厚生労働科学研究費補助金　（難治性疾患等実用化研究事業）
家族性 LCAT 欠損症患者に対する細胞加工医薬品
「LCAT 遺伝子導入ヒト全脂肪細胞」の早期実用化にむけた非臨床試験
平成２６年度　分担研究報告書

細胞加工医薬品「LCAT 遺伝子導入ヒト前脂肪細胞」に適した脂肪細胞の存在部位の解明と無菌的採取法の確立

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研究要旨
細胞加工医薬品「LCAT 遺伝子導入ヒト前脂肪細胞」にはヒト皮下脂肪から採取した脂肪組織が用いられる。(1) 腹部皮下脂肪組織では浅筋膜の浅層にある脂肪組織と深層にある脂肪組織の 2 層構造が明瞭である。浅層脂肪組織の方が深層脂肪組織と比べて天井培養由来増殖性脂肪細胞の回収効率、回収した天井培養脂肪細胞の脂肪分化能とも優れていた。(2) 脂肪吸引採取時の外科的衛生手順の徹底を追求することで無菌的な脂肪採取が可能であることが確認できた。これらの結果は、細胞加工医薬品「LCAT 遺伝子導入ヒト前脂肪細胞」を用いた治療に特化した最適な移植法の確立に繋がる。

A.　研究目的

細胞加工医薬品「LCAT 遺伝子導入ヒト前脂肪細胞」を用いた治療においてはヒト皮下脂肪組織から吸引採取した脂肪組織から天井培養法によって分離・培養した脂肪細胞が用いられる。脂肪吸引及び脂肪移植法は形成外科領域の日常診療で行われる比較的普及した治療手技である。安全性や生着率の面では近年提唱された微量分割注入法により大幅にその成績が向上しつつある。脂肪移植は組織増量のみならず、脂肪細胞の高い分泌能を活用した組織統合性の回復による創傷治癒促進、瘢痕などの組織性状の改善といった機能面の改善にも応用が広がりつつある。

しかし、細胞加工医薬品「LCAT 遺伝子導入ヒト前脂肪細胞」を用いた治療ではこれまで形成外科領域で確立されてきた脂肪吸引術および脂肪細胞移植術とは異なる工程があり、その点に格段の配慮が必要である。異なる点は、以下の 2 点である。

① コラゲナーゼ処理による細胞単離と天井培養の工程があるため高い無菌性を要求されること。
② 天井培養法に適した脂肪採取とはどういうものかが明らかではないこと。

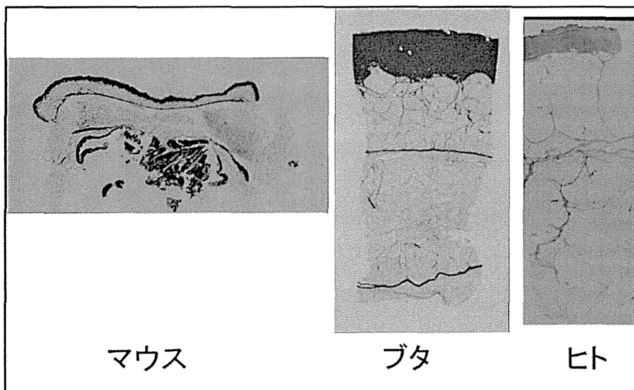
われわれはこれまでに確立された脂肪吸引および脂肪移植法の基本をもとに細胞加工医薬品「LCAT 遺伝子導入ヒト前脂肪細胞」を用いた治療に適した方法を明らかにする必要があると考えた。

本研究の目的は、細胞加工医薬品「LCAT 遺伝子導入ヒト前脂肪細胞」における天井培養に最適な脂肪採取法の特定と、仕様を満たす衛生水準の脂肪採取法の確立である。

B. 研究方法

① 皮下脂肪細胞の解剖学的構築差と天井培養脂肪細胞の特性の違い

皮下脂肪組織は浅筋膜または肉様膜と呼ばれる構造によって、それらより浅い層に存在する皮下脂肪組織（浅層脂肪組織）と、それらより深い層に存在する皮下脂肪組織（深層脂肪組織）の2層に分けられる。皮下組織の2層構造は哺乳類では種を超えて比較的普遍的にみられる構造である。しかし、これらの皮下組織 2 層構造の成り立ちや意義については、解剖学的にも生理学的にも殆ど分かっていない。



ヒトでは皮下組織 2 層構造は体の部位により明瞭な部位と不明瞭な部位が存在するが、体幹では明瞭であり、特に細胞加工医薬品「LCAT 遺伝子導入ヒト前脂肪細胞」で用いられる腹部では肉眼的にもはっきりと区別できる解剖学的構造差がある。

われわれは腹部皮弁を用いた手術時に通常廃棄される脂肪組織の浅層と深層各々から別個に天井培養法を行い回収効率の比較を行った。また、浅層と深層それぞれから回収された天井培養脂肪細胞の脂肪分化能を比較した。

② 無菌的吸引脂肪採取法の確立

形成外科領域で通常行われる脂肪移植法は吸引採取した脂肪組織を採取直後に注入するもので、外来処置室等で行うことが可能であり、また、通常、感染のリスクは殆ど無いと考えられている。一方、細胞加工医薬品「LCAT 遺伝子導入ヒト前脂肪細胞」においては、採取した脂肪組織から脂肪細胞をコラゲナーゼで単離した後数週間インキュベーターで培養を行うという通常の脂肪移植では行われない手順が加わる。そのため、衛生面に関して、細胞加工医薬品「LCAT 遺伝子導入ヒト前脂肪細胞」における脂肪採取を通常の脂肪移植の場合と同一に捉えることは適切でない。

細胞加工医薬品「LCAT 遺伝子導入ヒト前脂肪細胞」に用いられる脂肪細胞について衛生水準を満たすため以下の方法で、開腹・開頭・開胸手術と同等の外科的衛生を行い可能な限り最大限の清潔を保って脂肪吸引を行い結果を検証した。

- 術前外来診察の時点で腹部・臍部の診察を行い、オイルと綿棒を用いた臍部清掃を１日１回手術前日まで行うよう指導した。
- 脂肪採取を外来処置室でなく大学病院中央手術室で行った
- 術者は手術用ウエア、帽子、マスクを着用したうえで7.5%ポビドンヨード液で2回外科手洗いを行い滅菌ガウンを着用した。滅菌手袋を二重に着用した。
- 患者側の処置として脂肪吸引管の挿入予定部位である臍傍部を中心に10%ポビドンヨード液で2回消毒後5分間待機した。滅菌ドレープで腹部以外の体表面を完全に覆った。採取予定部位である腹部をイソジンドレープを貼付した。
- 局所浸潤麻酔注射の後、脂肪吸引管を皮下に挿入し手動で陰圧をかけて採取した。採取した脂肪はシリンジから出さずシリンジに栓をした。
- シリンジごと清潔手術野からおろし、シリンジ内の脂肪組織を外注微生物検査で

