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

Yasuyuki Aoyagi[1,2,3], Masayuki Kuroda[1,2,3,*], Sakiyo Asada[1,2,3], Hideaki Bujo[2], Shigeaki Tanaka[3], Shunichi Konno[3], Masami Tanio[3], Itsuko Ishii[4], Masayuki Aso[3], and Yasushi Saito[5]

[1]Center for Advanced Medicine, Chiba University Hospital, Chiba University, Chiba, Japan

[2]Department of Genome Research and Clinical Application, Graduate School of Medicine, Chiba University, Chiba, Japan

[3]CellGenTech, Inc., Chiba, Japan

[4]Graduate School of Pharmaceutical Sciences, Chiba University, Chiba, Japan

[5]Chiba University, Chiba, Japan

***Corresponding author:** Masayuki KURODA, Ph.D.

Current address: Center for Advanced Medicine, Chiba University Hospital, Chiba University, Chiba, Japan

1-8-1, Inohana, Chuo-ku, Chiba, 260-0856, Japan.

Tel.: +81-43-222-7171, or +81-43-441-4121

Fax: +81-43-226-2095

E-mail: kurodam@faculty.chiba-u.jp

Running title: Fibrin glue scaffold for gene-transduced adipocytes

Abbreviations: ceiling culture-derived proliferative adipocytes, (ccdPA); lecithin:cholesterol acyltransferase, (LCAT); Peroxisome Proliferator-Activated Receptor, (PPAR)

Abstract

The development of clinically applicable scaffolds is important for the application of cell transplantation in various human diseases. The aims of this study are to evaluate fibrin glue in a novel protein replacement therapy using proliferative adipocytes and to develop a mouse model system to monitor the delivery of the transgene product into the blood and the fate of the transduced cells after transplantation. Proliferative adipocytes from mouse adipose tissue were transduced by a retroviral vector harboring the human lecithin-cholesterol acyltransferase (*lcat*) gene, and were subcutaneously transplanted into mice combined with fibrin glue. The *lcat* gene transduction efficiency and the subsequent secretion of the product in mouse adipocytes were enhanced using a protamine concentration of 500 μg/ml. Adipogenesis induction did not significantly affect the *lcat* gene-transduced cell survival after transplantation. Immunohistochemistry showed the ectopic enzyme production to persist for 28 days in the subcutaneously transplanted gene-transduced adipocytes. The increased viability of transplanted cells with fibrin glue was accompanied with the decrease in apoptotic cell death. The immunodetectable serum LCAT levels in mice implanted with the fibrin glue were comparable with those observed in mice implanted with Matrigel, indicating that the transplanted *lcat* gene-transduced adipocytes survived and functioned in the transplanted spaces with fibrin glue as well as with Matrigel for 28 days. Thus, this *in vivo* system using fibrin is expected to serve as a good model to further improve the transplanted cell/scaffold conditions for the stable and durable cell-based replacement of defective proteins in patients with LCAT deficiency.

Keywords: fibrin glue, adipocyte, transplantation, protein replacement therapy, lecithin:cholesterol acyltransferase, scaffold

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; Patrick, 2000; Patrick, 2001). Recent studies have shown that the preadipocytes in aspirated fat are multipotential and implicated in the source of cell-based therapies (Stashower *et al.*, 1999; Zuk *et al.*, 2001; Gimble *et al.*, 2007). One such potential is the high capability for exogenous gene transduction and the secretion of transgene products (Ito *et al.*, 2005). We have recently identified human ceiling culture-derived proliferative adipocytes (h-ccdPA) in subcutaneous adipose tissue, and proposed the application of gene-transduced h-ccdPA to the long-lasting replacement therapy for a variety of inherited or acquired gene-defective diseases (Kuroda *et al.*, 2011; Asada *et al.*, 2011).

