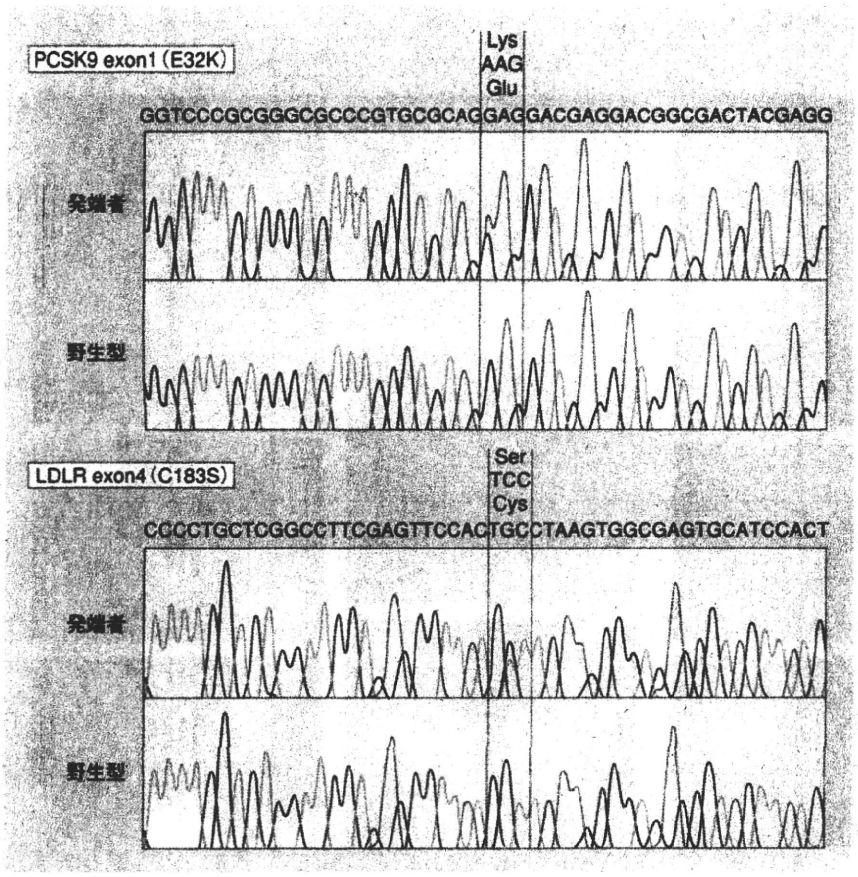


図❸ 本症例の遺伝子解析

(a) 34.2mg/dL

頸動脈エコー所見：内膜中膜複合体の肥厚 (0.4mm) が観察された.

遺伝子解析

末梢白血球から抽出したDNAを用いたLDLR遺伝子解析にて，母方由来のLDLR C183S (c.611G＞C) 変異が確認されたが，ダイレクトシークエンスによる全エクソンの精査，およびMultiplex Ligation-dependent Probe Amplification (MLPA) 法による大規模欠失・重複変異の検索でも，父方由来のLDLR遺伝子変異は見出されなかった．また，FDBの病因変異も認められなかったため，さらにPCSK9遺伝子の解析を行ったところ，父方由来の機能亢進型PCSK9 E32K (c.94G＞A) 変異が確認された (図❶，❸).

治療経過

2005年6月からピタバスタチン4mg内服加療を開始，2007年8月よりエゼチミブ10mgを追加し，LDL-Cは271mg/dLまで改善した (図❹)．これに伴い，皮膚黄色腫は著明に改善した (図❷).

考 察

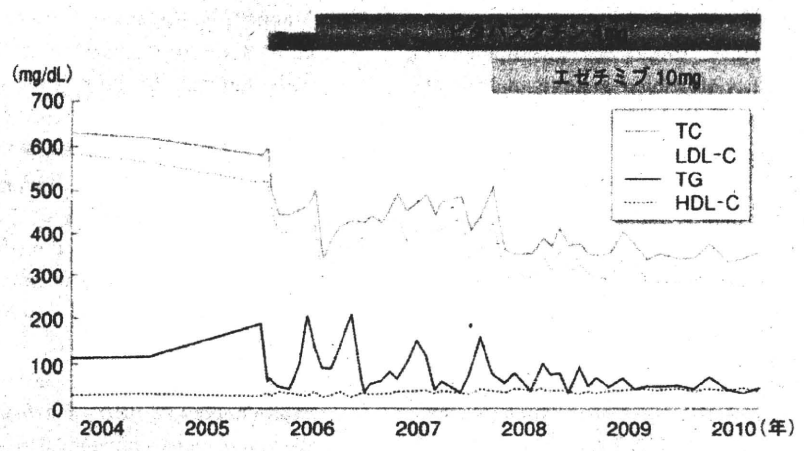
ホモ接合体性FHは，約100万人に1人の頻度でみられるまれな疾患であるが，FHの極型ともいえる重篤な臨床症状を呈する．わが国ではこれまで，LDLR遺伝子変異1種類のホモ接合体，もしくはLDLR遺伝子変異2種類の組み合わせによる複合ヘテロ接合体のみが報告されてきた．今回，われわれはPCSK9 E32K変異とLDLR C183S変異のダブルヘテロ接合体でもホモ接合体性FHの臨床像を呈することを見出した.

