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分担研究報告書

原発性高脂血症に関する調査研究

高トリグリセリド血症の多様性に関する研究

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研究要旨

高トリグリセリド血症の成因について、特にカイロミクロン血症の場合にはリポタンパクリパーゼ（LPL）の異常が頻度として高いが、この背景にはLPLの蛋白量や活性測定が確立していることがある。カイロミクロン血症の発現時期も関与する遺伝子異常により異なることがあり、治療方法・効果や予後を観察するうえで、成因分類は重要と考えられる。LPL に異常が認められない場合は、候補遺伝子アプローチの蓄積だけでは成因診断に至らないこともあるので、成因解明に向けてのブレイクスルーが期待される。

A. 研究目的

高トリグリセリド（TG）血症を呈する、I・V型高脂血症では、アポB含有リポタンパクの水解過程の障害がその原因として第一にあげられる。その中でもリポタンパクリパーゼ（LPL）欠損症やアポC-II欠損症は代表的な疾患で、遺伝子異常とともにその機能が明らかにされてきた。しかしながら、カイロミクロン血症をきたす原因として、LPLやアポC-IIに変異が同定されないことも少なからずある。近年になって、アポA-V、ANGPLT4/3、GPIHBP-1、LMF1などがアポB含有リポタンパクの水解過程に重要な役割を担っていることが、ヒトの変異・欠損症や欠損動物モデルから明らかにされている。本研究では、候補遺伝子の異常の有無を緻密に解析することにより、高TG血症の成因診断を行うことを目的とする。遺伝子診断に基づいた治療効果や予後を調査し、比較検討することにより治療ガイドライン整備の基礎データの構築を目指す。

B. 研究方法

1）本研究は、自治医科大学と東京大学による共同研究である。

2）対象者：各施設を受診された脂質異常症症例を対象とし、TG 値が 500 mg/dL 以上、リポタンパク電気泳動でカイロミクロンを認める脂質異常症の症例。

3）生化学検査：ヘパリン 30 単位/kg・体重を静注し 15 分後に採取した血漿を用い、LPL mass および活性を測定した。また、血清アポC-II およびA-V を測定した。

4）遺伝子解析：LPL mass あるいは活性、

アポ C-II、A-V の低値を示す症例に関して、末梢白血球より抽出した DNA を用いて、それぞれの遺伝子のシークエンシングを行い、変異の有無について調べた。

C. 研究結果

今回、これまでにサンプルを収集できた38名の症例についてまずは生化学的検査を行った。図1－3に TG と LPL mass、活性、アポ A-V の分布を示す。

1）Case 1

この症例では、LPL mass は 118 ng/mL、LPL 活性は 0 μmol FFA/h/mL であった。LPL 蛋白が検出できるので酵素活性に障害を起こす変異が期待された。DNA シークエンスの結果、ASP204GLU の既知の変異のホモ型であった（Gotoda T et al. J Clin Invest 88:1856, 1991）。

2）Case 2

この症例では、LPL mass は 27 ng/mL、LPL 活性は 0 μmol FFA/h/mL であった。DNA シークエンスの結果、ASN43SER の既知の変異のホモ型であった（Kobayashi J et al. Biochem Biophys Res Commun 205:506, 1994）。

3）Case 3

この症例では、LPL mass は 52 ng/mL であった。LPL 活性は未測定である。DNA シークエンスの結果、既知の変異である LPL-ARITA（916delG）と SER447TER の複合ヘテロ型であった（Takagi A et al. J Clin Invest 89:581, 1992, Kobayashi J et al. Biochem Biophys Res Commun 182: 70, 1992）。

4）Case 4

この症例は、血清 TG 値が 28,500 mg/dL を示した新生児であった。LPL mass は 132 ng/mL、アポ C-II は 10.4 mg/dL であり、LPL 欠損症やアポ C-II 欠損症ではなかった。だたし、アポ A-V が図3に示すように、TG 値に比して圧倒的に低値であった。このためアポ A-V 欠損症が疑われた。DNA シークエンスの結果、VAL153MET と THR184SER を認めた。症例の父親は THR184SER を有し、母親は VAL153MET を有していたが、高 TG 血症は認めなかった。