A key factor in the protein delivery system via the autotransplantation of various types of gene-transduced cells is the regulation of the survival and the secretory function of these 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 the gene expression of adipocytes in the fat graft after subcutaneous transplantation in mice (Matsumoto *et al.*, 2002). In addition, the secretion of vascular endothelial growth factor (a bioactive molecule secreted from the vascular system) around the transplanted graft in recipients is also important for long-term cell survival (Yamaguchi *et al.*, 2002). Particularly, recent studies have highlighted the importance of various cytokines for the regulation of cell function and the surrounding matrix conditions (Kuramochi *et al.*, 2008; Cho *et al.*, 2001; Kimura *et al.*, 2003; Torio-Padron *et al.*, 2007; Ning *et al.*, 2009). Together with the consideration of cytokine delivery for the transplanted cells, the development of scaffolds for transplantation contributes to the early construction of the surrounding matrix around the transplanted site. Insulin gene-transduced cells transplanted with Matrigel as a scaffold have been shown to survive as insulin-secreting adipocytes for three months after transplantation (Ito *et al.*, 2005). It is therefore critical to set up an appropriate clinically applicable scaffold for the adipocyte transplantation into patients, which allows not only a longer survival of the implanted cells but also guarantees a longer-lasting secretion of the therapeutic gene product into the blood stream.

In this study, we have optimized the gene transduction conditions for the most effective retroviral 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 the expanded human enzyme gene-transduced m-ccdPA for the evaluation of protein delivery in the serum of the mice. Using an *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.

Results

Establishment of human *lcat* gene transduced m-ccdPA (m-ccdPA/*lcat*)

We have recently established h-ccdPA, which secretes functionally active hLCAT, a key circulating enzyme for serum cholesterol esterification, and proposed a novel cell-based gene therapy by the subcutaneous transplantation of the cells for the long-lasting replacement of the protein in the patients with LCAT deficiency (Kuroda *et al.*, 2011). In order to establish the most suitable mouse model for the evaluation of the effect of the scaffold on the survival and function of the transplanted adipocytes, we first prepared m-ccdPA for the *lcat* gene transduction as donor cells for the recipient mice. The biochemical characterization showed that the prepared m-ccdPA have morphological features and surface antigen expression patterns similar to those of h-ccdPA (unpublished data). Our preliminary experiments showed that the transduced m-ccdPA secreted a much lower amount of hLCAT than the h-ccdPA when the average copy number 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: ref. 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 *et al.*, 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

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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.

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 (Ito *et al.*, 2005; Piasecki *et al.*, 2008; Kitagawa *et al.*, 1999). 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 (Figure 3A-F). 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 apoptotic cell death in the cells with fibrin glue was significantly decreased in comparison to those without fibrin glue (Figure 3G, 3H and 3K). On the other hand, there was no significant difference in Ki67 staining between the cells with and without fibrin glue (Figure 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 (Neuss *et al.*, 2008; Malafaya *et al.*, 2008; Mano *et al.*, 2007) 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.*, 2007). 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 (Figure 3A-F). 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 (Figure 3G-K), 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 x 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 TrueBlotTM anti-Rabbit Ig IP Beads (eBioscience, San Diego, CA) was added and incubated with rotation for 2 hrs at 4°C. Beads/proteins complex was washed with PBS-NP40, and treated by boiling in 10 μl of 2 x 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 x 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 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 C57BL/6J mice experiments, blood samples were taken from tail without sacrifice to monitor the hLCAT 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).

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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Figure Legends

Figure 1. Enhanced gene transduction efficiency in m-ccdPA. Integrated copy numbers (A) and LCAT activity in the culture medium (B) after retroviral vector- mediated human *lcat* gene transduction were analyzed. Single round (b) and two rounds (a, c) of exposure to CGT_hLCATRV in the presence of 8 (a) or 500 μg/ml (b, c) of PS. Transductions with 8 μg/ml (a) and 500 μg/ml (b, c) of PS were performed overnight and one hour, respectively. Data are presented as the mean ± SD (n=3). *$p<0.05$.

Figure 2. Detection of hLCAT and survival of human *lcat* gene after transplantation of *lcat* gene-expressing m-ccdPA. Human *lcat* gene-transduced mouse ccdPA (5 x 10^6 cells) were subcutaneously transplanted in nude mouse with fibrin glue as a scaffold. **A.** Existence of hLCAT protein in mice sera was detected by IP-Western experiments. 15 μg human high density lipoprotein (HDL) was loaded for quantification of signals (H). Mouse serum with (PC) or without (NC) 15 μg HDL were subjected to IP-Western. The gene-transduced (T) m-ccdPA were transplanted with (+) or without (-) fibrin glue. Sera (100 μl) from the mice and mice transplanted with un-transduced (C) m-ccdPA were subjected to IP-Western analysis. **B.** Human *lcat* gene-transduced mouse ccdPA (5 x 10^6 cells) were transplanted after three days of culture with (open bars) or without (closed bars) adipogenic differentiation medium. The serum concentrations of the hLCAT protein were quantified by densitometric analysis (left), and the human *lcat* gene was quantified in excised implants (right).