本症例のLDL-C値は581mg/dLで，それぞれのヘテロ接合体である両親 (平均LDL-C 290mg/dL) の2倍，変異非保有同胞 (LDL-C 103mg/dL) の約5.6倍であった．未治療時の黄色腫の部位および程度は，LDLR遺伝子変異ホモ接合体の小児と同様であり，また，3歳時ですでに内膜中膜複合体の肥厚 (0.4mm) が観察された．ホモ接合体性FHでは冠動脈硬化症や大動脈弁上狭窄症が生命予後を規定し，十分なコレステロール低下療法が行われない場合には，平均33歳で心臓死に至る[8]．したがって，ホモ接合体性FHの生命予後を改善するに

は，早期からの有効なコレステロール低下療法の導入が必要である．

LDLR遺伝子変異ホモ接合体は正常なLDLRを発現しないため，肝細胞でのLDLR発現誘導を介してLDL-Cを低下させるスタチンなどの薬物療法では，有効なコレステロール値の低下は困難であることが多い．例外的に，臨床像が軽症であるとされるLDLR L547V変異[9]との組み合わせでは，LDLR遺伝子変異複合ヘテロ接合体であっても薬剤治療反応性が比較的良好な場合がある．一般的には，ホモ接合体性FHの治療には体外循環によりLDLなどのアポB含有リポ蛋白粒子を直接除去するLDLアフェレーシス療法が絶対適応とされている．

一方，本症例ではピタバスタチン4mgの内服加療によりLDL-Cは300mg/dL程度まで低下し，エゼチミブ10mg追加によりさらに低下して初期値の50%以下の271mg/dLまで改善した(図❹)．コレステロール値の低下とともに，全身性に認められた皮膚黄色腫の大部分が退縮・退色しており(図❷)，この点からも薬剤による治療の有効性が示された．われわれは本症例のほかにも3例のPCSK9 E32K/LDLR遺伝子変異ダブルヘテロ接合体を見出しており，また，世界でも報告のない機能亢進型PCSK9変異ホモ接合体も2例見出した(表)．いずれの症例でも，本症例と同様に薬剤に対する反応性は良好であった．FDBとLDLR遺伝子変異のダブルヘテロ接合体でもLDLR遺伝子変異ホモ接合体に比して軽症であり，薬剤にも反応するという報告があることから[10]，正常LDLR



図❹ 臨床経過図

表 PCSK9遺伝子異常症の自験例

No.	遺伝子変異		性	年齢(歳)	黄色腫	TC (mg/dL)	TG (mg/dL)	HDL-C (mg/dL)	LDL-C (mg/dl)
1	PCSK9 E32K	LDLR C183S	男性	2	重度の皮膚黄色腫	629	111	26	581
2	PCSK9 E32K	LDLR C292X	男性	33	重度のアキレス腱黄色腫(右32mm，左30mm)	520	287	38	425
3	PCSK9 E32K	LDLR K790X	男性	1	重度の皮膚黄色腫	345	226	52	248
4	PCSK9 E32K	LDLR IVS15-3C/A	男性	47	アキレス腱黄色腫(右15mm，左16mm) 骨膜下黄色腫	504	234	41	416
5	PCSK9 E32K	PCSK9 E32K	女性	52	アキレス腱黄色腫(右10mm，左11mm)	420	153	50	339
6	PCSK9 E32K	PCSK9 E32K	女性	43	アキレス腱黄色腫(右11mm，左9mm)	322	173	53	246

を発現しうることが良好な治療反応性に結びついていると考えられる．本症例のように薬物療法で十分な効果が期待できるのか，あるいはLDLアフェレーシスが必要なのかを判断し，適切な治療法を選択する上で，臨床的にホモ接合体性FHと診断された症例の病因遺伝子変異の特定は非常に重要である．

われわれの検討において，PCSK9 E32K変異保有者では，PCSK9蛋白の機能増強および血中濃度上昇の双方を介して高脂血症となることが示唆されている[6]．PCSK9の異常が高脂血症の原因となっているこれらの症例では，近年開発が進んでいるアンチセンスオリゴヌクレオチド[11]，RNAi[12]，抗PCSK9抗体[13]，epidermal growth factor-like repeat Aペプチド[14]などのPCSK9阻害薬が，根本的な治療法として奏効する可能性がある．スタチンによりPCSK9の血中濃度が上昇するという報告もあり[15]，スタチン服用時の有力な併用薬としての可能性も併せ，PCSK9阻害薬の実用化が待たれる．

おわりに

ホモ接合体性FHの臨床像を呈するPCSK9 E32K/LDLR C183Sダブルヘテロ接合体を経験し，薬剤による治療効果について評価した．本症例はLDLR遺伝子変異ホモ接合体に比して薬剤に良好に反応し，血清TC値の低下とともに皮膚黄色腫の著明改善が観察された．FHの遺伝子診断の意義を考察する上でも，遺伝子診断が治療選択と結びつく可能性を示唆する重要な症例と考え報告した．

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The E32K variant of PCSK9 exacerbates the phenotype of familial hypercholesterolaemia by increasing PCSK9 function and concentration in the circulation

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ABSTRACT

Objective: Proprotein convertase subtilisin/kexin type 9 (PCSK9) regulates cholesterol trafficking by mediating degradation of cell-surface LDL receptors (LDLR). Gain-of-function *PCSK9* mutations are known to increase plasma LDL-C levels. We attempted to find gain-of-function *PCSK9* mutations in Japanese subjects and determine the frequency and impacts of these mutations, especially on circulating PCSK9 and LDL-C levels.

Methods: PCR-SSCP followed by direct sequence analysis was performed for all 12 exons and intronic junctions of the *PCSK9* in 55 subjects with clinically diagnosed familial hypercholesterolaemia (clinical-FH), who were confirmed to have no *LDLR* mutations. Among the mutations detected, *PCSK9* E32K was likely to be a gain-of-function mutation, and screening was performed by PCR-RFLP in clinical-FH and general Japanese controls. The levels of PCSK9 in plasma from subjects and in media of HepG2 cells transfected with PCSK9 constructs were measured by ELISA.

