus), C011 CF7(7) (closed circle), and C011 CF7(14) (closed triangle). Lines are drawn with Pearson r-values of 0.991, 0.908, and 0.937 for C010 CF7(7) (solid line), C011 CF7(7) (broken line), and C011 CF7(14) (dotted line), respectively ($p<0.05$). (**b**) Integrated copy numbers (copies/cell, open bars) and ZsGreen-positive cells (%, closed bars) after a single round of exposure of 2.0×10^9 RNA copies/ml of virus vector are shown. The cells (C010 and C011) were exposed to the transduction mixture one day (Day 1) or two days (Day 2) after seeding. Data are presented as the mean ± SD (n=3). *$p<0.05$. (**c**) Secreted LCAT protein was detected by immunoprecipitation/immunoblotting in culture medium incubated for 3 days with 1×10^5 cells (C013). After a densitometric analysis of immunodetected signals for human LCAT protein (60-65kDa), the integrated copy number and LCAT level (arbitrary units) were plotted (Pearson r-value of linear coefficient, 0.953, $p<0.05$). (**d**) Culture medium incubated with 1×10^5 cells (C013) for 3 days were subjected to assay of LCAT activities. The activity was presented by esterified cholesterol production from the cholesterol in the medium of human ccdPAs (Pearson r value of linear coefficient, 0.954, $p<0.05$).

transduced human ccdPAs was analyzed (Fig. **3c** and **3d**). Seven days after gene transduction, 1 x 10^5 cells were seeded in a 12-well plate, grown for three days, and the supernatant was collected for subsequent assays. LCAT protein production and the LCAT activity were determined by immunoprecipitation/immunoblot (IP-Western) and a cholesterol esterifying assay in the medium, respectively. LCAT protein and activity significantly correlated with the integrated copy number (r=0.917 and 0.954, respectively, $p<0.05$). Therefore, the activity of the LCAT protein produced by the gene-transduced ccdPA was estimated by the integrated copy number. The *lcat* gene-transduced ccdPAs produced LCAT protein with a specific activity of 5.2 ± 0.5 fmol esterified-cholesterol/integrated copy/hr in the culture medium within 3 days.

Properties of the *Lcat* Gene-Transduced Human ccdPAs during the Manipulation Process

The effect of *in vitro* manipulation was evaluated on the ccdPA characteristics regarding adipogenic differentiation ability, expansion rate, cell surface marker expression, transgene stability, and anchorage-independent cell growth. The cells were stimulated to differentiate and Oil Red O staining demonstrated the transduced cells had clearly differentiated into adipocytes (Fig. **4c**), and their appearance as well as that without differentiation stimulation, was not obviously different from cells without gene transduction (Fig. **4a-f**). The triglyceride contents showed no significant differences between transduced and control cells in C014 samples (1.30 ± 0.43 vs. 1.25 ± 0.27 mg/mg protein). The proliferating cell number and the resultant doubling time were not signifi-