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). *$p<0.05$.

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 IP-Western experiments (B).

Figure 1.

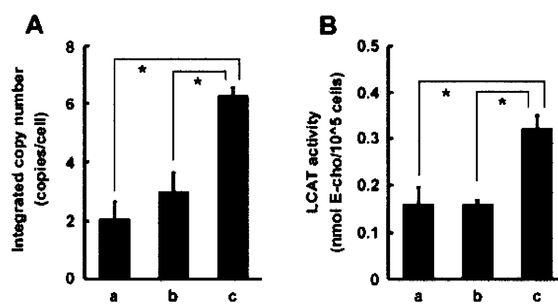


Figure 2.

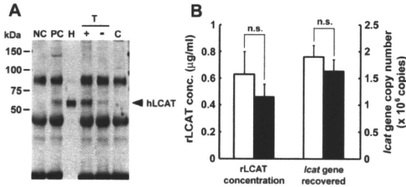


Figure 3.

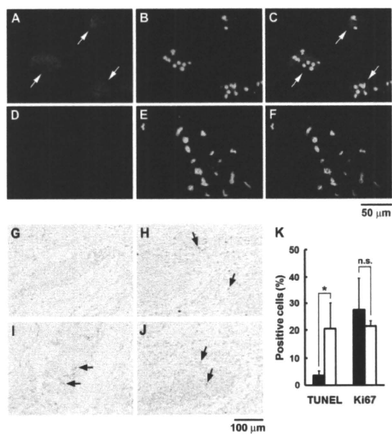
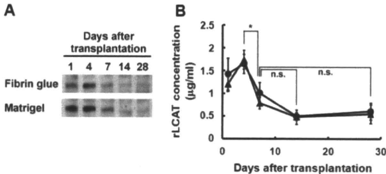


Figure 4.



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

Masayuki Kuroda[1,2,3,*], Yasuyuki Aoyagi[1,2,3], Sakiyo Asada[1,2,3], Hideaki Bujo[2,*], Shigeaki Tanaka[3], Shunichi Konno[3], Masami Tanio[3], Itsuko Ishii[4], Kazuhiko Machida[5], Fumiaki Matsumoto[6], Kaneshige Satoh[6], Masayuki Aso[3] and Yasushi Saito[7]

[1]*Center for Advanced Medicine, Chiba University Hospital, Chiba University, Chiba, Japan*

[2]*Department of Genome Research and Clinical Application, Graduate School of Medicine, Chiba University, Chiba, Japan*

[3]*CellGenTech, Inc., Chiba, Japan*

[4]*Graduate School of Pharmaceutical Sciences, Chiba University, Chiba, Japan*

[5]*Central Institute for Experimental Animals, Kawasaki, Japan*

[6]*Department of Plastic and Reconstructive Surgery, Graduate School of Medicine, Chiba University, Chiba, Japan*

[7]*Chiba University, Chiba, Japan*

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.

*Address correspondence to these author at the Current address: Center for Advanced Medicine, Chiba University Hospital, Chiba University, 1-8-1, Inohana, Chuo-ku, Chiba, 260-0856, Japan; Tel: +81-43-222-7171, or +81-43-441-4121; Fax: +81-43-226-8130; E-mail: kurodam@faculty.chiba-u.jp and Department of Genome Research and Clinical Application (M6), Graduate School of Medicine, Chiba University, 1-8-1, Inohana, Chuo-ku, Chiba, 260-8670, Japan; Tel: +81-432227171; Fax: +81-43-226-8130; E-mail: hbujo@faculty.chiba-u.jp

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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'-ATCGGATCCAGGGCTGGAAA TGGGGCCGCCC-3' (forward) and 5'-ATCGGATCC GTCGACGGAAGGTCTTTATTCAGGAGGCGGGGG-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 x 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