Results: We detected 7 *PCSK9* variants, including E32K. The frequency of *PCSK9* E32K in clinical-FH (6.42%) was significantly higher than that in controls (1.71%). Three cases representing homozygous FH phenotypes were double heterozygous for *PCSK9* E32K and *LDLR* C183S, C292X or K790X. Two cases were true homozygous for *PCSK9* E32K; to our knowledge, these are the first true homozygotes for gain-of-function *PCSK9* mutations reported to date. The *PCSK9* E32K mutant had over 30% increased levels of PCSK9 in plasma from the subjects and in media of transiently transfected HepG2 cells as compared with those in controls. Furthermore, LDL-C levels in the *PCSK9* E32K true homozygotes and heterozygotes were 2.10- and 1.47-fold higher than those in controls with comparable circulating PCSK9 levels, respectively, suggesting enhanced function of *PCSK9* E32K.

Conclusions: We found 2 true homozygotes for *PCSK9* E32K and 3 double heterozygotes for *PCSK9* E32K and *LDLR* mutations associated with autosomal dominant hypercholesterolaemia. This study provided evidence that *PCSK9* E32K significantly affects LDL-C levels *via* increased mass and function of PCSK9, and could exacerbate the clinical phenotypes of patients carrying *LDLR* mutations.

1. Introduction

Proprotein convertase subtilisin/kexin type 9 (*PCSK9*) is the ninth member of the subtilisin-like serine convertase superfamily [1,2]. PCSK9 regulates plasma levels of LDL-cholesterol (LDL-C) by directing cell-surface LDL receptors (LDLR) to the lysosomes for degradation, resulting in reduced clearance and accumulation of LDL-C in the circulation [3]. *PCSK9* mutations that increase the degradation of LDLR are referred to as gain-of-function mutations and cause autosomal dominant hypercholesterolaemia (ADH) and premature coronary artery disease (CAD) [4]. Conversely, loss-of-function mutations in *PCSK9*, which decrease LDLR degradation, are associated with hypocholesterolaemia and less frequencies of CAD [5,6].

In the Japanese population, 60–70% of cases of ADH are caused by *LDLR* mutation, and no apolipoprotein B mutations have been identified to date [7,8]. Thus, gain-of-function *PCSK9* mutations could explain a part of the remaining 30% of ADH cases in the Japanese population.

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The only known function of PCSK9 as a convertase is in autocatalytic processing at the VFAQ152↓SIP site [2], and unlike the other proprotein convertases, PCSK9 does not appear to undergo a second cleavage event to release an active convertase [9,10]. The prodomain of PCSK9, which binds non-covalently to the mature convertase, acts as a chaperone to assist the folding of the mature convertase and also blocks access to the catalytic site of the mature convertase [1]. It is suggested from these mechanisms that mutations in the prodomain of *PCSK9* can markedly affect the function of PCSK9; in fact, many gain-of-function mutations and loss-of-function mutations in the prodomain region have been reported with almost the same frequency as those in the catalytic domain and C-terminal region [11–13].

Enzyme-linked immunoassays for measuring plasma PCSK9 have been established recently, and circulating levels of PCSK9 as well as sequence variations in *PCSK9* have been shown to affect plasma levels of LDL-C [4,14]. However, the relationship between mutations and circulating PCSK9 levels *in vivo* has not been fully elucidated [15,16]. Characterisation of the effects of naturally occurring gain-of-function mutations in *PCSK9* on circulating PCSK9 and LDL-C levels may provide important insight into the mechanism by which PCSK9 degrades LDLR.

In this study, we identified a gain-of-function *PCSK9* mutation in the prodomain region and clarified their lipid profiles and circulating PCSK9 levels in comparison to *LDLR* mutation heterozygotes. We also present data on gain-of-function *PCSK9* mutation true homozygotes and double heterozygotes with *LDLR* mutations, providing meaningful perspectives regarding the function of PCSK9.

2. Materials and methods

2.1. Subjects

In this study, we analysed 514 unrelated patients with clinically diagnosed familial hypercholesterolaemia (clinical-FH) attending the lipid clinic of Kanazawa University Hospital or the affiliated clinics in the Hokuriku district and 351 general Japanese men attending the medical clinic for their annual health examinations in the same district as controls. The diagnosis of FH was made according to the following criteria: (1) primary hypercholesterolaemia (total cholesterol above 230 mg/dL) with tendon xanthomas or (2) primary hypercholesterolaemia with and without tendon xanthomas in first-degree relatives of hypercholesterolaemic patients [17].

Screening was performed in the 514 subjects with clinical-FH, and 262 (51.0%) were confirmed to have mutations in *LDLR*. The two most common *LDLR* mutations were c.2431A>T mutation in exon 17 (K790X, 16.8%) and c.2312-3C>A mutation in the splice acceptor region of intron 15 (IVS15-3C/A, 4.5%). Of the remaining 252 subjects whose mutations were unidentified, 55 subjects were randomly assigned to a *PCSK9* mutation screening study to detect gain-of-function *PCSK9* mutations. Among the sequence variations detected, the relatively common c.94G>A variant (E32K) in the prodomain region of *PCSK9* was likely to be a gain-of-function mutation, and a larger scale screening was performed by restriction fragment length polymorphism (RFLP) in 514 clinical-FH and 351 general Japanese controls to determine the frequency of the *PCSK9* E32K variant. Relatives of *PCSK9* E32K carriers were further screened for *PCSK9* E32K and *LDLR* mutations.