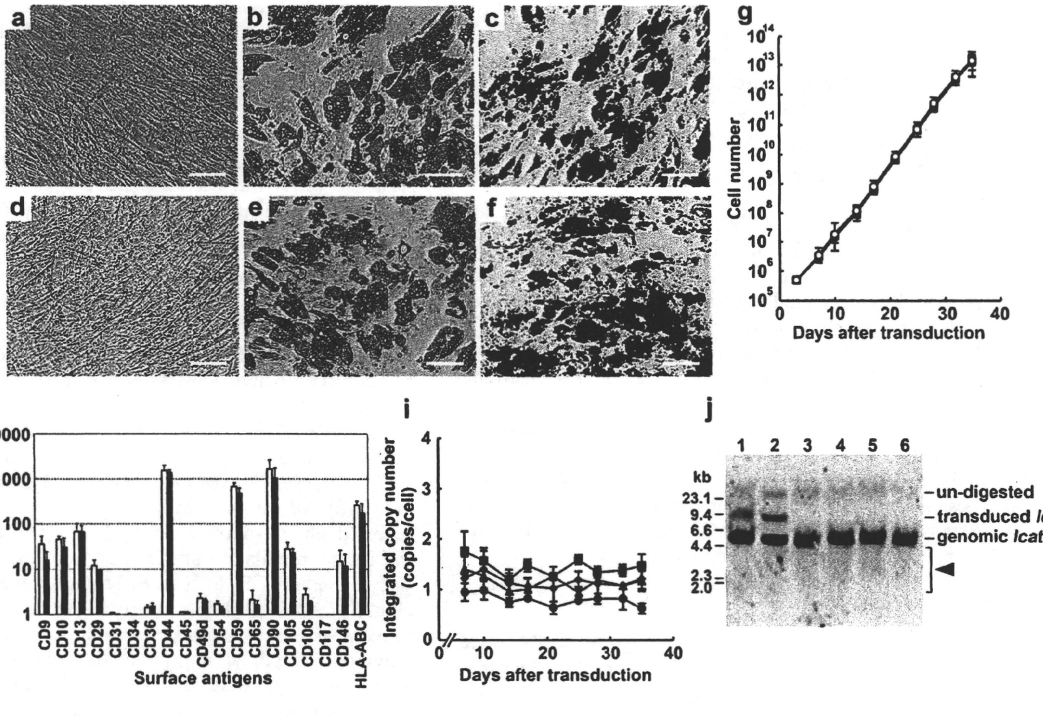

Fig. (4). Characterization of *lcat*-transduced ccdPA in culture. The *lcat*-transduced (**a, b, c**) and non-transduced (**d, e, f**) cells of C013 were incubated for two weeks with (**b, c, e, f**) or without (**a, d**) differentiation stimulation. The appearance of cells was observed with (**c, f**) or without (**a, b, d, e**) Oil Red O staining (magnification bar, 100 µm). (**g**) C013 cells were transduced, and the resultant cells were passaged. The cell numbers were counted during proliferation for 35 days. The cells were transduced by the conditions of 1.3 x 10^9 RNA copies/ml on Day 2 (closed circle), 1.3 x 10^9 RNA copies/ml on Day 1 (closed triangle), 2.0 x 10^9 RNA copies/ml on Day 1 (closed rhombus), or 3.1 x 10^9 RNA copies/ml on Day 1 (closed square). Doubling times were 32.2 ± 5.8 (closed circle), 31.5 ± 4.0 (closed triangle), 31.6 ± 3.9 (closed rhombus), and 31.3 ± 4.4 hrs (closed square), respectively. The doubling time of the control (non-transduced) cells (open circle) was 31.5 ± 4.7 hrs. Data are presented as the mean ± SD (n=3). No significant differences were observed in comparison to the control cells. (h) The *lcat*-transduced cells (closed bars) and non-transduced cells (open bars) were expanded in MesenPRO medium for two weeks after gene transduction. The values of Geo/mean for 19 different surface antigens were examined by a flow cytometry analysis. Data are presented as the mean ± SD (n=3). (**i**) The integrated copy number of *lcat*-transduced ccdPAs was followed during *in vitro* culture. Symbols are same as shown in Fig. **4G**. Data are presented as the mean ± SD (n=3). (**j**) A clonal analysis was performed by Southern blotting in C013 cells. C013 genomic DNA samples were prepared from the cells 18 days after gene transduction. Lanes 1 and 2, *lcat* gene-transduced clones obtained by transduction of 293 cells; lanes 3, 4, and 5, *lcat* gene-transduced human ccdPAs with different integrated copy number (lane 3; 0.90 ± 0.20; lane 4, 1.65 ± 0.12; and lane 5, 1.79 ± 0.23 copies/cell); lane 6, non-transduced (control) cells. A smeared faint signal was observed in the *lcat*-transduced ccdPAs (shown by arrow).

cantly different between the transduced cells and control cells (Fig. **4g**). In addition, no significant differences were observed in the cell surface marker expression levels between transduced and control cells (Fig. **4h**). The integrated copy number in the transduced ccdPAs was monitored to assess the fate of the transgene during the culture period for 35 days (Fig. **4i**). The integrated copy number did not significantly change after gene transduction. A Southern blot analysis using the human *lcat* gene as a probe revealed that only a faint signal was present independent of the genomic *lcat* locus, indicating that no amplification of a specific clone had occurred during the expansion process (Fig. **4j**). A soft agar assay showed that no anchorage-independent colony formation was present in the gene-transduced human ccdPAs (data not shown). These results demonstrated that the effect of gene transduction was negligible (or denied) on the characteristics of the obtained human ccdPAs regarding the differentiation, cell surface marker expression, transgene stability, and cell growth, in comparison to the non-transduced cells.

Circulating LCAT Supplementation by the Implantation of *Lcat* Gene-Transduced ccdPA in Mice

The capacity of human ccdPAs to be recipient cells for *lcat* gene product delivery was assessed in mice. A cell suspension containing 1.5 x 10^6 cells was transplanted into the fat tissue of immuno-deficient mice, and the levels of LCAT protein secreted into the serum was determined by the IP-Western method. Human LCAT was clearly detected in the sera of all transplanted mice at Day 1 (Fig. **5a**), and was detectable after a month in mice (Fig. **5b**). A densitometric analysis revealed that the concentration of human LCAT was approximately 0.26 ± 0.19 μg/ml at Day 1. The real-time PCR quantification of the adipose tissue transplanted with *lcat*-gene-transduced ccdPA showed that the *lcat* gene was present at 42.9 ± 27.1% (Day 1), 1.0 ± 1.0% (1 month), and 1.2 ± 0.7% (3 months) compared to transplanted cells at Day 0. These results suggested that approximately 1% of the *lcat* gene-transduced ccdPAs survived for 3 months after the transplantation of cells into the fat tissue of mice.