5）Case 5

この症例は図1－3には示されていないが、30代半ばで高脂血症を指摘され、精査でアポ C-II が測定感度以下のため、アポ C-II 欠損症が疑われた。しかし、高感度のイムノブロットでは正常と同じ分子量の蛋白が症例からも検出された。症例のアポ C-II 濃度は 0.6 mg/dL と計算された。DNA シークエンスを行ったが、エクソン及びその境界、5'-UTR、3'-UTR には変異が認められず、サザンブロットでも大きな欠失は認められなかった。

D. 考察

カイロミクロン血症を呈した5症例について遺伝子解析を行った。LPL 欠損症と診断しえたのは3例であった。いずれも LPL 活性は極めて低値であったことが手がかりとなっている。見いだされた変異は全て既報のものであった。204 残基の ASP は種間で保存されるアミノ酸で酵素活性に必須とされている。43 残基の ASN は糖鎖修飾されるもので、LPL の成熟に重要であり、その変異により分泌障害が起こるとされている。エクソン5の 916 番目のヌクレオチドの G が欠損することでフレームシフトによる早期停止が起こる LPL-ARITA では mRNA は検出

されないので、蛋白も当然検出されない。Case 3 は複合ヘテロ型なので、SER447TER の変異型の LPL が検出されている。

アポ A-V の VAL153MET についてはレムナントの量には影響を及ぼさないことが報告されているので (Arterioscler Thromb Vasc Biol 26:1236, 2006)、THR184SER が原因との可能性はあるが、父親も同じ遺伝子型にもかかわらず、TG 値は正常であった。アポ A-V はその後 435 ng/mL (TG 831 mg/dL) までに回復していたので、アポ A-V のエクソン領域には異常があるとは考えにくい。母乳という高脂肪負荷により一過性にカイロミクロン血症が出現したとも考えられる。但し、THR184SER が影響を及ぼさないかどうかについて、アポ A-V 欠損マウスを用いて THR184SER をアデノウィルスで導入する実験を準備している。

Case 5 はアポ C-II 欠損症と診断されていたが、実際には完全欠損ではなく微量なアポ C-II が検出された。臨床検査で一般に使用されている免疫比濁法による測定の感度が悪いため、微量なアポ C-II が検出できなかった。糖鎖や分子量は、正常対照と区別することはできず、エクソンには変異を見い出すことはできなかった。アポ C-II の発現量を低下させる原因として、プロモーター領域も調べているが、現在のところ変異は見つかっていないので、trans-element の異常の可能性も考慮して、今後解析する必要がある。全ゲノムシークエンスによるアプローチも必要かもしれない。

E. 結論

今回の研究により、カイロミクロン血症の原因検索には、LPL 活性の測定が先決となること、LPL に異常が認められない場合の原因の同定は困難であることからカイロミクロン血症の成因は多様であることが再確認された。