調査した。

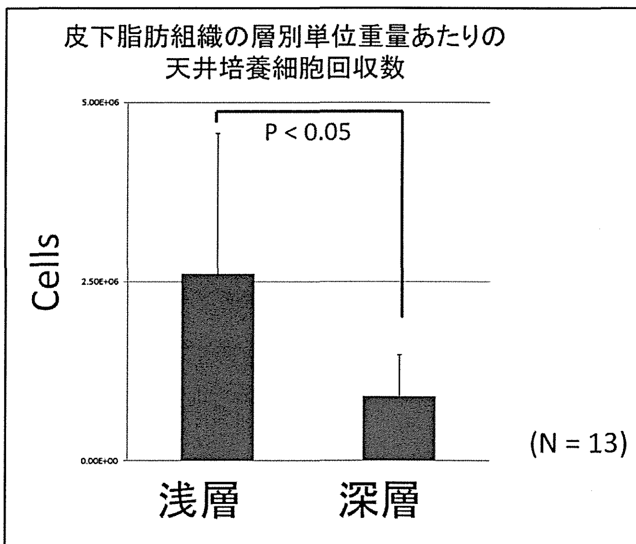
（倫理面への配慮）

千葉大学倫理委員会の承認の下、患者に対し、術前に文書を併用した説明を十分に行い、書面での同意が得られた患者のみを対象とした。

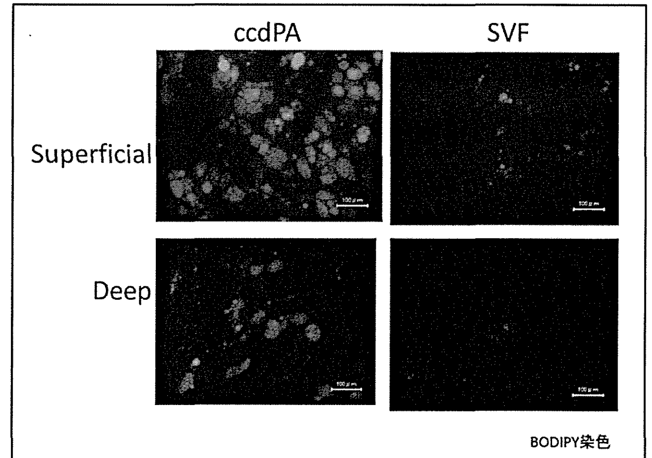
C. 研究結果

①脂肪採取部位による回収効率、脂肪分化能の差

採取脂肪組織１ｇあたりの 天井培養由来増殖性脂肪細胞の回収数は浅層皮下脂肪由来のものが深層皮下脂肪由来のものと比べて有意に高値だった。



浅層皮下脂肪由来の天井培養由来増殖性脂肪細胞は深層皮下脂肪由来のそれと比較し、高い脂肪分化能を示した。また、天井培養由来増殖性脂肪細胞と Stromal vascular fraction (SVF)の脂肪分化能の比較では浅層、深層ともに天井培養由来増殖性脂肪細胞が SVF と比べて高い脂肪分化能を示した。



②無菌的脂肪採取

採取した脂肪組織から微生物は検出されなかった。

D. 考察

浅層脂肪組織からは深層脂肪組織と比べて有意に多くの天井培養由来増殖性脂肪細胞が回収できた。脂肪吸引時に浅層からの吸引を心がけることで細胞加工医薬品「LCAT 遺伝子導入ヒト前脂肪細胞」用の細胞の回収効率を向上できる可能性が示された。また、浅層脂肪組織から回収された天井培養由来増殖性脂肪細胞は深層脂肪組織から回収されたそれと比べて脂肪分化能が高いことが明らかになった。遺伝治療の安全性の点から脂肪分化能が高い方が良いと考えられており、この点からも浅層脂肪組織からの脂肪採取の方が深層脂肪組織からの回収と比べて望ましいと考えられた。

外科的衛生手順を遵守し最大限の清潔操作を行うことで臍傍部から無菌的に吸引脂肪組織を採取できた。通常の脂肪移植では吸引された脂肪は、採取直後に移植される上に、数 mm の脂肪組織として存在し組織統合性がある程度保たれているため感染が問題になることは殆どない。しかし、細胞加

工医薬品「LCAT 遺伝子導入ヒト前脂肪細胞」では細胞は単離されて数週間培養されるため培養液中の抗生物質を除けば有害微生物に対抗する力は細胞自身にはほとんどない。よって、採取時点から微生物を混入させないことが衛生管理上最大の目標となる。今回、無菌的採取が可能であることを確認できたことで細胞加工医薬品「LCAT 遺伝子導入ヒト前脂肪細胞」の衛生面での大きな課題の一つがクリアできたことになる。

E. 結論

皮下組織に存在する浅層と深層の 2 層構造のうち細胞加工医薬品「LCAT 遺伝子導入ヒト前脂肪細胞」には浅層からの採取が望ましいと考えられた。

外科的衛生手順を厳密におこなうことで無菌的な吸引脂肪採取が可能だった。

研究発表

1. 論文発表

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(2) Mitsukawa N, Morishita T, Saiga A, Omori N, Kubota Y, Akita S, Satoh K. A case of Crouzon syndrome treated by simultaneous bimaxillary distraction. J Plast Reconstr Aesthet Surg. 2014 Jan;67(1):124-5.

2. 学会発表

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F. 知的財産権の出願・登録状況

該当無し

厚生労働科学研究費補助金（難治性疾患等実用化研究事業）
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「LCAT 遺伝子導入ヒト前脂肪細胞」の早期実用化にむけた非臨床試験
平成２６年度　分担研究報告書

科学的・倫理的配慮に基づく遺伝子治療臨床研究への円滑な橋渡しに関する研究

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研究要旨：遺伝子治療を実施するにあたって適切な臨床研究基盤を整備する必要性がある。医師主導治験が実施できるように、その実施体制整備に取り組んだ。

A. 研究目的

治験実施計画書作成のため、遺伝子治療臨床研究と今後予定される医師主導治験の実施体制について比較検討する。

B. 研究方法

本研究においては、以下の３項目につき研究に取り組んだ。

(1) プロジェクト管理
(2) GMP 準拠環境整備
(3) データ管理体制

（倫理面への配慮）

本研究は試験実施の準備のため、直接被験者への影響はない。実施される臨床研究は遺伝子治療臨床研究に関する倫理指針に基づいて実施される。

C. 研究結果

(1) プロジェクト管理

試験期間内の適切な症例の組み入れ等、試験遂行のためプロジェクト管理は不可欠である。臨床試験部プロジェクトマネジメントにより、細胞調製の基礎研究者、臨床担当の内科医師、脂肪細胞摘出・移植担当の整形外科医師及び千葉大学発ベンチャー（セルジェンテック）が合同で概略月１回会議を重ね、臨床研究における具体的な手順や役割分担について検討した。

