厚生労働科学研究費補助金（難治性疾患等克服研究事業）
家族性LCAT欠損症患者に対する細胞加工医薬品
「LCAT 遺伝子導入ヒト前脂肪細胞」の早期実用化にむけた非臨床試験
平成２４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。そこで、治験実施計画書作成のため、先行する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した。

A. 研究目的

治験実施計画書作成のため、先行する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４項目につき検討を行った。

(1)プロジェクト管理

(2)安全性評価

(3)モニタリング

(4)DM

（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。実施される試験については臨床研究に関する倫理指針に基づいて実施する必要がある。

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の手法は欠かせない。そこで、プロジェクトマネジメントに関する手順書に従い先行研究を実施することとした。

(2)安全性評価

先行研究では安全性が未確立のため、安全性情報の収集、報告・評価の対応が試験の要といえる。医師主導治験においては安全性情報の収集に関する手順書があり、先行研究もその手順書に準じて実施することとした。

(3)モニタリング

遺伝子治療臨床研究に関する倫理指針ではモニタリングに関する特に記載はないが、正しいデータ取得等の品質管理のためにはモニタリングが重要である。医師主導治験においてはモニタリングに関する手順書があり、先行研究も中央モニタリングだけでなく、当該手順書に準じ出張モニタリング等も検討することとした。

(4)DM

CRF について、電子的又は書面による取得方法がある。医師主導治験においては、①データマネジメント業務に関する手順書、②データマネジメント業務に関する手順書、③アカウント管理および eDC トレーニングに関する手順書、④DM 計画書及び DM 報告書の作成に関する手順書（以上 EDC）、⑤データマネジ

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G.　知的財産権の出願・登録状況

該当無し

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
黒田正幸、武城英明	家族性LCAT欠損症	遠藤文夫他	先天代謝異常ハンドブック	中山書店	東京	2013年	398-399

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
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Fukaya Y, Kuroda M, Aoyagi Y, Asada S, Kubota Y, Okamoto Y, Nakayama T, Saito Y, Satoh K, Bujo H.	Platelet-rich plasma inhibits the apoptosis of highly adipogenic homogeneous preadipocytes in an in vitro culture system.	Exp Mol Med.	44(5)	330-339	2012
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Ⅳ. 研究成果の刊行物・別冊

Clinical Research

Amelioration of circulating lipoprotein profile and proteinuria in a patient with LCAT deficiency due to a novel mutation (Cys74Tyr) in the lid region of LCAT under a fat-restricted diet and ARB treatment

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Running title: A patient with a novel mutation in the lid region of LCAT.

Abstract

Familial lecithin-cholesterol acyltransferase (LCAT) deficiency is a hereditary disease characterized by an abnormal lipid profile, corneal opacity, anemia and progressive renal disease. We report a patient with complete loss of LCAT activity due to a novel *lcat* gene mutation of Cys74Tyr in the lid region of LCAT protein. Esterification of cholesterol in this patient was disturbed by disruption of a substrate binding loop of Cys50-Cys74 in LCAT protein. She had progressive renal dysfunction, proteinuria, corneal opacity, anemia and an abnormal lipid profile. Her serum lipids showed a significant increase in abnormal lipoproteins at the original position in agarose gel electrophoresis and VLDL-cholesterol, and a severe decrease in serum HDL-cholesterol. Lipoprotein analyses also revealed the presence of an abnormal

midband lipoprotein, and a maturation disturbance of HDL particles. Renal function and proteinuria improved following the adoption of a fat-restricted diet and administration of an angiotensin II receptor blocker. The abnormal lipoproteins also decreased after this treatment.

(Abstract: 156 Words)

Introduction

Lecithin-cholesterol acyltransferase (LCAT) deficiency is an uncommon autosomal recessive disorder which results from a gene mutation of LCAT. Since the first identification of LCAT as a unique plasma enzyme (Glomset et. al., 1962), 86 mutations in the LCAT gene have been described. Patients with LCAT deficiency show an abnormal circulating lipoprotein profile as a result of the disturbed esterification of free cholesterol incorporated into high-density lipoproteins. Increased plasma concentrations of unesterified cholesterol, triglyceride (TG) and phosphatidylcholine result in lipid deposition in the tissue. LCAT deficiency develops as two clinically distinct syndromes, familial LCAT deficiency (FLD) and fish eye disease (FED). Patients with FLD show corneal opacities, hemolytic anemia, and progressive renal disease (Santamarina-Fojo et al., 2001). Renal disease occurs as a result of the loss of enzyme activity against β-lipoproteins rather than against α-lipoproteins. Meanwhile, FED patients develop corneal opacities as a result of a partial deficiency in LCAT activity.