2.2. Lipids and PCSK9 measurements

Serum total cholesterol (TC), triglyceride (TG) and HDL-cholesterol (HDL-C) concentrations were determined at accredited clinical laboratories using routine clinical methods. LDL-cholesterol (LDL-C) concentrations were calculated using the Friedewald equation as there were no subjects with serum triglycerides >400 mg/dL in the present study population. To distinguish the effects of gene mutations on circulating PCSK9 and LDL-C levels, plasma PCSK9 concentrations were determined using an enzyme-linked immunosorbent assay (ELISA) kit targeting human PCSK9 (CycLex, Nagano, Japan) in 2 *PCSK9* E32K true homozygotes, 15 *PCSK9* E32K heterozygotes, 30 *LDLR* mutation heterozygotes (20 K790X and 10 IVS15-3C/A) and 20 control subjects. Lipid profile analysis and PCSK9 ELISA were performed using fasting blood samples collected when subjects were not taking any lipid-lowering drugs. Written informed consent was obtained from each of the subjects prior to participation in the study. The study protocol was approved by the Ethics Committee of the Graduate School of Medical Science, Kanazawa University.

2.3. DNA analysis

Genomic DNA was prepared from white blood cells using a Genomic DNA Purification Kit (Gentra Systems, Minneapolis, MN, USA). Primers covering all of the exons and exon–intron boundary sequence of *LDLR* and *PCSK9* were designed using Primer3 online software (http://frodo.wi.mit.edu/). *LDLR* mutations were identified using the Invader assay method (Third Wave Technologies, Inc., Madison, WI, USA) for 32 point mutations previously identified in Japan, the multiplex ligation-dependent probe amplification (MLPA) method for large rearrangements using a P062B LDLR MLPA kit (MRC Holland, Amsterdam, Netherlands) and DNA sequencing method using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) for the other mutations. MLPA and direct sequencing were performed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Mutations in *PCSK9* were detected by polymerase chain reaction (PCR) single-strand conformational polymorphism (SSCP) followed by direct sequence analysis. SSCP analysis was performed as described previously [18]. *PCSK9* E32K mutation in exon 1 of the PCSK9 gene was determined using a PCR-RFLP method with primers 5′-TGAACTTCAGCTCCTGCACA-3′ and 5′-AACGCAAGGCTAGCACCA-3′. PCR products were digested at 37 °C overnight with 1 U of the restriction enzyme BslI (New England Biolabs, Ipswich, MA, USA). RFLP assay was designed such that the normal *PCSK9* sequence would be cut twice to generate fragments of 34, 49 and 171 bp and the E32K sequence would be cleaved once to generate fragments of 34 and 220 bp, to check that the restriction enzyme was digesting properly. PCR conditions for SSCP, RFLP and DNA sequencing were as follows. Each 25-mL reaction mixture contained 60 ng of DNA, 10 pmol of each primer, 0.25 mM of each dNTP and 1 U Taq polymerase (Biotech International, Perth, Australia) in PCR buffer containing 1.5 mM $MgCl_2$. Cycling conditions were 95 °C for 5 min followed by 5 cycles of step-down PCR consisting of 95 °C for 5 s, annealing temperature +5 °C for 30 s (decrease 1 °C each cycle) and 72 °C for 1 min, then 30 cycles of 95 °C for 5 s, annealing temperature for 30 s and 72 °C for 1 min, with a final extension for 5 min at 72 °C.

2.4. In vitro expression of PCSK9

The WT-PCSK9 plasmid (pCMV-PCSK9-FLAG) containing the sequence of the FLAG epitope tag fused to the 3′ end of the PCSK9 coding sequence, was a generous gift from Dr. Jay D. Horton, University of Texas Southwestern Medical Center, Dallas, TX, USA. To make the E32K-PCSK9 construct, PCR fragments containing the *PCSK9* E32K mutant sequence were generated from the genomic DNA of a *PCSK9* E32K homozygote with the primers used for RFLP.

Table 1
Distribution of *PCSK9* E32K mutation in a general Japanese population by LDL-cholesterol quintile.

	LDL-C quintile						ANOVA *P* value
	1 (n=69) LDL-C 47.8–88.1	2 (n=70) LDL-C 88.2–104.3	3 (n=72) LDL-C 104.4–122.5	4 (n=70) LDL-C 122.6–138.9	5 (n=70) LDL-C 139.0–222.4	Total (n=351)	
Number of PCSK9 E32K carrier	0	0	0	4	2	6	
%	0	0	0	5.71	2.86	1.71	
Age (years)	43.3±12.2	47.3±9.1	43.1±9.4	46.8±9.8	49.0±7.4	45.9±9.9	<0.001
TC (mg/dL)	146.3±18.0	170.5±13.9	184.6±14.0	203.1±12.3	225.2±19.4	186.0±31.2	<0.001
TG (mg/dL)	111.2±73.9	118.2±59.0	112.3±62.9	110.2±46.4	125.6±53.3	115.5±59.7	0.517
HDL-C (mg/dL)	51.9±11.6	50.3±14.0	49.9±11.7	51.4±11.4	46.0±11.2	49.9±12.1	<0.05
LDL-C (mg/dL)	72.2±10.9	96.5±5.4	112.2±5.4	129.7±4.4	154.1±14.0	113.0±29.2	<0.001

HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglyceride.

Amplified fragments and pCMV-PCSK9-FLAG were digested at 37 °C overnight with the restriction enzymes NheI and SacII (New England Biolabs), purified with a QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) and ligated using T4 DNA ligase (Nippon Gene, Toyama, Japan). The whole coding sequence of the E32K-PCSK9 plasmid except for the G to A substitution at the position of E32K was confirmed to be identical to the WT-PCSK9 plasmid by DNA sequencing method. An empty plasmid, pcDNA3.1/myc his-c (Invitrogen, Carlsbad, CA, USA) was used as a control in the transfection experiments.