特に遺伝子を扱う治療であるため、遵守すべき法令に基づいて、手技が行われる場所や方法について多角的な検討を行った。

(2) GMP 準拠環境整備

医師主導治験を実施するためには GMP 対応の CPC 整備が必要である。昨年度末に新たな CPC を創設し、今年度は GMP の整備をハード・ソフト面で行った。

CPC の運営管理および SOP の整備が大きな課題であるため、専任の運営管理担当者を本年 4 月より採用した。

組織管理体制の見直し

運営会議体と GMP 体制を区分し、会議体の下に GMP 体制を構築する

現行基準書以外にも手順書中にセンター長等の承認・サインが必要な部分が多く、迅速な対応が難しく、再生医療安全性確保法や GMP 基準に対応した責任者群の規程がないため、全体的な見直しを行った。

責任者群の見直し

再生医療等安全確保法及び GMP 基準を準用した責任者群の設置

各 Project の責任者と施設管理に関わる責任者群を区分

SOP の見直し

SOP 体系の見直しと既存の三管理基準書、各種手順書の改訂と新規作成

再生医療等安全性確保法への対応

1) 総則の新規作成：1 sop
2) 三管理基準書の改訂：3 sop
3) ゼネラル SOP の改訂及び新規作成（新法に記載のある手順書）：9 sop
4) 各種手順書の改訂・新規作成（上記以外の規程）：9 sop
5) 運用マニュアル（統一的な運用を図るためにマニュアル）：4 manuals

その結果 CPC 施設としてバリデーションもでき、施設として可動が可能となった。

(3) データ管理体制

データの管理については、臨床試験部に新しく設置されたデータセンターにおける電子データシステムによりデータを保管する。症例報告書の作成に向けて、基礎研究者、臨床担当医師、データ管理責任者等がより詳細な必要項目の精査を行った。

(4) 品質管理関連

センター内での迅速測定法の採用に向けた検討

エンドトキシン試験：Endosafe-PTS による簡易同定（所要時間　約 20 分）

測定装置を導入し、反応干渉因子試験の実施中（一部終了）

マイコプラズマ否定試験：RT-PCR 法による測定試薬メーカーによるデモ及び品質リスクアセスメントによる微生物学的な汚染防止

細胞調製室（重要区域、重要区域に隣接する清浄区域）に搬入する資材の三重包装化（微生物の持ち込みを防止）

ピペット、環境モニタリング用培地市販の三重包装品に切替済み

その他の資材（遠心チューブ、注射筒、注射針、無塵紙、その他のプラスチック製品）　自ら三重包装としてガンマ線滅菌を依頼するルートを確保し、実施中

調製室内から検出される微生物の同定を行い、バイオバーデンの把握を行う　　検査部（院内）の協力にもと、依頼書の内容と手順を確認し、MALDI-TOF MS による同定を実施中。現在、検出菌は *Bacillus subtilis* と同定され、環境由来菌であることを確認した。

細胞調製手順書・記録書は、CPC の内外を行き来するため汚染リスクが高い。かつ、その都度のガンマー線滅菌は不可である。　ペーパーレス化を指向し、電子システムの導入に向けて検討中である

D. 考察

遺伝子治療臨床研究は「遺伝子治療臨床研究に関する指針」に従い実施されるが、正しいデータを取得し、正しい評価を行うため、プロジェクトマネジメント、モニタリング、データマネジメント、CRC 業務等を取り入れ、可能な限り治験の実施体制と同様とすべきであると考える。特に、安全性を確保するために CPC における細胞調製において GMP 準拠の管理は不可欠であり、そのためのハードおよびソフト面の充実を図った。また、基礎研究者、内科医、外科医、データ管理及びこれらを取りまとめるプロジェクトマネージメントの協働体制を構築し、今後さらに関わる担当者を広げて大きく、また強固なチーム構築に取り組んで行く。

E. 結論

治験実施計画書作成のため、遺伝子治療臨床研究と医師主導治験の実施体制について比較検討した。治験は薬事法のもとで実施するため、CPC は GMP 対応が必須であるが、GMP 準拠で行う臨床試験においては GMP 準拠の SOP の整備が必要である。そのため SOP の作成を行った。今後、非臨床試験で得られたデータを適切に治験実施計画書に反映し、質の高いプロトコール作成を目指すものである。

F. 研究発表

なし

Ⅲ. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Kuroda M, Hollebboom AG, Stroes E, Asada S, Aoyagi Y, Kamata K, Yamashita S, Ishibashi S, Saito Y, Bujo H.	Lipoprotein subfractions highly associated with renal damage in familial LCAT deficiency.	Arterioscler Thromb Vasc Biol.	34	1756-62	2014
Akita S, Mitsukawa N, Kuriyama M, Hasegawa M, Kubota Y, Tokumoto H, Ishigaki T, Hanaoka H, Satoh K.	Suitable therapy options for sub-clinical and early-stage lymphoedema patients.	J Plast Reconstr Aesthet Surg.	67(4)	520-5	2014
Mitsukawa N, Morishita T, Saiga A, Omori N, Kubota Y, Akita S, Satoh K.	A case of Crouzon syndrome treated by simultaneous bimaxillary distraction.	J Plast Reconstr Aesthet Surg.	67(1)	124-5	2014

Ⅳ．研究成果の刊行物・別冊

Arteriosclerosis, Thrombosis, and Vascular Biology

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Lipoprotein Subfractions Highly Associated With Renal Damage in Familial Lecithin:Cholesterol Acyltransferase Deficiency

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Lipoprotein Subfractions Highly Associated With Renal Damage in Familial Lecithin:Cholesterol Acyltransferase Deficiency

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Objective—In familial lecithin:cholesterol acyltransferase (LCAT) deficiency (FLD), deposition of abnormal lipoproteins in the renal stroma ultimately leads to renal failure. However, fish-eye disease (FED) does not lead to renal damage although the causative mutations for both FLD and FED lie within the same *LCAT* gene. This study was performed to identify the lipoproteins important for the development of renal failure in genetically diagnosed FLD in comparison with FED, using high-performance liquid chromatography with a gel filtration column.

Approach and Results—Lipoprotein profiles of 9 patients with LCAT deficiency were examined. Four lipoprotein fractions specific to both FLD and FED were identified: (1) large lipoproteins (>80 nm), (2) lipoproteins corresponding to large low-density lipoprotein (LDL), (3) lipoproteins corresponding to small LDL to large high-density lipoprotein, and (4) to small high-density lipoprotein. Contents of cholesteryl ester and triglyceride of the large LDL in FLD (below detection limit and 45.8±3.8%) and FED (20.7±6.4% and 28.0±6.5%) were significantly different, respectively. On in vitro incubation with recombinant LCAT, content of cholesteryl ester in the large LDL in FLD, but not in FED, was significantly increased (to 4.2±1.4%), whereas dysfunctional high-density lipoprotein was diminished in both FLD and FED.