A number of approaches to the treatment of LCAT deficiency have been proposed. LCAT replacement therapy by plasma transfusion produced a marked improvement in the deranged composition of TG-rich lipoproteins and Apo-E concentrations (Norum et. al., 1968, Maruyama et. al., 1984). Recent advances in gene therapy have allowed the transplantation of *ex vivo* lcat gene-transduced adipocytes and subsequent production of human LCAT protein in circulating plasma (Kuroda et al., 2011 [5],[6]). Further, a clinical trial of synthetic LCAT in patients with coronary arterial disease is also currently underway at NIH (NCT01554800). In contrast, a fat-restriction diet improves the hypertriglyceridemia in these patients by reducing TG-rich lipoproteins. The lipid-lowering drugs nicotinic acid and finofibrate have been shown to ameliorate renal function and proteinuria (Yee et al., 2009), and corticosteroids and renin-angiotensin-aldosteron (RAA) system blockers such as ACE inhibitors and angiotensin II receptor blockers (ARB) also decreased proteinuria (Aranda et al., 2008, Miarka et al., 2011, Holleboom et. al.2011).

Here, we report a novel LCAT gene mutation in a patient which resulted in

disruption of the disulfide bridges essential to the enzyme lid region. This patient had a complete deficiency in LCAT activity and renal insufficiency. We also investigated the effects of a fat-restriction diet and administration of an ARB on plasma lipoprotein profiles and proteinuria in this patient.

Materials and Methods

Biochemical and genetic analysis

Biochemical and urine samples were analyzed by enzymatic methods using a chemical autoanalyzer (Hitachi Co., Tokyo, Japan). Esterified cholesterol concentrations were calculated as the difference between total and free cholesterol. LDL-cholesterol was determined a Determiner-L LDL-C (Kyowa Medex, Tokyo, Japan). LCAT activity in serum was measured using a colorimetric method for analyzing cholesterol esterification rate (CER) with synthetic dipalmitoyl lecithin sol (Nagasaki et al. 1976). Alpha-LCAT activity was also measured using Anasolb LCAT® (Sekisui Medical, Tokyo, Japan). Genomic DNA was purified from plasma with a QIAamp DNA kit (QIAGEN, Hilden, Germany). Genomic fragments were amplified by PCR, followed by agarose gel purification and direct sequencing. The entire sequence of the *lcat* gene locus thereby obtained was compared with a reference sequence (NM_000229) to identify nucleotide substitution. The study was approved by the Ethics Committee of Chiba University School of Medicine, and informed consent was obtained from the patient. Both parents were deceased, and informed consent for genetic analysis was not obtained from her younger sister, who shows corneal opacity.

Lipoprotein analysis

The serum lipoprotein profile was determined by polyacrylamide gel disc electrophoresis (Narayan et al., 1965). Two-dimensional electrophoresis was performed as described previously (Asada et al., 2011). Lipoproteins were also evaluated by agarose gel electrophoresis using the rapid electrophoresis system (Winkler et al., 1995, Contois et al., 1999, Kido et al., 2001, Zhang et al., 2004). After electrophoresis, cholesterol and triglycerides in the plates were separately stained and analyzed with a Cho/Trig COMBO kit according to the manufacturer's instructions (Helena Laboratories, Saitama, Japan). Using the serum total cholesterol and TG concentrations in the blood samples, concentrations of cholesterol and TG in each fraction were determined using the detected ratio of cholesterol and TG after automatic densitometric analysis.