HepG2 cells obtained from the Health Science Research Resources Bank (HSRRB) were cultured in Dulbecco's modified Eagle's medium with low glucose and L-alanyl-glutamine (Gibco, Carlsbad, CA, USA) containing 1× penicillin-streptomycin solution (Wako Pure Chemical Industries, Osaka, Japan) and 10% foetal bovine serum (Gibco) in a humidified atmosphere (37 °C, 5% CO_2). HepG2 cells were then transiently transfected with the different PCSK9 constructs or empty plasmid using Lipofectamine 2000 Reagent (Invitrogen) in accordance with the manufacturer's instructions for the 24-well plates. PCSK9 levels in the media were determined using an ELISA kit after 24 h of incubation. The experiment was repeated 3 times with 4 wells in each group. Data were collected as the ratio to the WT-PCSK9 plasmid, and mean values were calculated.

2.5. Statistics

All data in the text and figures are expressed as means ± S.D. The frequency distribution of genotype was compared using standard χ^2 test. For multiple comparisons, the Tukey–Kramer HSD test was performed for variables with $P<0.05$ on F-test in one-way ANOVA. Linear correlations were analysed using Pearson's correlation coefficient analysis. JMP 5.1.2 software (SAS Institute, Cary, NC, USA) was used for statistical analyses. $P<0.05$ was considered statistically significant.

3. Results

3.1. Identification of the LDLR and PCSK9 mutations

Screening of *LDLR* mutations in 514 clinical-FH subjects resulted in detection of 52 point mutations and 11 large rearrangements in 210 and 52 patients, respectively. Twenty-two of 55 randomly selected subjects who were free from *LDLR* mutations had the following *PCSK9* sequence variants: c.61_63dupCTG (L21dup, 8 heterozygotes), c.94G>A (E32K, 1 true homozygote and 2 heterozygotes), c.158C>T (A53V, 1 heterozygote), c.787G>A (G263S, 2 heterozygotes), c.1420A>G (I474V, 4 heterozygotes), c.2004C>A (S668R, 1 heterozygote) and c.2009A>G (E670G, 3 heterozygotes). Silent mutations that do not result in a change in the amino acid sequence of the protein product or occur within intronic regions were omitted. The *PCSK9* E32K true homozygote showed markedly higher plasma LDL-C level than the 2 heterozygotes (339 mg/dL *vs.* 222 and 248 mg/dL).

3.2. Screening of E32K mutation in exon 1 of the PCSK9 gene

Genotyping of the *PCSK9* E32K mutation in the general population indicated that *PCSK9* E32K occurred in 1.71% of subjects (n=6) and was detected in the highest and the second highest quintiles divided according to LDL-C level (Table 1). The frequency of *PCSK9* E32K variants in clinical-FH was significantly higher (6.24%, $P<0.01$) than that in the general population. Further analysis in the relatives of *PCSK9* E32K carriers showed that LDL-C concentrations in *PCSK9* E32K carriers were higher than those in the general population and lower than those in *LDLR* mutation heterozygotes (Fig. 1). The *PCSK9* E32K variant was associated with a 75% increase in LDL-C (197 mg/dL *vs.* 113 mg/dL, $P<0.01$) and a 31% increase in triglyceride (TG, 151 mg/dL *vs.* 115 mg/dL, $P<0.01$), and did not influence HDL-C level (Table S1).

3.3. PCSK9 E32K true homozygote and double heterozygote with LDLR mutation

There were 5 rare cases among the *PCSK9* E32K carriers in clinical-FH and their relatives as follows: subject I, a true homozygote for the *PCSK9* E32K with the highest LDL-C level in her family pedigree at 339 mg/dL (Fig. 2A); subject II, another *PCSK9* E32K true homozygote with a modest increase in LDL-C level at 246 mg/dL; subject III, a double heterozygote for c.611G>C (C183S) mutation of *LDLR* and *PCSK9* E32K (age: 1 year, LDL-C: 581 mg/dL and severe cutaneous xanthomatosis, Fig. 2C); subject IV, a double heterozygote for c.939C>A (C292X) mutation of *LDLR* and *PCSK9* E32K (age: 30 years, LDL-C: 425 mg/dL and severe tendon xanthomatosis); subject V, a double heterozygote for *LDLR* K790X and *PCSK9* E32K (age: 2 years, LDL-C: 248 mg/dL and severe cutaneous xanthomatosis, Fig. 2D). No mutations in *LDLR* were detected in *PCSK9* E32K carriers except for the 3 double heterozygotes described above.

3.4. Analysis of circulating PCSK9 levels in PCSK9 E32K carriers and LDLR mutation heterozygotes

Plasma PCSK9 levels in the *PCSK9* E32K heterozygotes were significantly higher than those in controls (349±90 ng/mL *vs.* 266±112 ng/mL, $P<0.05$), and those in *PCSK9* E32K true homozygotes (363 ng/mL) were similar to those in heterozygotes. Strong positive correlations were observed between PCSK9 and LDL-C levels in both the *PCSK9* E32K heterozygotes ($r=0.69$, $P<0.01$) and in control subjects ($r=0.64$, $P<0.01$, Fig. 3A). Further, the slope of the regression curve between plasma PCSK9 and LDL-C in *PCSK9* E32K heterozygotes ($y=0.383x+54.1$) was far steeper than that in controls ($y=0.186x+61.6$). Consequently, relative LDL-C levels were 1.47-fold higher in *PCSK9* E32K heterozygotes and 2.10-fold higher in true homozygotes than in controls with comparable cir-

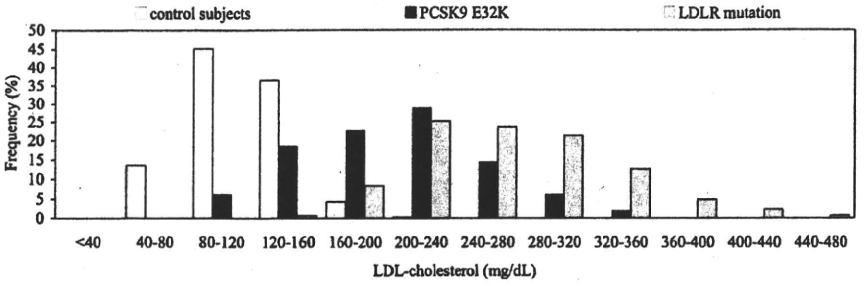

Fig. 1. Plasma LDL-C distribution in control subjects, *PCSK9* E32K carriers and *LDLR* mutation heterozygotes. Bars show the percentage of subjects in each group.