Conclusions—Our novel analytic approach using high-performance liquid chromatography with a gel filtration column identified large LDL and high-density lipoprotein with a composition specific to FLD, but not to FED. The abnormal lipoproteins were sensitive to treatment with recombinant LCAT and thus may play a causal role in the renal pathology of FLD. **(*Arterioscler Thromb Vasc Biol.* 2014;34:1756-1762.)**

Key Words: chromatography, gel ■ LDL ■ lecithin acyltransferase deficiency ■ renal insufficiency

Lecithin:cholesterol acyltransferase (LCAT)–deficiency syndromes are rare autosomal recessive diseases, characterized by hypo-α-lipoproteinemia and corneal opacity.[1,2] They are caused by mutations in the *LCAT* gene, of which 88 have been reported to date.[3] Severe mutations lead to familial LCAT deficiency (FLD), mild mutations lead to fish-eye disease (FED). In FLD, the mutant LCAT enzyme is either absent in plasma (not secreted from the hepatocyte or rapidly degraded on secretion) or exhibits no catalytic activity on any lipoprotein; in FED, LCAT cannot esterify cholesterol on high-density lipoprotein (HDL; loss of α-activity) but retains its activity on lipoproteins containing apolipoprotein B (β-activity).[1,2] Likely, the molecular difference is causal to the major clinical difference between FLD and FED: patients with FLD develop renal failure, whereas patients with FED do not.[2,4]

To prevent renal failure in patients with FLD, replacement therapy with recombinant enzyme is currently being developed.[5–8] Alternatively, we are developing a long-lasting gene therapy by transplantation of human *LCAT* gene-transduced autologous adipocytes.[7,9] Recombinant LCAT (rLCAT) secreted by the *LCAT* gene-transduced adipocytes corrected abnormal HDL subpopulations in sera of FED patients in vitro.[10]

LCAT catalyzes the esterification of cholesterol with acyl groups hydrolyzed from phospholipids, predominantly on HDL particles. This leads to mature lipoproteins with cores filled with cholesterol ester. LCAT dysfunction leads to decreased maturation of the HDL particle and to increased levels of both its substrates: unesterified cholesterol and phosphatidylcholine. In the absence of LCAT activity, abnormal lipid particles have been observed throughout lipoprotein fractions.[11–14] The HDL fraction contains disk-shaped particles in rouleaux and small spherical particles. Density-gradient ultracentrifugation followed by electron microscopy

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Nonstandard Abbreviations and Acronyms	
CE	cholesteryl ester
FC	free cholesterol
FED	fish-eye disease
FLD	familial lecithin:cholesterol acyltransferase deficiency
GFC	gel filtration column
HDL	high-density lipoprotein
HPLC	high-performance liquid chromatography
LCAT	lecithin:cholesterol acyltransferase
LDL	low-density lipoprotein
Lp	lipoprotein
LpX	lipoprotein-X
rLCAT	recombinant LCAT

revealed that the low-density lipoprotein (LDL) fraction contains 3 abnormal particles with different sizes, lipid composition, and associated apolipoproteins,[11,12] which were proposed to be important in the pathogenesis of renal manifestation in patients with FLD.[15–18] Of these, lipoprotein-X (LpX)[19,20] have been postulated to accumulate in glomeruli, potentially causing the renal damage observed in patients with FLD.[16–18] In 1 patient with FLD, lipid-lowering therapy led to a reduction of LpX and a concomitant reduction in proteinuria.[21] LpX is phospholipid (PL)-rich and free cholesterol (FC)–rich but triglyceride (TG)-poor particle without apolipoproteins, ranging in size between very low density lipoprotein and large LDL.[22]

To characterize the abnormal lipoproteins associated with the renal pathology of FLD, we characterized lipoprotein fractions by analyzing patients with different mutations and manifestations in comparison with another LCAT-deficiency syndrome, FED. We applied high-performance liquid chromatography with a gel filtration column (HPLC-GFC) for the first time to characterize the above abnormal lipoproteins and in fact identified lipoprotein subfractions specific to FLD. The lipid contents and particle size were biochemically determined, and the responsiveness of the lipoproteins against incubation with rLCAT was investigated in vitro.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Lipoprotein Subfractions Specific to LCAT-Deficiency Syndromes

Five patients with FLD (1–5) and 4 patients with FED (6–9) were compared with 4 nonaffected normolipidemic controls. Clinical and molecular characteristics and lipid profiles of the patients are given in Tables 1 and 2, respectively. Ultracentrifugation fractionation followed by determination of lipid contents was performed in patients 1, 2, and 5 (Table I in the online-only Data Supplement). LCAT α-activities in the patients' sera were all <2% of reference. As expected in LCAT deficiency, mature HDL particles found at fraction (Fr.) 16 and 17 of unaffected controls were absent in the 9 patients (Figure 1). Although the lipid profiles of patients were heterogeneous, HPLC-GFC showed 4 lipoprotein fractions in sera of patients with FLD and FED that were not present in sera of unaffected controls: large lipoproteins (>80 nm) in Fr. 1 (Lp1), lipoproteins corresponding to large LDL in Fr. 8 (or Fr. 7–10; Lp8), lipoproteins corresponding to very small LDL and large HDL in Fr. 12 to 16 (Lp12–16), and lipoproteins corresponding to small HDL in Fr. 18 to 20 (Lp18–20). The levels of cholesterol, TG, and PL in these specific fractions varied among the 9 patients (Figure 1). Serum apolipoprotein analyses of Fr. 7 to 10, Fr. 13 to 15, and Fr. 18 to 20 in 3 patients (1, 2, and 5) showed that Fr. 13 to 15 and Fr. 18 to 20 were rich in apolipoprotein A as normolipidemic control although varied among patients (Figure I in the online-only Data Supplement). Apolipoprotein Cs were also rich in Fr. 18 to 20 but not in Fr. 13 to 15. Apolipoprotein B was mostly distributed in Fr. 8 to 10 among the 3 fraction categories. Apolipoprotein E was abundant in all 3 fraction categories when compared with that in the control.

Abnormal Lipoproteins Are Present in FLD Regardless of Degree of Proteinuria

To study the relationship between lipoproteins and the degree of proteinuria in patients with FLD, lipoproteins between 2 sibling patients with FLD homozygous for the C337Y mutation in LCAT were compared (Figure 1, patients 1 and 3). Patient 1 had proteinuria in the nephrotic range (6 g/24 h), whereas patient 3 had only mild proteinuria (0.45 g/L).[23] All 4 abnormal lipoproteins were present in both patients (Figure 2A), although 3 lipoproteins (Lp1, Lp8, and Lp18-20) were lower in the younger patient.