Results

Patient

A 61-year-old Japanese woman was transferred to the Department of Nephrology, Kitasato University Hospital. She complained of dyspnea on walking for the preceding five months, and eyelid and pretibial edema for one month. Family history showed her married parents were cousins, and that her younger sister had corneal opacity and mental retardation. She had anemia (Hemoglobin 9.5 g/dl) bilateral corneal opacities (Fig 1A) and pitting edema. Urinalysis revealed proteinuria at 2 g/day, 1+ microscopic hematuria, and an N-acetyl-ß-D-glucosaminidase (NAG) level of 29.1 unit/L (normal range: 1-4.2). Blood chemistry showed total protein 6.4 g/dL, albumin 3.4 g/dL, total cholesterol 235 mg/dL, TG 235 mg/dL, HDL-cholesterol 22 mg/dL, LDL-cholesterol 39 mg/dL, urea nitrogen 40 mg/dL, creatinine 1.83 mg/dL, and uric acid 8.2 mg/dL. CER of normal sera without heat inactivation was 96.3 ± 10.4 nmol/ml/hr (n=3), indicating normal LCAT activity. In contrast, CER of patient sera without heat-inactivation was 16.3 nmol/ml/hr, which was below the CER of heat-inactivated normal sera (21.3 ± 1.8 nmol/ml/hr, n=3), indicating the total loss of LCAT activities in the patients. The 84 units of alpha-LCAT activity in patient serum measured by Anasorb LCAT® was also markedly low as compared with a standard level of 382 – 512 units. No abnormalities on chest X-ray, electrocardiography or echography findings were detected in either kidney. The renal biopsy specimen showed glomerular mesangial expansion, and foam cell infiltrates into glomerular tufts and mesangium. PAM staining revealed irregular thickening, double contouring, and vacuolation of the glomerular basement membrane (GBM) (Fig. 1B). Electron microscopic findings revealed numerous small vacuoles and granular structures within the vacuoles in the GBM and mesangial matrix (Fig. 1C). Immunofluorescence revealed negative staining for immunoglobulins of IgG, IgA and IgM, and for complement components of C1q, C4 and C3. These findings are consistent with the findings of LCAT deficiency.

Gene analysis

Direct sequencing of the *lcat* gene and comparison with a reference sequence (NM_000229) showed that the proband had a novel homozygous G to A nucleotide substitution in exon 2 resulting in Cys74Tyr [c.293 G>A (p.Cys74Tyr)]. The amino acid substitution was a novel mutation in the lid region of the LCAT protein. The substituted cysteine was one of four cysteine amino acid residues which formed the disulfide bonds in construction of the the enzyme lid structure (Yang et al., 1987).

Lipid and lipoprotein profiles

Serum TG and free cholesterol values were higher than normal, while cholesterol ester, LDL-cholesterol and HDL-cholesterol values appeared lower (Table 1). Densitometric analysis for lipid staining of lipoproteins on disc polyacrylamide gel electrophoresis of serum showed a significant decrease in α and preβ-β positions, and a tiny abnormal "midband" localized on preβ-β position (Fig. 2A). Two-dimensional gel electrophoresis followed by immunodetection for apoprotein showed that distribution of apoprotein was shifted to the smaller HDL particles, indicating that the maturation of HDL particles was impaired (Fig.2B).. The production of LDL particles was thus severely disturbed, as evidenced by the presence of an abnormal "midband" lipoprotein and the disturbed maturation of HDL particles resulting in a severe decrease in HDL lipoprotein.

Cholesterol and TG staining for lipoproteins which migrate to the α-position on agarose gel electrophoresis could not be seen in the serum at admission (Fig. 3A). Lipoproteins which migrate to the preβ-position were also decreased on both cholesterol and TG staining at admission (Fig. 3A), whereas lipoproteins which migrate to the β-position showed broad bands in both cholesterol and TG staining. The amount of cholesterol and TG in each lipoprotein fraction of serum at admission is shown in Table 2. Apolipoproteins AI and AII, which are predominantly contained in HDL-lipoprotein, were decreased at admission (Table 2), whereas apolipoprotein CII and E were increased (Table 2).

Effects of a fat-restriction diet on LCAT deficiency-induced lipid profile and kidney disease.

The patient was prescribed a fat-restricted diet consisting of meals containing 10 g of fat, 45 g of protein and 1570 kcal of energy during admission, and treatment with losartan, an ARB, was started by single daily administration at 50 mg from 14 days after admission. This treatment was continued following discharge, and follow-up at 8 months showed that compliance with both the diet and medication was good. Her body weight decreased from 64 to 52 kg at 6 month after the start of the fat-restricted diet, and was thereafter maintained. At one year of treatment, her proteinuria had decreased from 2.04 g/g·creatinine(Cr) to 0.62 g/g·Cr, and her serum Cr level had decreased from 1.83 mg/dl to 1.10 mg/dl.

Changes in lipid and apolipoprotein fractions in sera samples collected at admission and 8 months of treatment are shown in Tables 1 and 2, respectively. Surprisingly, adoption of the fat-restriction diet resulted in a decrease in cholesterol,

while TG contents and abnormal lipoproteins migrated to their original position (Table 2, Figure 3 A&B). The cholesterol content of lipoprotein at the β-position was also substantially decreased (Table 2, Figure 3 A&B). These data showed that the decrease in abnormal lipoproteins at the original position and change in lipid content in β-lipoproteins were the result of the fat-restriction diet and administration of an ARB.