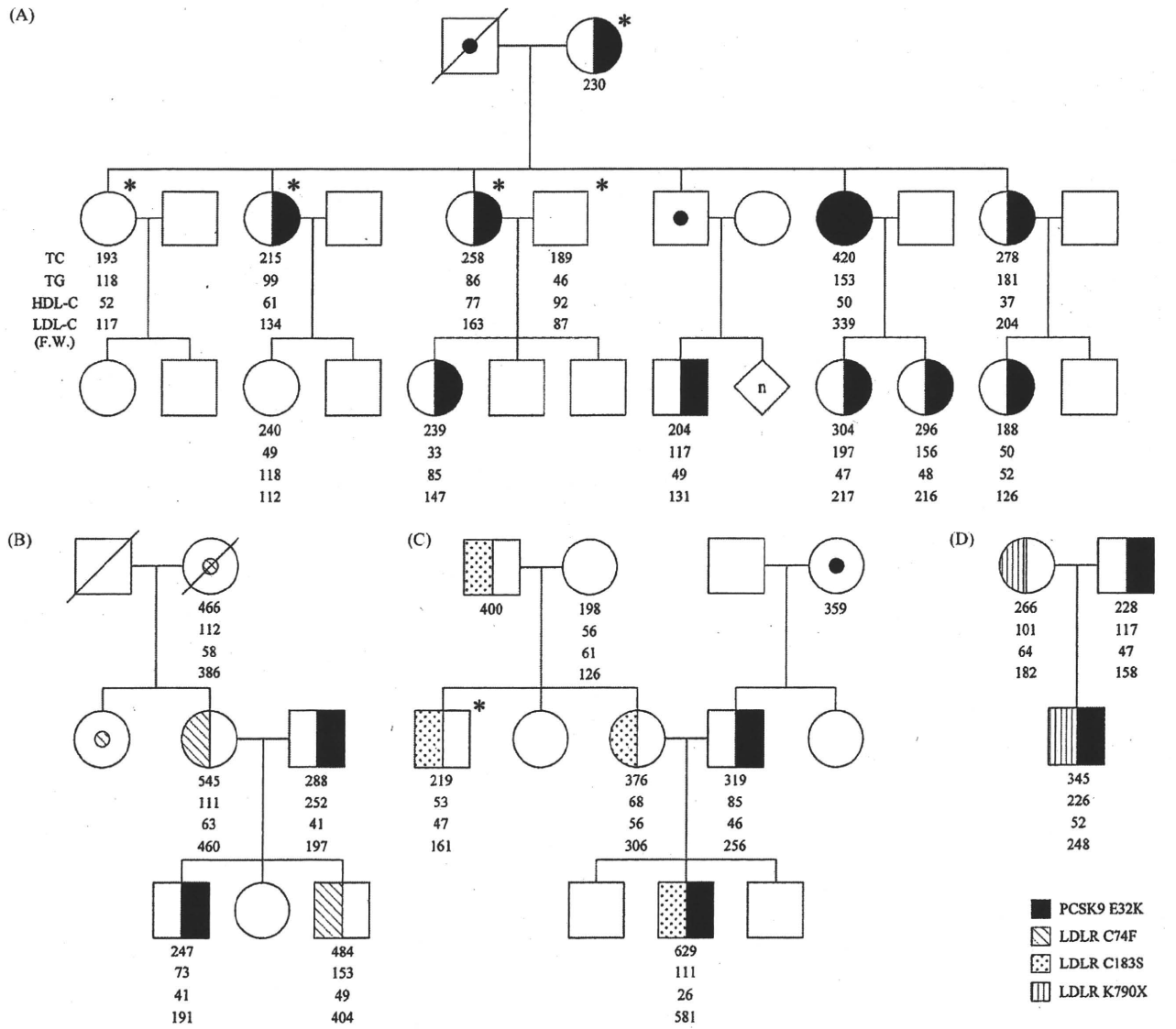

Fig. 2. Pedigrees of gain-of-function *PCSK9* mutation families caused by the *PCSK9* E32K. (A) Pedigree of subject with true homozygous *PCSK9* E32K mutation. (B) Pedigree affected by a single mutation of *PCSK9* E32K or *LDLR* C74F in each subject. (C) Family with a double heterozygote of *PCSK9* E32K and *LDLR* C183S mutations. (D) Pedigree of a double heterozygote with *PCSK9* E32K and *LDLR* K790X mutations. Relatives of another *PCSK9* E32K true homozygote and a double heterozygote with *PCSK9* E32K and *LDLR* C292X mutations did not participate in this study. Half-shaded symbols indicate carriers of a single mutation of *PCSK9* or *LDLR* and filled symbol indicates true homozygote. Plasma TC, TG, HDL-C and LDL-C values (mg/dL) are shown below each symbol. Asterisks indicate the subjects treated with lipid-lowering drugs at the time of the study. Hatched lines represent deceased family members. Diamond symbols labelled "n" indicate number and sex of individuals unknown.