Table 1. Clinical and Molecular Characteristics of Patients With Lecithin:Cholesterol Acyltransferase Deficiency

Patient	Sex	Age, Y	Race	Renal Failure/Proteinuria	Corneal Opacity	Anemia	CAD	Phenotype	AA Substitution	References
1	F	17	White (Morocco)	6 g/24 h	+	11.4 g/dL	–	FLD	C337Y	23
2	F	61	Japanese	2 g/24 h	+	9.5 g/dL	–	FLD	C98Y	24
3	F	12	White (Morocco)	0.45 g/L	+	9.2 g/dL	–	FLD	C337Y	23
4	F	63	Japanese	0.23 g/24 h	+	10.3 g/dL	–	FLD	G203R	25
5	M	68	Japanese	0.5 g/L	+	6.6 g/dL	–	FLD	G54S	26
6	M	38	Japanese	–	+	–	–	FED	T147I	10
7	M	58	White (Dutch)	–	+	–	–	FED	T147I	None
8	M	36	White (Dutch)	–	+	–	–	FED	W99S/T147I	27
9	F	30	White (Dutch)	–	+	–	–	FED	T147I/V333M	28

Patients 8 and 9 are compound heterozygotes; others are homozygotes for the indicated mutations. AA indicates amino acid; CAD, coronary artery disease; F, female; FED, fish-eye disease; FLD, familial lecithin:cholesterol acyltransferase deficiency; and M, male.

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Table 2. Lipid Profiles of Patients With Lecithin:Cholesterol Acyltransferase Deficiency

Patients	TC	TG	HDL-C	LDL-C	CE/TC
1	109	179	5.8	67	0
2	123	307	9.3	52	0.13
3	47	56	10.1	26	0
4	47	89	6.3	23	0.13
5	56	59	2.0	42	0
6	85	120	4.0	57	0.57
7	133	120	4.7	104	0.54
8	144	205	3.9	99	0.57
9	98	118	4.9	70	0.39

Values for LDL-C were calculated according to Friedewald et al.[29] CE/TC indicates cholesteryl ester/total cholesterol ratio; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; TC, total cholesterol; and TG, triglyceride.

Next, lipoprotein profiles of a patient with FLD with homozygous for the C98Y[24] mutation before and after a fat-restricted diet, which led to a reduction of proteinuria from 2.0 g/gCr to 0.6 g/gCr, were compared (Figure 1, patient 2). All 4 lipoproteins remained present after the diet although Lp1 and Lp8 were decreased to some extent (Figure 2B).

Lp8 and Lp12 to 16 Are Specific to FLD and Not to FED

Next, composition of the 4 Lps was analyzed (Figure II in the online-only Data Supplement). In all lipoproteins, cholesteryl ester (CE) was absent in FLD and low in Lp1, Lp12 to 16, and Lp18 to 20 in FED (panel A). PL in Lp8 was significantly lower in FLD when compared with that in FED (panel D). PL and FC were increased in Lp12 to 16 in FLD when compared with that in FED (panels B and D). FC, TG, and PL in both Lp1 and Lp18 to 20 did not differ between FLD and FED.

Lp8 Is a Large LDL, Rich in FC, PL, and TG, and Different From LpX

In comparison with unaffected controls and to patients with FED, CE in the LDL fractions of FLD sera was significantly decreased, whereas TG was increased (Figure 3A). In patients with both FLD and FED, FC, TG, and PL in Fr. 8 were significantly higher than in Fr. 9, whereas in controls, FC, TG, and PL in Fr. 8 were significantly lower than in Fr. 9 (Figure 3B). As a result, average sizes of Lp8 (Fr. 7–10) in FLD were significantly increased when compared with normal, whereas averaged particle size in FLD was lower than those in FED because of the severe deficiency of CE (Figure 3C). The composition of Lp8 in our patients with FLD is consistent with the previously reported FLD-LDL, and not consistent with the lipid characteristics of LpX.

Abnormal Lipid Compositions of FLD-Specific Lps Are Ameliorated by In Vitro Incubation With rLCAT

In vitro rLCAT incubation was performed followed by HPLC-GFC analyses (Figure III in the online-only Data Supplement). Incubation of patients' sera with rLCAT increased CE, TG, and PL in Fr. 16 to 18 in both FLD and FED (Figure IV in

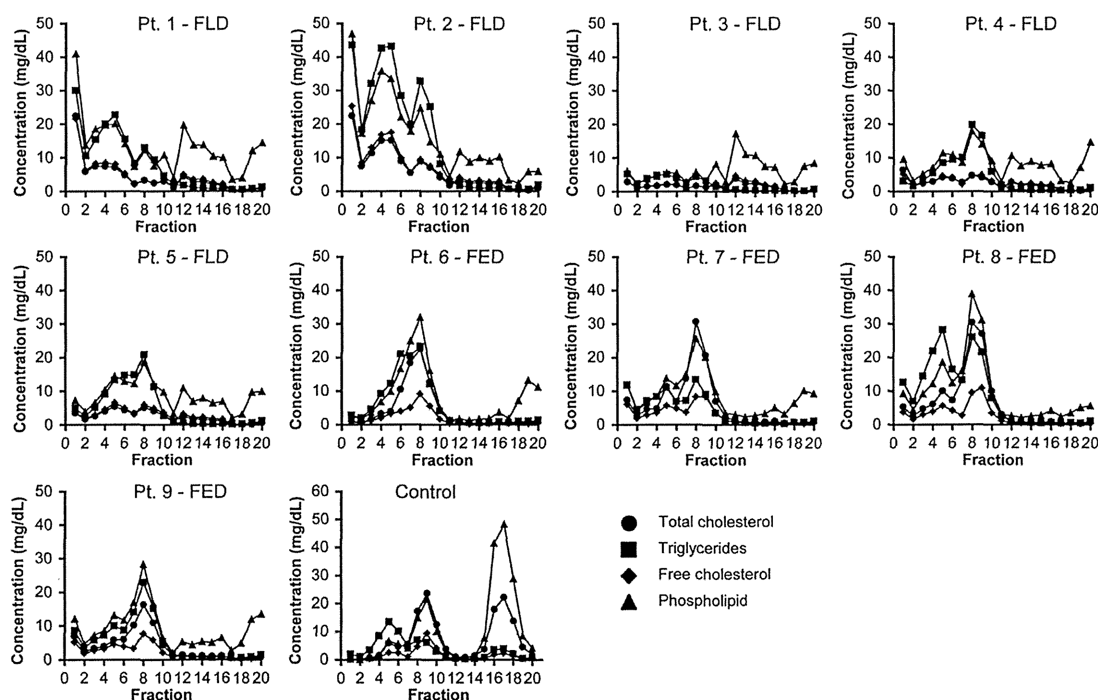

Figure 1. Lipoprotein profiles in patients with familial lecithin:cholesterol acyltransferase deficiency (FLD) by high performance liquid chromatography (HPLC) with gel filtration column (GFC). Sera from patients with 5 FLD (patients [Pts.] 1–5) and 4 Fish-eye disease (FED; Pts. 6–9) were subjected to lipoprotein size fractionation with concomitant determination of lipid concentrations in each fraction by high-performance liquid chromatography-GFC analyses. Representative result is shown for normolipidemic subjects. Concentrations of total cholesterol (●), triglyceride (■), free cholesterol (♦), and phospholipid (▲; *y* axis) in each fraction (*x* axis) are shown.