Discussion

In this study, we report a patient with LCAT deficiency who experienced progressive renal dysfunction, proteinuria, anemia and corneal opacity. Her renal biopsy specimen showed many foam cell infiltrates into glomerular capillary tufts and mesangium, and numerous clear vacuoles containing granular structures within the GBM and mesangial matrix. Glomerular foam cells infiltrates are a characteristic feature of LCAT deficiency (Chevet et al., 1978, Gjone et al., 1973, Hovig et al., 1973). The numerous clear vacuoles within the GBM and mesangial matrix are also consistent with the findings of a previous study of LCAT deficiency (Magil et al., 1982). However, the findings in our patient were not consistent with the structures of odd-shaped electron-dense materials with a membranous profile within clear vacuoles described in that study (Magil et al., 1982). Differences in structure in clear vacuoles may be related to patient age or lipid composition in the vacuoles. It is conceivable that the glomerular lipid deposits are fully or partially composed of LpX, a cationized lipoprotein (Magil et al., 1982). The glomerular charge barrier is composed of negatively-changed proteins and may be influenced by deposited cationized lipoproteins, resulting in the exacerbation of proteinuria. Indeed, agarose gel electrophoresis of serum from our patient at admission revealed a substantial amount of abnormal cationized lipoproteins suggestive of LpX at the original position (Figure 3B-1). Proteinuria in this patient might have been induced by the deposition of this abnormal cationized lipoprotein into glomeruli.

Our patient had a novel mutation of Cys74Tyr in the lid region of LCAT protein. LCAT protein contains two functional disulfide bridges, Cys50-Cys74 and Cys313-Cys356 (Yang et al., 1987). It appears likely that the Cys50-Cys74 bond was disrupted in our patient. In previous study, disruption of the Cys313-Cys356 bond by an amino acid substitution was shown to result in LCAT deficiency and early onset renal disease (Holleboom et al., 2011). The former loop region in the LCAT protein, consisting of Tyr51-Asp73, has binding capacity for HDL- and LDL-cholesterol (Jonas, 2000), and truncation of Lys53-Gly71 or Asp56-Leu68 from LCAT protein abolished the ability of LCAT protein to bind HDL- and LDL-cholesterol *in vitro* (Jin et al., 1999,

Peelman et al., 1999). Our patient also showed a complete loss of LCAT activity, indicating that the former loop region spanned by Cys50-Cys74 is essential for substrate recognition of LCAT in the esterification process (Adimoolam et al., 1997). Further *in vitro* and *in vivo* investigation of the effect of partial transformation of the Tyr51-Asp73 loop region on the cholesterol esterification process may contribute to our understanding of the biochemistry of enzyme-lipid interactions as well as the pathophysiology of LCAT deficiency.

Although sequential ultracentrifugation is a standard method in lipoprotein analysis, we applied agarose gel electrophoresis to detect abnormal LpX and LDL-like lipoproteins and for visualization of lipoproteins. Our patient had a significant increase in abnormal lipoproteins at the original position and a severe decrease in HDL-lipoprotein. Her serum also showed an abnormal midband lipoprotein and a disturbance in the maturation of HDL particles. We suggest that these lipoprotein abnormalities were due to the disturbance in esterification resulting from the loss of LCAT activity.

We also evaluated the effect of a fat-restriction diet and administration of an ARB on lipid profile, proteinuria and renal function in this patient. This treatment decreased proteinuria and resulted in a delay in the deterioration of renal function. A fat restriction diet obviously decreased her serum total-cholesterol and TG, except HDL-cholesterol and LDL-cholesterol at 8 months of treatment. The abnormal cationized lipoproteins at original positions, suggestive of LpX, disappeared after treatment. This disappearance of abnormal cationized lipoproteins in her serum after treatment may have induced a decreased in the amount of deposited cationized lipoproteins within the GBM, thereby resulting in restoration of the charge barrier in the GBM and decrease in proteinuria. The lipid content of lipoproteins at the β-position and the cholesterol content of the midband lipoprotein decreased also after treatment. Lipoproteins accumulating in the kidney are thought to be abnormal apoprotein E-rich lipoproteins which have migrated from the β-position (Gröne et al., 1994), suggesting that the decrease in serum apoprotein E after treatment may be associated with the decrease in accumulated lipoproteins in the kidney. A fat-restriction diet in combination with ARB treatment may contribute to decrease in proteinuria and result in a delay in the deterioration of renal function in patients with LCAT deficiency.

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Figure legends.

Figure 1. Ocular and renal pathology

(A) Corneal opacity in the patient.

(B)-1) PAS staining of a renal biopsy specimen. 400X. An increase in cell number and matrix expansion are seen in the mesangial area of the glomerulus. Foam cell infiltration into capillary loops and the mesangial area are seen in the glomerulus. (B)-2) PAM staining shows irregular thickening and double contours in the glomerular basement membrane (GBM). Foam cells within granular structures are present in the capillary lumen.