DISCUSSION

The current study evaluated autologous ccdPAs, the mature adipocye-derived cells, prepared from the subcutaneous fat of patients as a vehicle for therapeutic protein replacement therapy. Adipose tissue contains two major sources of proliferative cell populations, the floating (mature adipocytes) and pellet fractions (SVF), following the centrifugation of collagenase-digested fat tissue. This cell-based gene therapy was developed from the mature adipocyte cultures, since SVF consists of a heterogeneous cell population, including blood cells, fibroblasts, and endothelial cells [15, 16] and has some risks in yielding a cell population with an abnormal phenotype in long-term culture *in vitro* [26, 27]. The ceiling culture of the SVF-removed floating fraction can further enrich the cells derived (or dedifferentiated) from mature adipocytes by the buoyant property of adipocytes during the ceiling culture periods. Our ceiling culture excludes CD31- and CD45-positive cells, and our ccdPAs were negative for CD34, the marker for which adipose-derived stem cells are positive [28-30].

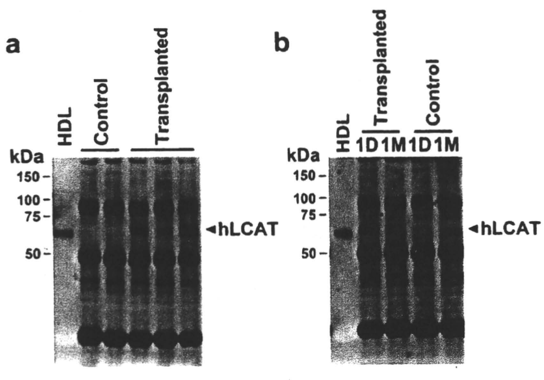

Fig. (5). Circulating human LCAT in NOG mice transplanted with *lcat*-transduced human ccdPAs. The cell suspension containing 1.5 x 10^6 *lcat*-expressing human ccdPA cells (C014, Transplanted) and Ringer's solution containing 0.5% HSA (Control) were injected into the fat tissue of NOG mice. After one day (**a, b**) or one month (**b**), the mice were sacrificed and serum samples were collected at each time point. D1, next day of injection; M1, 1 month after injection; H, 15 μg of HDL (control). At 1 month after transplantation, LCAT was detected in the serum of two mice out of six. At 3 months or later, LCAT was barely detectable in serum (data not shown).

MesenPRO medium, which is optimized for mesenchymal stem cells, provided some advantages in the preparation of ccdPAs through the higher expansion capacity in comparison to DMEM/FBS (Fig. **1b**). On the other hand, the MesenPRO medium was less effective for the propagation of human ccdPAs in ceiling culture than DMEM/FBS. Therefore, MesenPRO medium appears to be unsuitable for the proliferation of mature adipocytes in ceiling cultures. The FACS analyses showed that the obtained ccdPAs had a similar profile of surface markers with that of the previously reported adipose-derived cells [31, 32] (Fig. **2a**). In addition, the certain population of the ccdPAs retained a mature adipocyte marker (CD36) at an early stage and eventually lost it (Fig. **2b**). ccdPA exhibits clearly higher adipogenic potential in comparison to stromal vascular fraction derived cells, commonly used as multi-potential adipose tissue-derived stem cells, suggesting the advanced differentiation levels of ccdPA committed to mature adipocytes (manuscript in preparation). These adipogenic properties are sufficient for the cells to survive in fat tissue and to keep producing therapeutic protein for a long period after transplantation.

Previous reports described mature adipocyte-derived cells that were utilized and evaluated after primary culture for 2 weeks, and these cells were suggested to be a source of regenerative medicine [31, 32]. We demonstrated that 7-day primary cultures resulted in substantially better transduction properties than 14-day primary cultures for gene therapy applications. Simple exposure to the viral vector supernatant resulted in a 40-50% improved transduction efficiency (Fig. **3a** and **3b**) using ccdPAs after 7-day primary culture, thus suggesting that human ccdPAs serve as an excellent recipient for retroviral vector-based therapeutic applications, in contrast to cell populations in which efficient transduction requires drug selection [3, 33] or multiple rounds of transduction [34, 35]. Therefore, a single exposure to 2.0 x 10^9 RNA copies/ml of CGT_hLCATRV was selected to minimize the transgene copy number in each cell. Furthermore, the transduction efficiency was correlated with the integrated copy number (Fig. **3a** and **3b**). The *lcat*-expressing retroviral vector was constructed using pDON-AI, developed by Yu *et al.* [36], as a backbone vector. The risk of replication-competent retrovirus (RCR) occurrence was minimized by eliminating all the unnecessary structural genes from the MoMLV genome in this vector. In fact, no RCR was detected in the vector preparations (data not shown). The integration sites seemed to be randomly distributed since no clonal expansion was detected by a Southern blot analysis of the transgene following expansion culturing (Fig. **4j**), and no increase in the integrated copy number was observed in the preparations (Fig. **4i**). No evidence of transformation was observed in the soft agar assay, either at the time of implantation (after three weeks from fat tissue removal) or after long-term extended culture (data not shown). Furthermore, *in vivo* tumor formation assay by nude mice model revealed no abnormal cell growth after transplantation (unpublished observation). The safety issue of our therapeutic strategy will be carefully evaluated in future clinical studies.