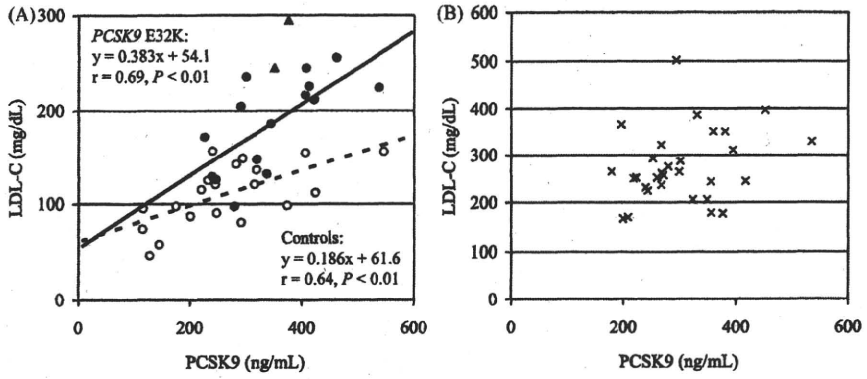

Fig. 3. Relationships between circulating levels of PCSK9 and LDL-C in (A) 20 control subjects (open circles), 15 *PCSK9* E32K heterozygotes (closed circles), 2 *PCSK9* E32K true homozygotes (closed triangles) and (B) 30 *LDLR* mutation heterozygotes (20 K790X and 10 IVS15-3C/A, crossed symbols).

culating PCSK9 levels (Table S2). In heterozygous *LDLR* mutation carriers, there were slight increases in plasma PCSK9 levels at 304 ± 83 ng/mL and the correlation between plasma PCSK9 and LDL-C levels was not significant ($r = 0.25$, $P = 0.40$, Fig. 3B). The results were similar when the relation was analysed in *LDLR* K790X carriers ($r = 0.12$, $P = 0.64$) and in *LDLR* IVS15-3C/A carriers ($r = 0.33$, $P = 0.34$), respectively. Plasma PCSK9 and TG levels were positively correlated in control subjects ($r = 0.48$, $P < 0.05$) and *LDLR* mutation heterozygotes ($r = 0.40$, $P < 0.05$), whereas the correlation did not reach statistical significance in *PCSK9* E32K carriers ($r = 0.44$, $P = 0.10$).

3.5. Effects of PCSK9 E32K mutation on secretion of PCSK9

To examine whether the *PCSK9* E32K mutation affects the secretion of PCSK9, the amounts of PCSK9 were measured in media of HepG2 cells transiently transfected with WT-PCSK9, E32K-PCSK9 or empty plasmid. Consistent with *in vivo* results, HepG2 cells transfected with E32K-PCSK9 secreted significantly larger amounts of PCSK9 into the media than those with WT-PCSK9 after 24 h of incubation (139 ± 13% *vs.* 100 ± 3%, $P < 0.01$, Fig. 4). PCSK9 levels in the media of HepG2 cells transfected with empty plasmid were almost undetectable (0.7 ± 0.3%) under our experimental conditions.

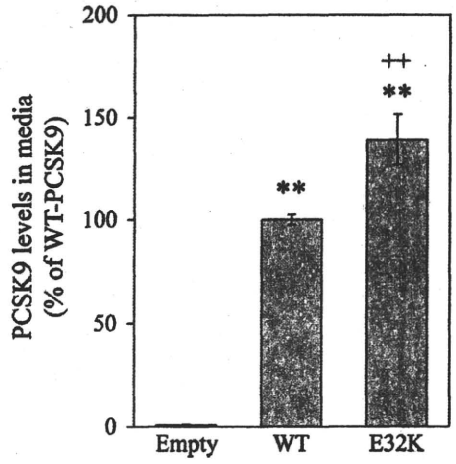

Fig. 4. Secretion of PCSK9 in media from HepG2 cells transiently transfected with WT-PCSK9, E32K-PCSK9 or empty plasmid. After 24 h of incubation, the media were collected and subjected to ELISA. The values are expressed relative to the amount of PCSK9 in the media of WT-PCSK9-transfected HepG2 cells and are given as means ± S.D. from three independent experiments. $^{**}P < 0.01$ *vs.* empty plasmid, $^{++}P < 0.01$ *vs.* WT-PCSK9.

4. Discussion

In this study, 55 clinical-FH subjects without *LDLR* mutations were screened for *PCSK9* mutations and 7 variants were detected in 22 subjects. One of the variant carriers was true homozygous for *PCSK9* E32K and showed markedly higher LDL-C levels than *PCSK9* E32K heterozygotes. On further screening for *PCSK9* E32K variant in the general population, 6 carriers of *PCSK9* E32K were detected among individuals with higher LDL-C levels (Table 1), which was similar to the results of Miyake et al. who detected *PCSK9* E32K variant in the higher LDL-C group and anti-hypercholesterolaemia treatment group [19]. The lipid profiles of *PCSK9* E32K carriers and the significantly higher frequency of *PCSK9* E32K in clinical-FH than that in controls of the same district suggested this should be classified as a gain-of-function mutation. The remaining 6 *PCSK9* variants detected here were also described in their study, and the capabilities of these variants to cause FH were considered to be very low.

Consistent with previous findings [14], plasma PCSK9 levels were positively correlated with LDL-C levels in both control subjects and *PCSK9* E32K heterozygotes (Fig. 3A). The slope of the regression curve in *PCSK9* E32K heterozygotes was almost double that in control subjects (Fig. 3A). Further, *PCSK9* E32K true homozygotes showed 2.10-fold higher LDL-C levels than controls with comparable PCSK9 levels and the value was almost the square of that in heterozygotes (1.47), suggesting the impact of circulating *PCSK9* E32K on LDL-C level (Table S2). Although the media of HepG2 cells transfected with E32K-PCSK9 plasmid as well as the plasma in *PCSK9* E32K carriers showed more than 30% higher PCSK9 concentrations compared with controls in our ELISA study (Fig. 4, Table S2), circulating PCSK9 levels in *PCSK9* E32K true homozygotes were similar to those in heterozygotes. In a recent study, a much more potent gain-of-function *PCSK9* mutation c.1120G>T (D374Y) was associated with lower plasma PCSK9 levels [16]. We presume that the increased function of PCSK9, which in turn enhances its own clearance, would in part lower the plasma PCSK9 concentration in *PCSK9* E32K true homozygotes like *PCSK9* D374Y carriers. The effect of *PCSK9* E32K was relatively mild, and thus age, gender and other metabolic markers, such as insulin and glucose, would considerably influence the plasma levels of PCSK9 [15,20,21].