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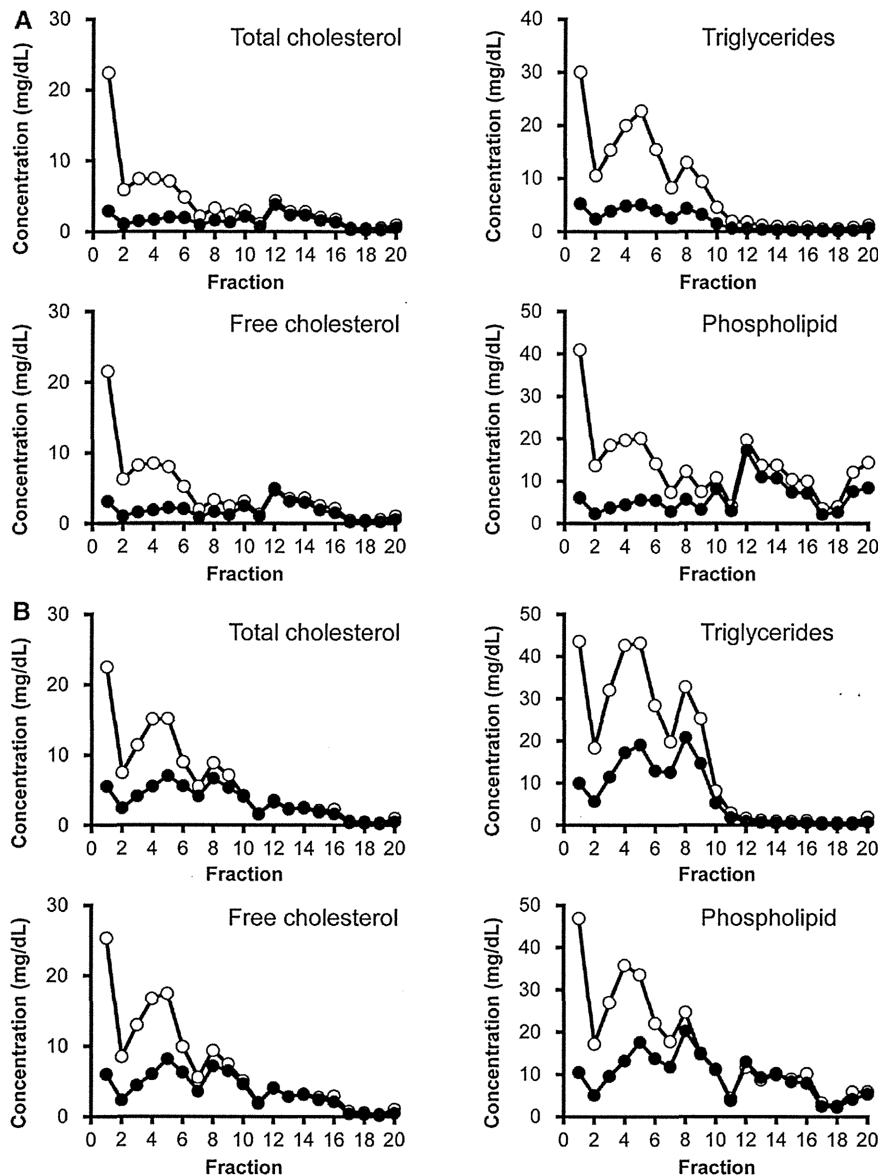

Figure 2. Differences in lipoproteins in patients with familial lecithin:cholesterol acyltransferase deficiency (FLD) with or without renal insufficiency. **A**, Lipoprotein profiles were compared between a patient with FLD with nephrotic range proteinuria (patient 1, ○) and patient 3 with mild proteinuria (●). **B**, Lipoprotein profiles were compared between before (○) and after (●) fat-restricted diet.

the online-only Data Supplement), indicating LCAT-mediated maturation of HDL. CE and PL contents of Lp8 were significantly increased and decreased, respectively, in FLD after incubation with rLCAT, whereas TG content was not significantly altered (Figure 4A and 4B). In FED, composition of Lp8 was not significantly altered by the treatment (Figure 4A and 4B). On incubation with rLCAT, Lp8 increased in size in FLD and it decreased in size in FED (Figure 4C). However, FC and PL in Lp12 to 16 decreased on incubation (Figure 4D).

Discussion

In this study, 4 lipoprotein fractions specific to LCAT-deficiency syndromes were identified by the HPLC-GFC analysis of samples from genetically diagnosed patients with different mutations and manifestations. Two of these had lipid compositions that were specific to FLD and thus may be involved in causing the renal damage that characterizes FLD. In vitro incubation with rLCAT corrected the abnormal fractions.

Lp1, one of the abnormal lipoproteins characteristic to LCAT-deficiency syndrome, was rich in TG and PL, and associated with the degree of proteinuria in 2 siblings with FLD, and was decreased on fat restriction in another patient with FLD (Figure 2). Indeed, abnormal lipoproteins with size of ≈100 nm corresponding to Lp1 have been identified in patients with LCAT deficiency with renal failure.[2,11,12,15] The lipid composition of Lp1 did not change on incubation with rLCAT (data not shown). Together, this suggests that Lp1 is most likely secondary to renal failure rather than directly caused by LCAT deficiency.