The human *lcat* gene-transduced ccdPAs yielded the glycosylated LCAT protein (data not shown) that had a molecular weight and *in vitro* enzymatic activity equivalent to that observed in human serum. An animal study indicated that the human LCAT protein secreted from the implanted transduced human ccdPAs was detectable in blood samples (Fig. **5**). The serum of familial LCAT-deficient patients contains less than 10% LCAT activity compared to that in healthy subjects [11]. Patients with partially inactive LCAT enzymes (8.3-15% activity of the normal enzyme) have no renal complications [37-39]. Plasma infusion in patients, which raises the plasma LCAT activity level from 9.4% to 17.4% compared to normal subjects, resulted in a significant improvement of lipoprotein profiles [13]. These observations suggest that addition of approximately 10% wild-type LCAT enzyme into patients can prevent the development of the symptoms. The circulating LCAT protein concentration is approximately 6 μg/ml [11] in normal plasma. Transplantation of 1.5 x 10^6 of *lcat*-expressing human ccdPAs achieved nearly 5% of the healthy control level on Day 1 in mice (Fig. **5**), but LCAT delivery and cell survival were significantly decreased. Our recent experiments using an autologous mouse transplantation model showed a substantial improvement in LCAT delivery and cell survival (unpublished data), implying that 10^9 cells would yield a therapeutic effect in patients based on the weight ratio between mice and human (1:3000). The fact that the *lcat* gene-transduced human ccdPAs could be expanded to nearly 10^{10} cells within two weeks after gene transduction from 1 g of fat tissue suggested that human *lcat* gene-transduced ccdPAs may rescue LCAT deficient patients. Considering the differences in the lipoprotein metabolism between mice and humans, a future strategy to investigate the efficacy of human LCAT replacement therapy may be to establish an *in vitro* evaluation system employing serum obtained from familial LCAT-deficient patients.

In summary, the present study has established a procedure to prepare *lcat* gene-transduced human ccdPAs for clinical application. These cells have the ability to differentiate into mature adipocytes and secrete functional human LCAT protein. Animal studies revealed that the implanted cells supplied a therapeutic level of LCAT into the serum. Because we confirmed the prolonged secretion of LCAT from *lcat*-transduced human ccdPAs over three months (data not shown), the significant reduction in LCAT delivery from transplanted cells at one month or later was probably due to the low cell survival rate at the site of transplantation. Therefore, future studies must focus on the improvement of the cell survival rate and prolong the production of the transgene product *in vivo*.

A clinical trial of an *ex vivo* gene therapy has shown that the implantation of autologous fibroblasts genetically modified to express human nerve growth factor into the forebrain improved the rate of cognitive decline in subjects with Alzheimer disease [40], indicating that the local delivery of therapeutic protein using autologous fibroblasts as a cell vehicle is clinically relevant. The establishment of clinically applicable procedures for the transplantation of gene-transduced human ccdPAs would be useful to obtain further applicable autologous cells for *ex vivo* gene therapy in patients with serum protein deficiencies who require long-term therapeutic protein supplements. In this study, we have analyzed the LCAT secretion property of *lcat* gene-transduced ccdPA from healthy volunteers. The propagated cells from different origins showed the LCAT protein secretion enough for our therapeutic strategy. To further expand our therapeutic strategy for the supplementation of other proteins, it is required to evaluate the characteristics of ccdPA from various kinds of fat diseases such as metabolic syndrome which may affect the secretion function of adipose tissues.

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Brief Communication

Disturbed apolipoprotein A-I-containing lipoproteins in fish-eye disease are improved by the lecithin:cholesterol acyltransferase produced by gene-transduced adipocytes *in vitro*

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ABSTRACT

We report the *in vitro* efficacy of recombinant LCAT produced by *lcat* gene-transduced proliferative adipocytes (ccdPA/*lcat*), which has been developed for enzyme replacement therapy. ApoA-I-specific immunodetection in combination with 1D and 2D gel electrophoreses showed that the disturbed high-density lipoprotein subpopulation profile was clearly ameliorated by the *in vitro* incubation with ccdPA/*lcat*-derived recombinant LCAT. Thus, these results using ccdPA/*lcat* strongly suggest the cell implantation could contribute the enzyme replacement for the patients with LCAT deficiency.

1. Introduction

Lecithin:cholesterol acyltransferase (LCAT) plays a central role in the formation and maturation of high-density lipoproteins (HDLs) [1]. Two classes of genetic deficiencies of LCAT have been identified: familial LCAT deficiency (FLD) and fish-eye disease (FED) [2]. We have been developing a long-lasting LCAT replacement therapy via the transplantation of human *lcat* gene-transduced autologous adipocytes in LCAT-deficient patients. In a previous study, we have described a cell preparation procedure and showed LCAT supplementation in mouse model [3]. However, the potential effect of secreted human LCAT on the improvement of disturbed lipoprotein profile and the mechanism how to remodel HDL *in vitro*, should be evaluated in the patient serum with LCAT deficiency. In this study, we examined the effects of the LCAT-containing culture supernatants from human *lcat* gene-transduced adipocytes on the HDL distribution in the FED patient's serum by apolipoprotein A-I (apoA-I) immunodetection in combination with non-denaturing gel electrophoresis.