(C) Electron micrograph of the glomerulus shows the presence of clear vacuoles containing granular structures in the mesangium (↓) and within the GBM (▼). 3000X. Bar=2μm.

Figure 2. Densitometoric analysis of lipoproteins on disc polyacrylamaide gel electrophoresis in the patient and a healthy control.

(A) Serum at admission shows an increase in original position lipoproteins, a decrease in α-position lipoproteins, and the appearance of midband lipoproteins instead of β-position lipoproteins.

(B) Two-dimensional disk electrophoresis consisting of charge separation for the first dimension and molecular weight separation for the second dimension followed by immunostaining for apolipoprotein. Apolipoprotein distribution was shifted towards the smaller HDL particles, indicating that the maturation of HDL particles in this patient was impaired.

Figure 3. Staining patterns for cholesterol and triglycerides in lipoproteins on agarose gel electrophoresis.

(A) In cholesterol staining, the lipoproteins at original position increased, while the lipoproteins at α- and β-position decreased in the patient at admission compared with those of a healthy control. After 8 months on a fat-restriction diet and administration of losartan 50 mg, a decrease in original position lipoproteins is seen. In triglyceride staining, the serum sample at admission shows a decrease in preβ-position lipoproteins and increase in β-position lipoproteins compared with levels of a healthy control.

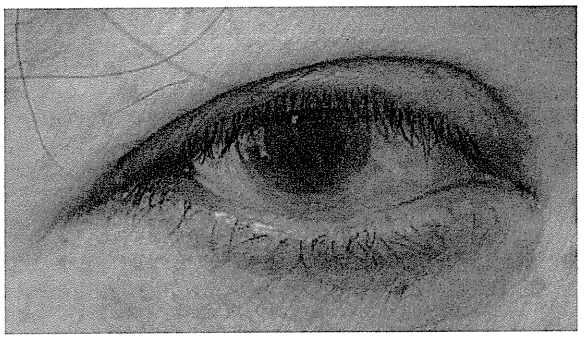
(B) Densitometric analyses for staining pattern.
Cholesterol staining is shown as a red area, and triglycerides staining as a blue area. (B)-1) Staining patterns for lipoproteins in the patient's serum at admission. (B)-2)

Staining patterns for lipoproteins in the patient's serum at 8 months after a fat-restriction diet and administration of losartan 50 mg. Original and β-position lipoproteins stained for cholesterol at admission have decreased after treatment for 8 months.

(abstract- figure legends: 3728 Words)

Figure 1. Naito et al.

(A)



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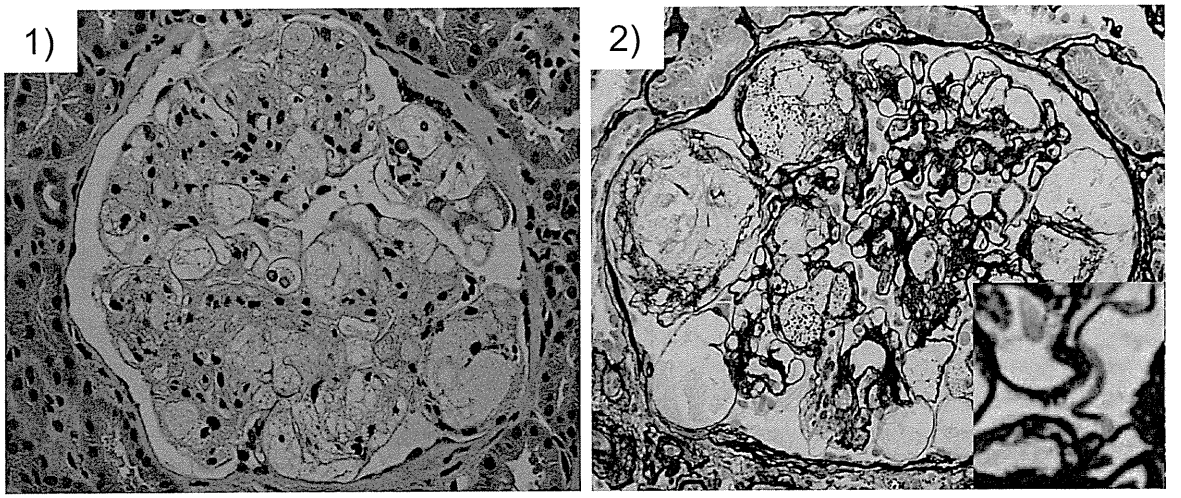

(C)