In the heterozygous FH subjects with *LDLR* mutations, plasma PCSK9 levels were similar to those in controls but plasma levels of LDL-C were twice those in controls after adjusting for circulating PCSK9 levels. A positive correlation between plasma PCSK9 and LDL-C levels was not observed in *LDLR* mutation carriers (Fig. 3B), and therefore we speculated that high LDL-C levels relative to PCSK9 levels in *LDLR* mutation carriers would reflect the far more

potent impact of *LDLR* mutations on LDL-C concentration than that of circulating PCSK9.

Although there is consensus regarding the mechanism of LDLR degradation by PCSK9, there is debate over its role in very low-density lipoprotein (VLDL) receptor (VLDLR) regulation. Poirier et al. demonstrated that PCSK9 induces degradation of VLDLR independent of the presence of LDLR [22], whereas Zhang et al. reported that the selectivity of PCSK9 for the LDLR is due to Leu318 in epidermal growth factor-like repeat (EGF)-A domain, which is not present in VLDLR [3]. With regard to VLDL production, fasting led to a marked decrease in LDLR levels in mice overexpressing PCSK9, which was associated with an increase in VLDL production rate [23]. In gain-of-function *PCSK9* S127R, the lipoprotein kinetic study using stable isotopes indicated a 3-fold increase in apolipoprotein B100 and VLDL production rate [24]. In the present study, there was a positive correlation between plasma PCSK9 and TG levels in controls, which was consistent with recent reports [15,20,21], whereas this correlation did not reach statistical significance in *PCSK9* E32K carriers despite marked increase in plasma PCSK9 and TG levels (Table S2). An *in vivo* lipoprotein kinetic study of the *PCSK9* E32K true homozygote using stable isotopes would provide more precise information on the lipoprotein kinetics in gain-of-function *PCSK9* mutations.

Previous studies and our results indicated that the gain-of-function mutation *PCSK9* E32K is relatively common and causes milder hypercholesterolaemia than *LDLR* mutations and other potent gain-of-function *PCSK9* mutations such as D374Y [19,25]. Interestingly, *PCSK9* E32K heterozygotes were clearly distinguished from *LDLR* C74F heterozygotes with regard to plasma LDL-C levels within the family (Fig. 2B). Similar to other gain-of-function mutations of *PCSK9* [25,26], *PCSK9* E32K could worsen the lipid profiles in subjects true homozygous or double heterozygous with *LDLR* mutation as in one patient in the present study (Fig. 2A, C and D), although the levels of plasma LDL-C varied widely from 246 to 581 mg/dL. There were no further mutations in *LDLR* in any of these subjects and they were apparently different from those homozygous for *LDLR* mutation in that their LDL-C levels could be reduced to values (<280 mg/dL) comparable to those in *LDLR* mutation heterozygotes by drug treatment.

In the family affected with *PCSK9* E32K and *LDLR* K790X, the presence of lipid-lowering gene mutation was suggested by the relatively low cholesterol levels considering their gene mutations, but at the same time, it is also possible that the combined gene mutations would interact with each other and affect the lipid profiles of double heterozygous subjects (Fig. 2D). The mutations encoded in 50-residue cytoplasmic domain of LDLR (amino acids 790–839), which is important in directing the receptors to coated pits and facilitating rapid endocytosis of bound LDL [27], are considered to be internalisation-defective alleles and we confirmed that T-lymphocytes of *LDLR* K790X heterozygotes showed defective uptake of DiI-LDL [28]. Recently, Bottomley et al. demonstrated that the EGF sub-fragments of the LDLR were able to counteract the inhibitory effect of PCSK9 in LDL uptake assays [29]. Likewise, in *LDLR* K790X heterozygotes, the intact EGF-AB domain of the internalisation-defective *LDLR* K790X may act as a decoy to protect the remaining half of normal LDLR against PCSK9-mediated degradation. In this regard, the weaker association between plasma levels of PCSK9 and LDL-C in subjects with *LDLR* K790X compared to subjects with *LDLR* IVS15-3C/A ($r = 0.12$ *vs.* $r = 0.34$) may support the inhibitory effect of *LDLR* K790X on the function of PCSK9, although neither relation reached statistical significance.

In summary, we found 2 true homozygotes for *PCSK9* E32K and 3 double heterozygotes for *PCSK9* E32K and *LDLR* mutations associated with ADH. To our knowledge, this is the first report of gain-of-function *PCSK9* mutation true homozygosity, providing evidence that *PCSK9* E32K significantly affects LDL-C levels *via* increased function and mass of PCSK9, and could exacerbate the clinical phenotypes of patients carrying *LDLR* mutations.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2009.11.018.

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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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Running header: LCAT replacement therapy by gene-transduced adipocytes

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.

Key words: 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.

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]. 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 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 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 (3.5×10^4 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 x 10^7 cells/ml. The cell suspension (50 μl) was injected into the adipose tissue between the shoulder-blades of NOD/Shi-*scid* IL-2Rγ^{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 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 3×10^4 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 ($99.1 \pm 0.3\%$ vs. $95.6 \pm 0.1\%$, $p<0.05$), 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 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**).