2. Materials and methods

The study was approved by the Ethics Committee of Chiba University School of Medicine and informed consent was obtained from the patient. Blood sample was obtained from a patient who had a homozygous mutation in the *lcat* gene causing T123I amino acid substitution in the LCAT protein which was described previously to cause the FED phenotype [4]. The patient and his parents profile were presented in Supplementary Table 1.

Human *lcat* gene was transduced into human ccdPA by retroviral vector. The resulting cells (ccdPA/*lcat*) [3] were seeded into T225 flask and grown to confluency in MesenPRO medium (Invitrogen). The medium was changed to 30 ml of OPTI MEM I (Invitrogen) and the cells were further incubated for seven days to collect culture supernatant. The culture supernatant was concentrated to one-fiftieth of the original volume by Amicon Ultra (MWCO = 50 kDa, Millipore). The amount of rLCAT in the concentrated culture medium (rLCAT/ccdPA/*lcat*) was determined by immunoblotting followed by densitometric analysis using commercially available rLCAT (Roar Biomedical, Inc.) as standard. LCAT activity of the concentrated medium was confirmed as described [3].

Abbreviations: LCAT, lecithin:cholesterol acyltransferase; FED, fish-eye disease; FLD, familial LCAT deficiency; apoA-I, apolipoprotein A-I; ccdPA, ceiling culture-derived proliferative adipocyte.

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Concentrated medium containing rLCAT/ccdPA/*lcat* was mixed and incubated at 37 °C with patient serum for 24 h. Inactivation of rLCAT was performed by incubation at 56 °C or addition of 5,5′-Dithiobis-(2-nitrobenzoic acid) [5] (DTNB, Sigma-Aldrich). Serum samples were diluted in 31% sucrose, 0.06% EDTA, and 0.01% BPB prior to gel electrophoresis. Samples corresponding to two micro-liters of serum and those corresponding to 0.25 µl of serum were subjected to non-denaturing two-dimensional (2D) gel electrophoresis [6,7] and 1D gel electrophoresis [8], respectively, with minor modifications. Separated serum proteins were transferred to PVDF membrane (Bio-Rad Laboratories Inc.) and apoA-I was detected by immunoblotting using specific antibodies (Calbiochem) followed by reaction with horseradish peroxidase labeled secondary antibodies. The signal was visualized by SuperSignal West Pico Chemiluminescent reagent (Thermo Fisher Scientific Inc.).

Total cholesterol (TC) and free cholesterol (FC) were quantified in the presence and absence of cholesterol esterase respectively using Cholesterol Quantification kit (BioVision). Cholesteryl ester (CE) contents of samples were then calculated by subtracting FC values from TC values.

Data are presented as means ± S.D. Statistical comparisons were made by ANOVA followed by the post hoc Tukey test using SPSS software. P-values of less than 0.05 were considered as significant.

3. Results

2D analysis showed that the HDL subpopulation distribution of FED patient serum is clearly different from that of healthy serum (Fig. 1A-(a), A-(b)). The patient serum was incubated with the cultured supernatant of ccdPA/*lcat* [3] at a final concentration of rLCAT (6.6 µg/ml), which is equivalent to that in a healthy subject [2,9,10]. The apoA-I-containing lipoprotein distribution in the patient serum was drastically shifted to the larger molecular weight region when the cultured supernatant of ccdPA/*lcat* was added (Fig. 1A-(c)) but not when the supernatant of ccdPA without *lcat* gene transduction was added (Fig. 1A-(d)). The effects were diminished by heat-inactivation of the cultured supernatant before incubation with the patient serum (Fig. 1A-(e)).

Using 1D analysis, a noticeable difference in the apoA-I-containing lipoprotein distribution appeared between the patient (Fig. 1B, lane 1) and the normal subject (Fig. 1B, lane 2). ApoA-I-containing HDL particles were shifted to larger sizes following the incubation with the cultured supernatant of ccdPA/*lcat* in a dose-dependent manner (Fig. 1C, lanes 5–7) as well as following the incubation with rLCAT (Roar Biomedical, Inc., Fig. 1C, lane 12). The incubation with the cultured supernatant of ccdPA (without transduced *lcat* gene, lane4) or PBS (lane 11) did not cause any change from the original serum pattern of the patient. The addition of DTNB (lane 8) or pre-heating the cultured supernatant (lane 9) diminished the effects on HDL particle shifting. The addition of the ccdPA/*lcat* cultured supernatant significantly elevated the CE levels in the HDL fractions (Fig. 1D, lane 7), as observed by the addition of rLCAT (Roar Biomedical, Inc., lane 12) and in agreement with the shift observed in 1D gel electrophoresis (Fig. 1C, lane 7). Taken together, the two kinds of gel electrophoresis analysis in combination with immunoblotting demonstrated that the disturbed HDL subpopulation distribution is ameliorated by *in vitro* incubation of the serum with the ccdPA/*lcat*-derived recombinant LCAT in FED patients.

