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-transduced human ccdPAs was analyzed (Fig. **3c** and **3d**). Seven days after gene transduction, 1×10^5 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 was not obviously different from cells without gene transduction (Fig. **4b**, **4c**, **4e**, and **4f**). 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, respectively). The proliferating cell number and the resultant doubling time were not significantly 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 1.5×10^6 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].

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 7-day culture ccdPAs, 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 2.0×10^9 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 1.5×10^6 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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FIGURE LEGENDS

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.

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.

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 x 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 x 10^5 cells (C013). After a densitometric analysis of immuno-detected 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 x 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).

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).

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).

Figure 1.

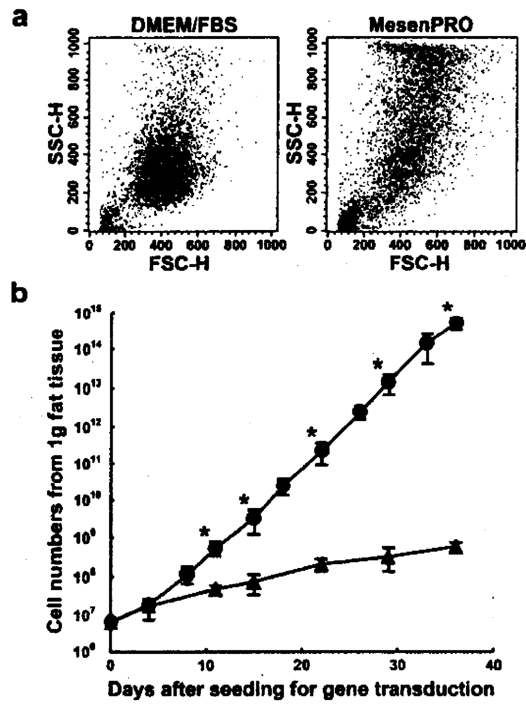

Figure 2.

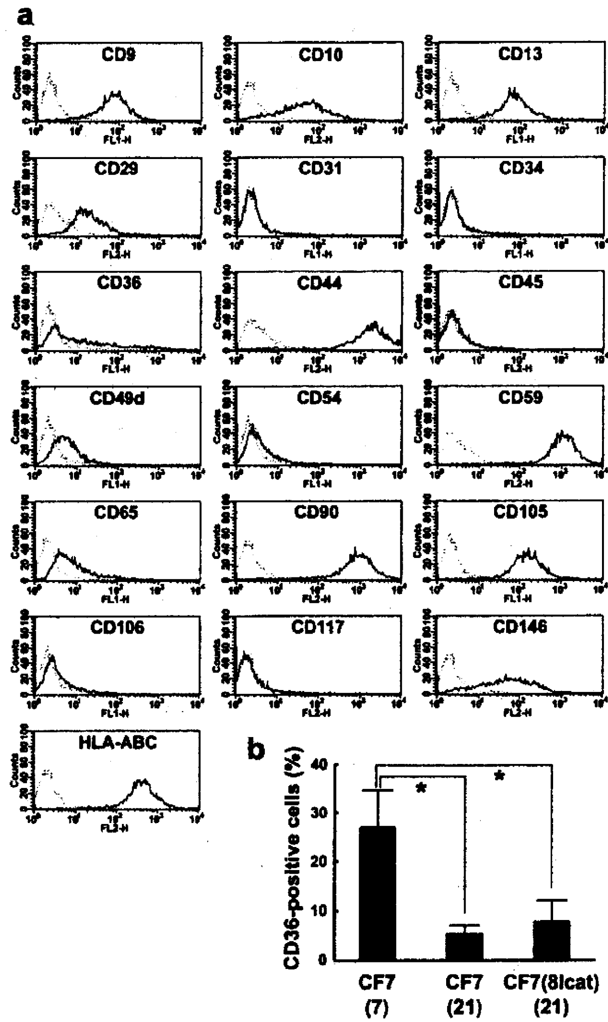

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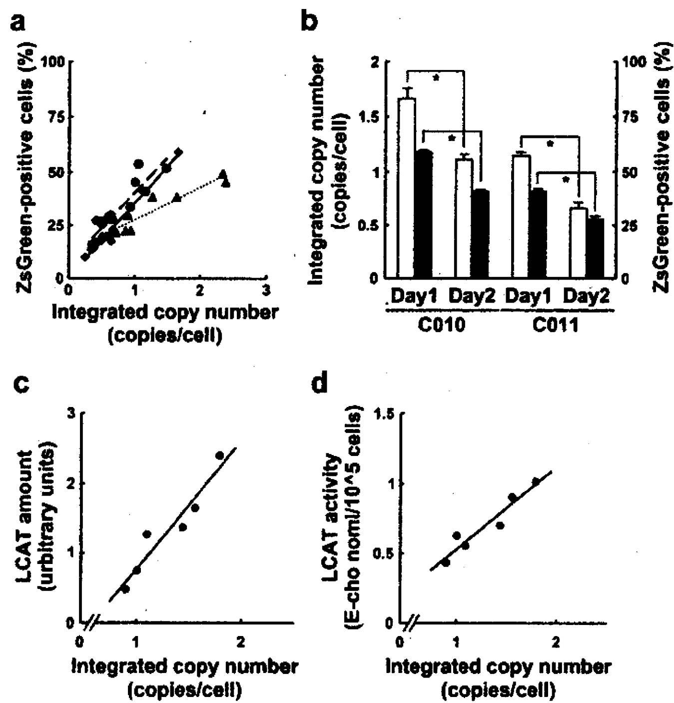
Figure 3.