As opposed to controls, Fr. 8 was richer in total cholesterol, TG, FC, and PL than Fr. 9 in the patients with LCAT

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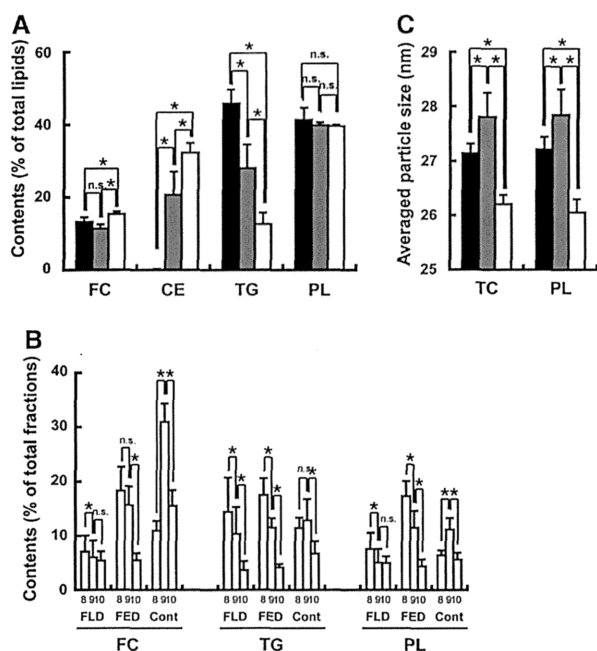

Figure 3. Characterization of lipid profiles in Lp8 of familial lecithin:cholesterol acyltransferase deficiency (FLD) and Fish-eye disease (FED). **A**, Lipid compositions of Fr. 7 to 10 fractions (Lp8) were compared among FLD (closed column), FED (gray column), and normal (open column). *$P<0.05$. **B**, Lipid concentrations of fractions 8, 9, and 10 were compared in FLD (n=5), FED (n=4), and controls (n=4). *$P<0.05$. Cholesteryl ester (CE) concentrations in FLD are not shown because levels were undetectable. **C**, Size distribution of lipoproteins in Lp8 (Fr. 7–10) was compared among FLD (closed column), FED (gray column), and normal (open column) based on total cholesterol (TC) and phospholipid (PL) concentrations. *$P<0.05$. FC indicates free cholesterol; and TG, triglyceride.

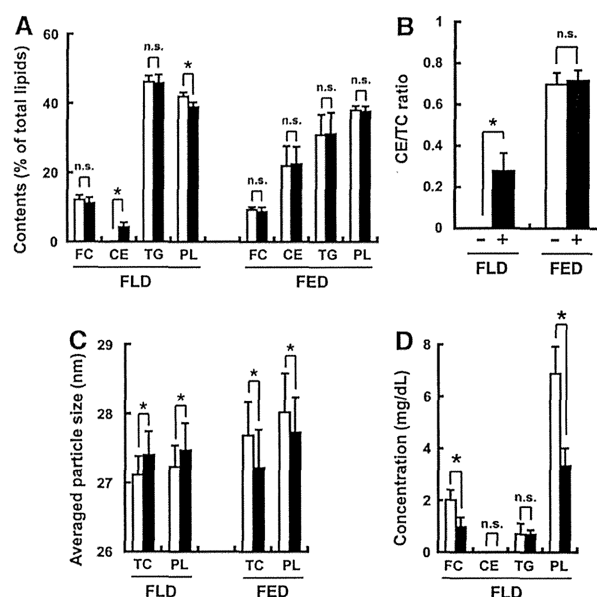

Figure 4. Effects of in vitro familial lecithin:cholesterol acyltransferase (LCAT) supplementation on the lipid profiles of abnormal lipoproteins in LCAT-deficiency syndrome. After analyses described in Figure II in the online-only Data Supplement, lipid composition (**A**), cholesteryl ester (CE)/TC ratio (**B**), averaged particle size based on total cholesterol (TC) and phospholipid (PL) concentrations (**C**), in Lp8, and lipid concentrations in Lp12 to 16 (**D**), were compared between culture media containing recombinant LCAT (rLCAT; closed column) and media without rLCAT (open column). *$P<0.05$. FC indicates free cholesterol; FED, Fish-eye disease; and TG, triglyceride.

deficiency (Figures 1 and 4B). Lp8 also differed in composition between FLD and FED: in FLD, it contained increased TG and decreased CE in comparison with FED (Figure 3A). Importantly, although the levels varied with the severity of renal damage as did those in Lp1, the buoyance of the peak at Fr. 8 did not vary with severity of renal damage (Figure 2), strongly suggesting that Lp8 directly results from a lack of LCAT and not from metabolic disturbances that occur during proteinuria and progressive renal failure.

In addition to the above-mentioned characteristics for Lp8 in LCAT-deficiency syndrome, HPLC-GFC analyses clarified novel unique lipid properties of Lp8 in FLD in comparison with that in FED; the averaged sizes of Lp8 are smaller in FLD than those in FED (Figure 3C). The lipid compositions of Lp8 in FLD were, in part, ameliorated by rLCAT incubation (Figure 4A). The averaged sizes of the Lp8 increased in FLD, whereas those in FED decreased (Figure 4C). rLCAT increased the CE formation in both LDL and HDL fractions in FLD sera. Thus, these findings indicated that the abnormal compositions were most likely caused primarily by the dysfunction of LCAT in the patients, and that the abnormal characteristics of Lp8 were not because of metabolic disturbances that occur during proteinuria and progressive loss of kidney function.

Previous extensive analyses using electron microscopy have identified 3 abnormal lipoproteins in the LDL fraction of FLD[12]: TG-rich and CE-poor particles of sizes similar to normal LDL (FLD-LDL); FC- and PL-containing particles of sizes distributing from 40 to 60 nm (LpX-like particle)[2]; particles with a diameter of 100 nm (designated as LM-LDL)[17,30] that were later reported to be identical to LpX.[15] LpX is FC- and PL-rich but TG-poor lipid particles (30%, 60%, and 2%, respectively)[22] without apolipoproteins, which range from very low density lipoprotein to large LDL fractions in fast performance liquid chromatography analysis.[31] The abnormal particles have been shown to be decreased by lipid-lowering therapy in a patient with FLD.[21] Lipoproteins in Lp8 were different from LpX in the lipid contents; the fractions were rich in FC and PL and also rich in TG (13.2±1.3%, 41.4±3.3%, and 45.8±3.8%, respectively). The composition analyses suggested that Lp8 corresponds to FLD-LDL, but the calculated sizes of Lp8 were larger than normal LDL using the data obtained by size fractionation with HPLC-GPC in the present study. Thus, the identified Lp8 in LCAT-deficiency syndrome was most likely not identical to LpX in the characteristics.

There is a limitation for the interpretation of the quantitative measurement of LpX in the frozen samples collected in our study because the abnormal lipoproteins were known to be labile to freezing-and-thawing treatment. In this context, fresh sera were collected from patients 2 and 4 and analyzed by agarose gel electrophoresis. The lipid staining of lipoproteins electrophoresed in agarose gel detected the abnormally slowly migrating TG-poor lipoproteins, LpX, at the expectedly migrating position, as well as TG-rich abnormal β-lipoproteins (LDL) in the once-frozen sample, as well as the fresh sample

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in patient 4, although the staining intensity tended to decrease in comparison with the fresh counterpart. However, LpX was not detected in either sample with or without freeze-and-thaw treatment from patient 2. Thus, LpX was indeed labile to freeze/thawing, and the frozen samples were not adequate for the quantitative measurement. However, the presence was still able to be evaluated after once-freezing treatment. On the basis of background data, HPLC-GFC analysis showed that lipid contents in Lp8 were not largely affected by once-freezing treatment in both patients 2 and 4: in contrast, the contents of TG and PL were slightly decreased in lipoproteins with peak of Fr. 5 (data not shown). Additional studies using fresh samples of patients with distinct mutations and manifestations are needed to interpret the significance of novel lipoproteins in comparison with LpX for the development of renal insufficiency in LCAT0deficiency syndrome quantitatively.