4. Discussion

We have been focusing on adipocytes as a therapeutic protein-secreting vehicle, since adipose tissue is well-vascularized and, secretes many cytokines systemically into the blood stream [11].

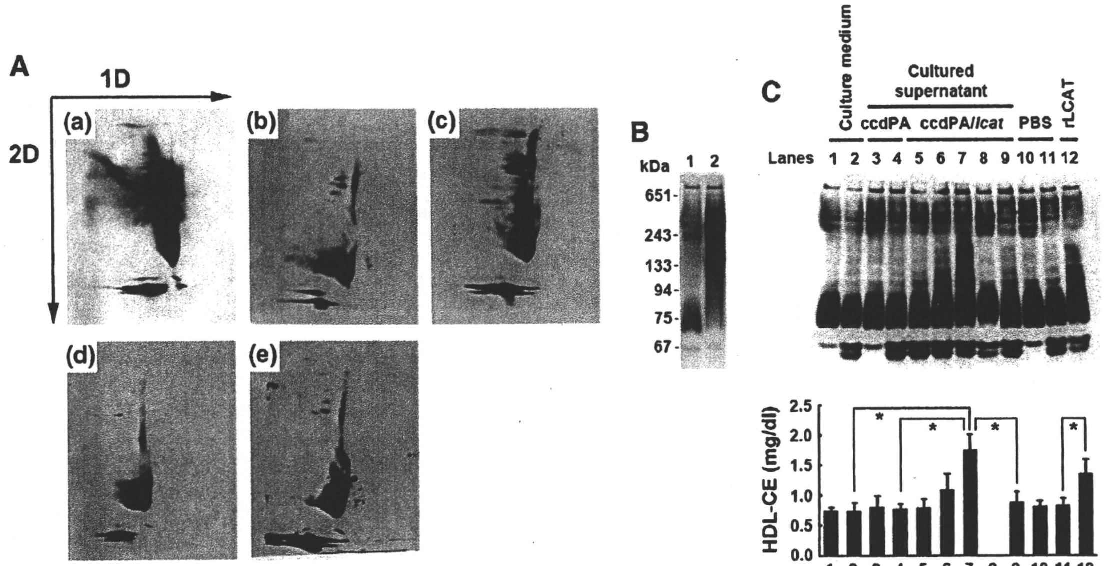

Fig. 1. Analysis of mobility changes in apoA-I-containing particles by *in vitro* incubation with rLCAT. A. Serum samples of normal subjects (a) and FED patient (b, c, d and e) were analyzed by 2D gel electrophoresis followed by immunoblotting against apoA-I. The patient serum without incubation (b). The patient serum was incubated at 37 °C for 24 h with cultured supernatant derived from *lcat* gene-transduced ccdPA (c) or with cultured supernatant from ccdPA (d), or with heat-inactivated cultured supernatant derived from *lcat* gene-transduced ccdPA (e). B. Serum samples of FED patient (lane 1) and normal subject (lane 2) were analyzed by 1D gel electrophoresis followed by immunoblotting against apoA-I. C. Culture medium (lanes 1 and 2), cultured supernatant of untransduced (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 Biomedical, Inc.) (lane 12) were added to the patient serum and incubated at 37 °C for 24 h (lanes 2, 4–9, 11 and 12). Samples without incubation (lanes 1, 3 and 10) were included as controls. Heat inactivated cultured supernatant of human *lcat* gene-transduced ccdPA was used (lane 9). DTNB (2 mM) was included in the reaction mixture (lane 8). The 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 in the reaction mixtures was quantified and shown in the bar graph at the bottom. The quantification of HDL-CE in the sample shown in lane 8 was not performed due to the interference of DTNB with the enzymatic determination of cholesterol [20]. *$p<0.05$.

The products of exogenous genes reach the circulation when it is overexpressed in the adipocytes after their transplantation in mice, although the precise mechanism is unknown [12–15]. The long-lasting blood glucose-lowering effect upon transplantation of insulin gene-transduced adipocytes by retroviral vector strongly suggested the stable expression of LTR-driven transgene expression in adipocytes [12]. Thus, we have developed retrovirally-*lcat* gene-transduced ccdPA (ccdPA/*lcat*) as a stably LCAT supplying vehicle *in vivo* [3]. The LCAT supplementation was indeed steadily detected in the serum after transplantation for 4 weeks in the adipocyte-transplanted mice [3].