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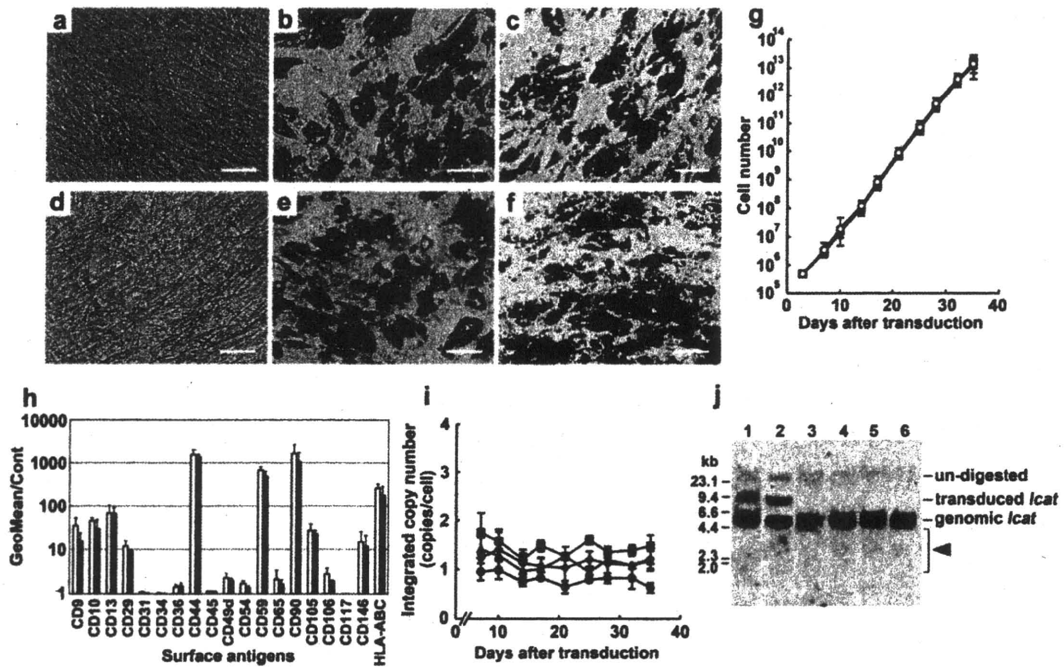
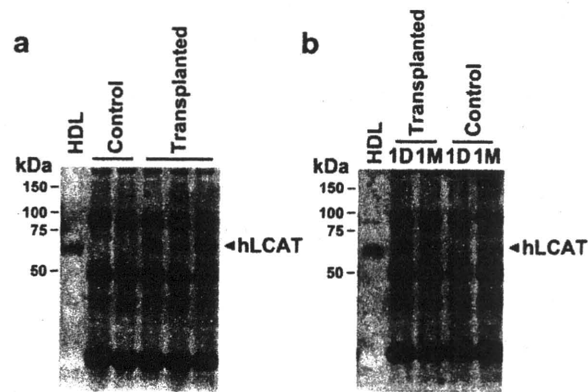


Figure 5.



Molecular Genetics and Metabolism 102 (2011) 229–231

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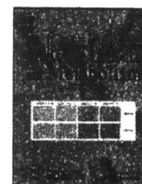

Brief Communication

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.

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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].