In FLD but not in FED, Lp12 to 16 were heterogeneous in size and rich in PL. rLCAT decreased PL in these fractions specifically (Figure 5D; Figure II in the online-only Data Supplement). This may suggest that the heterogeneous-sized PL-rich particles in Fr. 12 to 16 converge to normal-sized HDL (Fr. 16–18) on incubation with rLCAT, with concomitant esterification of FC.

In conclusion, 4 lipoprotein fractions specific to LCAT-deficiency syndromes were identified by the HPLC-GFC analysis of samples from genetically diagnosed patients with different mutations and manifestations. The composition of 2 of these was unique to only FLD; these were not likely compatible with the previously reported LpX. These abnormal lipoproteins may be causal to the renal pathology in FLD, the main cause of increased morbidity and mortality in this condition. The regular evaluation of these specific lipid fractions during LCAT enzyme replacement therapy in patients with LCAT deficiency may provide guidance for success of the intervention. The value of these lipid fractions for risk of future renal disease needs to be addressed in prospective follow-up studies in patients with FLD with various mutations in the *LCAT* gene before the onset of proteinuria.

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Disclosures

None.

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Significance

Lecithin:cholesterol acyltransferase-deficiency syndromes are classified into 2 forms: familial lecithin:cholesterol acyltransferase deficiency and fish-eye disease. Patients with familial lecithin:cholesterol acyltransferase deficiency develop renal failure, whereas fish-eye disease patients do not. This study was performed to identify abnormal lipoproteins associated with the renal damage of patients with different mutations and manifestations. Size fractionation with gel filtration of patients' sera and in vitro incubation experiments with recombinant lecithin:cholesterol acyltransferase showed abnormal lipoproteins associated with the renal damage. Thus, our novel analytic approach identified large low-density lipoprotein and high-density lipoprotein with a composition specific to familial lecithin:cholesterol acyltransferase deficiency but not to fish-eye disease. The identification of abnormal lipoproteins may shed light on the clarification of renal pathology and the development of treatment for the patients with familial lecithin:cholesterol acyltransferase deficiency.

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Materials and methods

Identification of FLD and FED patients

Patients were referred because of a clinical suspicion of LCAT deficiency due to the presence of corneal opacification and HDL deficiency, with or without proteinuria and/or renal insufficiency (Table 1). Lipid profiles of the patients are also given in Table 2. In our clinics, the definitive molecular diagnoses were established for all patients. The renal biopsy analyses were performed to make a diagnosis for renal damage in some patients. Patient(Pt)s 1 and 3 are Morroccan sister[24]. Other patients are unrelated. Pt.2 was treated with a fat-restricted diet (1570 kcal; fat 10 g and protein 45 g) during admission, with the prescription of losartan 50 mg/day for 8 months. Younger sister of Pt. 2 also shows corneal opacification. Informed consent from her was not obtained for genetic analysis and current study. Proteinuria for FLD patients were 6g/24h (Pt. 1), 2 g/24h (Pt. 2), 0.45 g/l (Pt. 3), 0.23g/24h (Pt. 4), and 0.5 g/l (Pt. 5).

Analysis of patient samples

This study was approved by the Ethics Committees of Chiba University School of Medicine and Academic Medical Center, University of Amsterdam, and informed consent was obtained from the participants including unaffected normolipidemic controls. Blood samples were obtained from participants, and serum was prepared and stored at -80 °C until use. LCAT activity (α-activity) was measured using artificial proteoliposomes as substrate[1]. Serum lipoproteins were fractionated by high-performance liquid chromatography with gel filtration column (HPLC-GFC)[2,3] and analyzed simultaneously by online enzymatic method to quantify total cholesterol (TC), free cholesterol (FC), triglyceride (TG), and phospholipid (PL) (Skylight Biotech, Akita, Japan). The resulting raw chromatograms (elution time versus lipid concentration) were further processed by computer program with the modified Gaussian curve fitting for resolving the overlapping peaks by mathematical treatment. Finally, the system subdivided the lipoprotein particles of normal subjects by size into the following 20 subclasses: chylomicron (CM, >80 nm, fractions 1-2), very low density lipoprotein (VLDL, 30-80 nm, fractions 3-7), low density lipoprotein (LDL, 16-30 nm, fractions 8-13), and high density lipoprotein (HDL, 8-16 nm, fractions 14-20). In this fractionation analysis, standard particle diameters have been reported to be >90, 75, 64, 53.6, 44.5, 36.8, 31.3, 28.6, 25.5, 23.0, 20.7, 18.6, 16.7, 15.0, 13.5, 12.1, 10.9, 9.8, 8.8, 7.6 nm for fraction 1 through 20, respectively[2]. Average sizes of lipoprotein

classes were calculated based on the particle diameter as follows: (sum of particle diameter x lipid concentration in each fraction)/(sum of lipid concentration of the fractions of interest). Ultracentrifugation fractionation followed by determination of lipid contents was performed in Pt. 1, 2 and 5 (Table I in data supplement). For some FLD sera, representative fractions were collected, concentrated by Vivaspin (MWCO=3,000, Sartorius, Weender Landstr., Germany) and subjected to determine apolipoprotein concentrations by Milliplex MAP Human Apolipoprotein Panel kit (Millipore, Billerica, MA) using BioPlex apparatus (BioRad, Hercules, CA).

Preparation of recombinant LCAT and *in vitro* incubation with patient's serum

Human *LCAT* gene was transduced into human preadipocytes by retroviral vector as described previously[1]. The resulting cells were seeded into T225 flask and grown to confluency in MesenPRO medium (Life Technologies, Carlsbad, CA). The medium was changed to 30 ml of OPTI MEM I (Life Technologies) 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)[4]. LCAT concentration was titrated by immunoblotting using commercially available human rLCAT (Roar Biomedical, Inc., Calverton, NY) as standard. After mixing with rLCAT-containing culture supernatant at the ratio of 29:71 (v/v), each patient's serum was incubated at 37 °C for 24 hr (final concentration of rLCAT was 6 μg/ml), and subjected to lipoprotein analysis. Culture supernatant of human preadipocytes without gene transduction was used as control.

Statistical analysis

Data are presented as means ± S.D. Comparisons were assessed for significant differences by paired Student's *t*-test, or by ANOVA followed by Tukey test, where appropriate. SPSS software was used for statistical analyses. In all cases, P values of less than 0.05 were considered significant.

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