ApoA-I is a cofactor of LCAT, and the proper interaction between them in the serum is required for the proper remodeling of HDL, and the mechanism of LCAT activation by apoA-I is not completely determined [16]. Here, we examined the functional issue to be dissolved before the subsequent clinical application, whether LCAT protein secreted by ccdPA/*lcat* improves the disturbed lipoprotein remodeling in human patient's serum. The 2D analysis of the apoA-I-containing HDL distribution profile showed that the rLCAT changed the abnormal HDL population sizes in the FED patient toward the pattern in the normal subject. This change in the HDL particles was also detected using 1D electrophoresis with the rLCAT-dependent formation of CE in HDL. Thus, the incubation with the rLCAT derived from ccdPA/*lcat* stimulated CE formation and the subsequent maturation of HDL subpopulations in the FED patient serum. Thus, rLCAT from ccdPA/*lcat* is functional in correcting the abnormal HDL distribution in the serum of FED patient. It is still assumed that the rLCAT supplied *in vivo* might not as effective in LCAT-deficient patients as the here shown *in vitro* results, since the tissue supplying the recombinant enzyme is adipocytes, and not the liver, original site producing LCAT, thus causing the presence of unexpected inhibitor(s), inefficient interaction with the patient apoA-I, or accelerated dynamics of the enzyme [17]. A clinical application of ccdPA/*lcat* transplantation is now in progress in Japan as a first clinical trial. Based on the *in vitro* study, the 1D and 2D gel electrophoresis examinations of the HDL profile in the sera of patients are expected to contribute to the clinical evaluation of the treatment efficacy after the cell transplantation, in addition to the *in vitro* functional examination of the patient's ccdPA/*lcat*-derived rLCAT against their own serum prior to the cell transplantation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.ymgme.2010.10.009.

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① 外傷性裂開創(一次閉鎖が不可能なもの)
② 外科手術後離開創・開放創
③ 四肢切断端開放創
④ デブリードマン後皮膚欠損創

とされるが，具体的には急性創傷ではデグロービング外傷，開放性骨折，術後創離開，術後開放創など，慢性創傷では褥瘡，糖尿病性足壊疽など，真皮よりも深い創傷でデブリードマン後の創傷が対象となる．

NPWT のシステムの特徴から創傷を密閉することになるので，感染創を対象とする際にはあらかじめ壊死組織除去や抗菌薬などを使用し，感染を制御しておくことが推奨される．

また，露出した血管，臓器に直接 NPWT を使用することは大出血や重大事故につながるおそれがあるため，禁忌とされる．

NPWTの応用

NPWT はコラーゲン使用人工皮膚や持続洗浄とともに用いても効果を発揮する．

NPWT に，ドレーン孔を有するコラーゲン使用人工真皮(テルダーミス真皮欠損用グラフト® 膜付きドレーン孔タイプ，東京，オリンパステルモバイオマテリアル社)を併用する方法では，肉芽形成をさらに促進することが可能である．

持続洗浄と NPWT の組合せでは洗浄によって感染を制御しながら陰圧を負荷できるので，感染を認める創傷における NPWT の可能性を拡大させる[3]．

褥瘡などの慢性創傷において皮弁移植術を計画する場合には，NPWT を術前に施行することで創傷を最適な状態へ変換し，周術期合併症の発生リスクを軽減することが可能となる[2]．

また，網状分層植皮術における固定用のドレッシング材としても有用である．

おわりに

V. A. C. に代表される NPWT は難治性潰瘍に対する確立された治療法として，いまや世界的に認知されている．わが国においても完成度の高い V. A. C. の登場は，創傷の臨床医に NPWT の効果を再認識させ，多くの難治性潰瘍をもつ患者に光明をもたらすことになるであろう．

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糖尿病・内分泌代謝学

脂肪細胞による新規蛋白質補充療法
――LCAT欠損症遺伝子治療臨床研究

Enzyme replacement therapy by gene-transduced adipocytes for LCAT deficiency

LCAT 欠損症やライソゾーム病などの難治性遺伝病には，根治療法が存在しない．これらの希少疾患の生命予後や生活を改善するための持続的な蛋白質補充を行う手段として，安全で普遍的，また医療経済に適合した細胞治療法があげられる．このような考えから著者らは，すでに日常臨床で安全に行われている皮下脂肪組織の摘出と脂肪細胞の移植技術を基盤にした新規の細胞治療法を開発した(図 1)[1]．この特徴は，これまでの多くの研究で得られてきた脂肪細胞の多彩な機能と生体における安定性を，遺伝子導入細胞として応用することにある．本稿では，本治療法による LCAT 欠損症治療実用化開発を概説する．

LCAT欠損症とは

レシチン：コレステロールアシルトランスフェラーゼ(LCAT)はリポ蛋白 HDL とともに存在し，血中コレステロールのエステル化を担う酵素である．LCAT 欠損症は，まれな常染色体劣性遺伝性疾患である．北ヨーロッパ，日本を中心に本疾患患者が同定され，原因となる 40 種類以上の LCAT 遺伝子異常が報告されている．低 HDL 血症とともに角膜混濁，溶血性貧血，腎不全などの臨床症状を呈し，根治療法は存在しない[2]．新鮮血漿の輸血により一時的に臨床症状が改善したという報告[3,4]があることから，長期間安定に持続する LCAT 補充療法が期待される．

自己移植用脂肪細胞 ccdPA[5]

形成外科領域で一般に行われる臨床技術である脂肪吸引により得られる脂肪組織から，遺伝子導入用脂肪細胞を調製することができる．脂肪細胞は油滴を含むために比重が小さいことがこの調製に利用される．脂肪組織をコラゲナーゼ処理し遠心後の沈渣(stromal vascular fraction：SVF)を除き，他の細胞などの含まれない成熟脂肪細胞分画を収集することができる．この油滴含有脂肪細胞分画を用いて天井培養法[6]により遺伝子導入用細胞(ceiling culture-derived