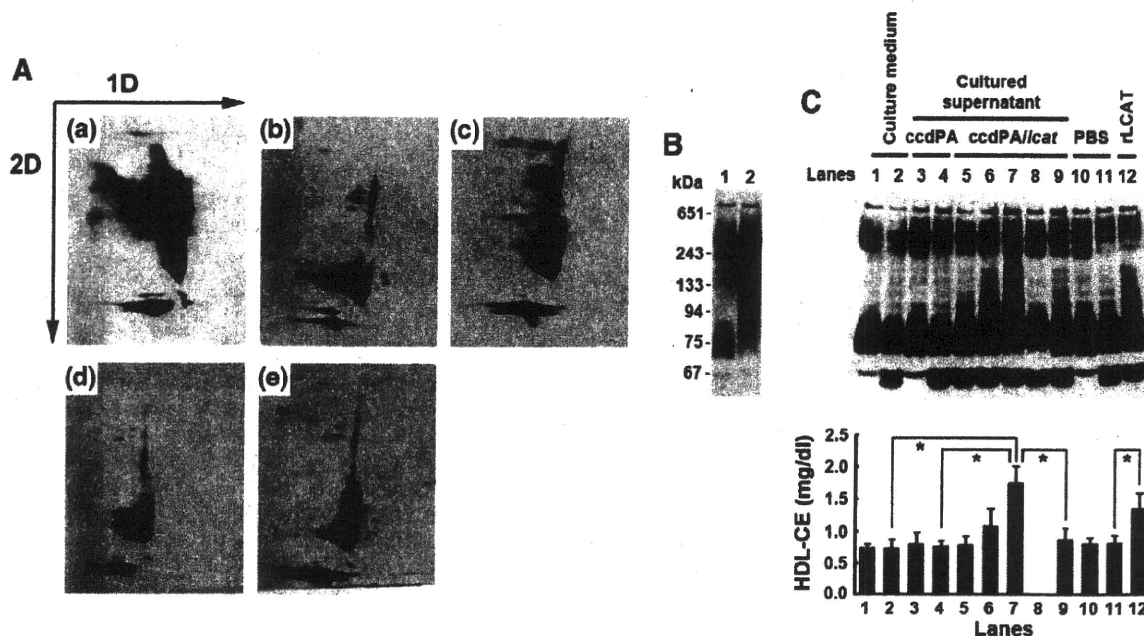

Fig. 1. Analysis of mobility changes in apoA-I-containing particles by *in vitro* incubation with rLCAT. A. Serum samples of normal subjects (a) and FED patient (b, c, d and e) were analyzed by 2D gel electrophoresis followed by immunoblotting against apoA-I. The patient serum without incubation (b). 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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Alcohol Consumption and Risk of Atrial Fibrillation

A Meta-Analysis

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Objectives The purpose of this meta-analysis is to summarize the estimated risk of atrial fibrillation (AF) related to alcohol consumption.

Background Results from observational studies examining the relationship between alcohol consumption and AF are inconsistent.

Methods A systematic electronic search of Medline (January 1966 to December 2009) and Embase (January 1974 to December 2009) databases was conducted for studies using key words related to alcohol and AF. Studies were included if data on effect measures for AF associated with habitual alcohol intake were reported or could be calculated. The effect measures for AF for the highest versus lowest alcohol intake in individual studies were pooled with a variance-based method. Linear and spline regression analyses were conducted to quantify the relationship between alcohol intake and AF risk.

Results Fourteen eligible studies were included in this meta-analysis. The pooled estimate of AF for the highest versus the lowest alcohol intake was 1.51 (95% confidence interval: 1.31 to 1.74). A linear regression model showed that the pooled estimate for an increment of 10 g per day alcohol intake was 1.08 (95% confidence interval: 1.05 to 1.10; $R^2 = 0.43$, $p < 0.001$). A spline regression model also indicated that the AF risk increased with increasing levels of alcohol consumption.

Conclusions Results of this meta-analysis suggest that not consuming alcohol is most favorable in terms of AF risk reduction. (J Am Coll Cardiol 2011;57:427–36) © 2011 by the American College of Cardiology Foundation

Atrial fibrillation (AF) is the most common sustained arrhythmia, representing a growing epidemic, and is accompanied by serious complications. Atrial fibrillation accounts for 45% of all embolic strokes and has a deleterious impact on longevity, with an approximate doubling of all-cause mortality (1). Although the etiology of AF is not fully understood, many epidemiological associations with AF, including both cardiac (e.g., valvular disease, cardiomyopathy, coronary artery disease) (2) and noncardiac conditions (e.g., aging, obesity, sleep apnea, diabetes mellitus, metabolic syndrome, heavy alcohol consumption) (3) have been vigorously investigated.

The association of episodic heavy alcohol use with the onset of AF has been recognized as "holiday heart syndrome" for a long time (4). Recently, it has been hypothesized that not only episodic but also habitual heavy alcohol consumption is associated with the risk of AF (5). However, results from epidemiological studies that aim to confirm this hypothesis have been inconsistent, although high alcohol consumption has been associated with several major disease groups such as neoplasms and cardiovascular diseases (6). It is also important to clarify the overall impact of any degree of alcohol intake on AF risk given that moderate alcohol consumption has been associated with a lower risk of cardiovascular disease (7) or all-cause mortality (8). Therefore, our aim of this meta-analysis of observational studies is to review the risk of AF in relation to alcohol consumption, focusing on determining if there is a dose-response relation-

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