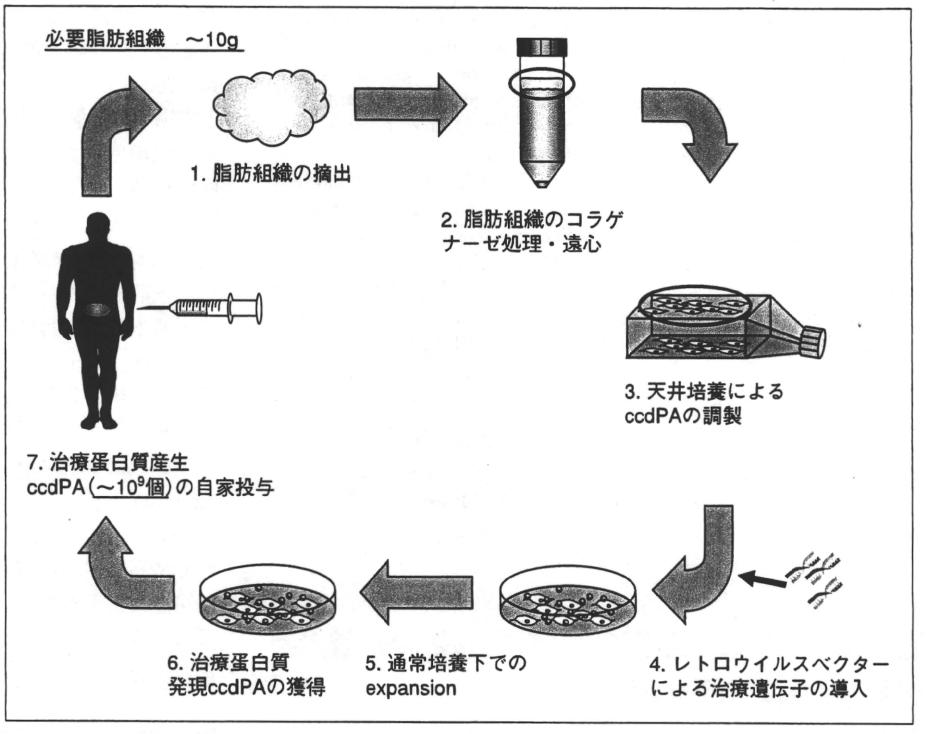

図 1　遺伝子導入脂肪細胞を用いた蛋白質補充療法の概略
患者腹部脂肪組織を摘出し，天井培養により遺伝子導入用脂肪細胞(ccdPA)を獲得する．レトロウイルスベクターによる治療用遺伝子導入の後，拡大培養した細胞を回収し，腹部脂肪組織に自己移植する．1 回の治療に必要な移植用 ccdPA は脂肪組織摘出後 3 週間で調製される．

proliferative adipocytes：ccdPA)が調製される．SVF には多分化能を有する幹細胞が存在するのに対し[7]，ccdPA はこれらが排除され，表面蛋白プロファイルの安定な細胞の集団となる．ccdPA は線維芽細胞様の形態を示し，脂肪細胞への成熟能が優れる．一部で SVF 由来幹細胞が長期 *ex vivo* 培養によりトランスフォーム(癌化)する報告があるのに対し[8]，ccdPA はこれまで異常増殖の所見は認められておらず，移植治療において安全性に優れた細胞と考えられる．

ccdPAによるLCAT補充

ccdPA にレトロウイルスベクターを用いて遺伝子導入を行うと，高陽性率(40～50%)，低平均導入コピー数(細胞当り約 1 コピー)と，遺伝子治療用の細胞として理想的な細胞であることが示される[5]．LCAT 遺伝子導入 ccdPA は，正常 LCAT と同等のコレステロールエステル化活性を有する酵素蛋白を培地中に分泌する[5]．LCAT 欠損症患者血清にこの分泌 LCAT を添加作用させるとエステル化障害による異常なリポ蛋白が是正され，正常な分布に近づく．LCAT 遺伝子導入 ccdPA をマウスに移植すると，全身のリポ蛋白代謝を改善することが期待できる LCAT 蛋白が血中に補充された．またこの効果は，すくなくとも数カ月持続することが予想される．

おわりに

LCAT 欠損症患者を対象にした遺伝子治療臨床研究“家族性 LCAT 欠損症を対象とした LCAT 遺伝子導入ヒト前脂肪細胞の自家移植に関する臨床研究”は，千葉大学医学部附属病院遺伝子治療臨床研究審査委員会での承認を得，平成 22 年 (2010) 4 月，厚生労働省へ実施申請書類を提出した．今後は LCAT 欠損症に加えて，さまざまな蛋白欠損症(酵素欠損症，血友病，糖尿病など)を対象に，その合併症が進行することを抑制できる新規細胞治療法として広く臨床応用されることが期待される．

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