F. 健康危険情報

特になし

G. 研究発表

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H. 知的財産権の出願・登録状況（予定を含む）。

1. 特許取得　なし
2. 実用新案登録　なし
3. その他　なし

図１. TG 値と血漿 LPL mass の分布

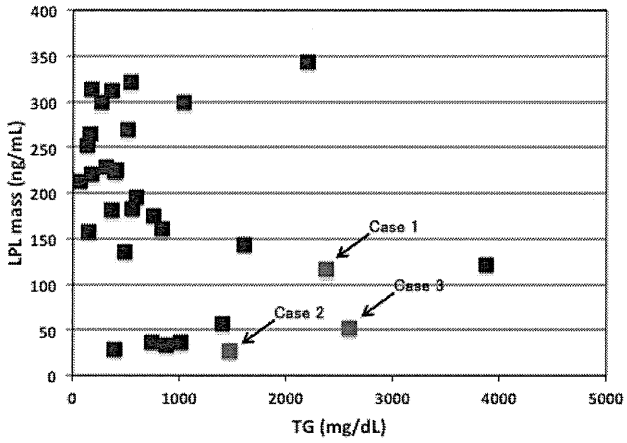


図２. TG 値と血漿 LPL 活性の分布

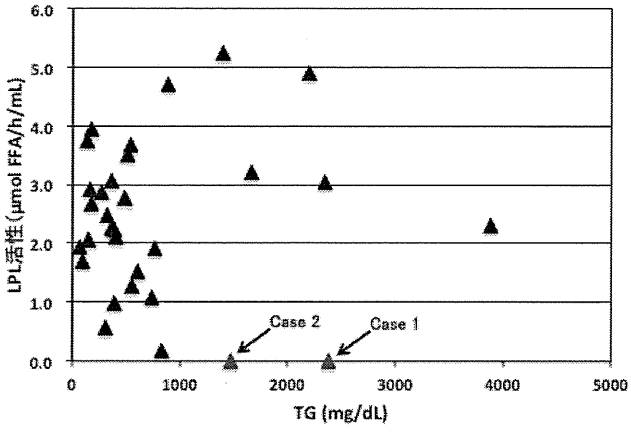
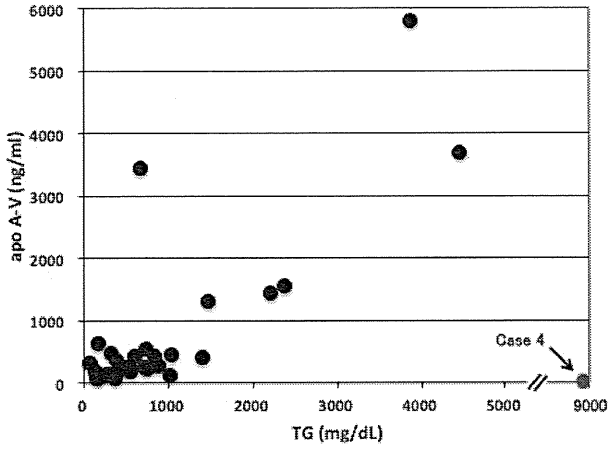


図３．TG 値と血清アポ A-V 値の分布



厚生労働科学研究費補助金（難治性疾患克服研究事業）
分担研究報告書
原発性高脂血症に関する調査研究
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研究要旨　家族性高コレステロール血症における動脈硬化の質的診断

A．研究目的

原発性高脂血症の代表的疾患である家族性高コレステロール血症（ＦＨ）は、早発性の動脈硬化をきたすことが大きな問題である。超音波で頸動脈を観察すると、ＦＨでみられる、いわゆる粥状動脈硬化はプラークの多発が特徴的である。一方、代表的な動脈硬化危険因子である２型糖尿病（ＤＭ）では、びまん性の頸動脈内中膜肥厚（ＩＭＴ）増大が主たる所見であり、形成される動脈硬化病変の性質がＦＨとは異なっていると考えられる。

我々の大学では、工学部と共同で新しい超音波測定法・位相差トラッキング法の臨床応用を進めている。本測定法は、血管壁における複数の計測点の、心拍毎の動きを数十μｍ単位で追跡することで、組織性状を非侵襲的に評価可能なことから、動脈硬化診断への有用性が期待されている。

本測定法を用いて、ＦＨの動脈硬化病変の性質を検討し、質的診断の体系を確立することで、ＦＨのイベント発症予測・予後向上に繋げることが、本研究の目的である。

B．研究方法

ＦＨ４９名を対象に位相差トラッキング法で測定した頸動脈性状、ＩＭＴ、血液検査指標を検討した。年齢、体重、血圧をマッチさせたＤＭ６８名をコントロールとした。尚、位相差トラッキング法は従来の医療用超音波機器を用いることから、被験者への有害事象の危険性は極めて低いと考えられる。また測定に関しては本学医学部倫理委員会の審査・承認を得ている(2011-335)。

C．研究結果

両群の検査データを比較すると、平均ＩＭＴはＦＨ vs ＤＭ(以下同様)：0.72mm vs 0.79mm（n.s.）、頸動脈プラークスコア 3.5 vs 1.4 ($p<0.01$)とＦＨで頸動脈プラークの多発を認めた。一方、位相差トラッキング法の測定値は39.9kPa vs 54.6 kPa ($p<0.01$)とＦＨで有意に低い値であった。

D．考察

位相差トラッキング法の測定値が高いほど、その部分の血管性状は硬めであると評価される。すなわち、これまでの途中経過では、ＦＨの血管性状はＤＭに比較して柔らかいと認識された。これは内膜へのコレステロール沈着を主とするＦＨの粥状動脈硬化と、膠原繊維の増生を主とするＤＭの中膜肥厚の血管性状の違いを評価しているものと考えられる。今後は症例数を更に増やす

とともに、プラークの性状も含めたＦＨの動脈硬化の特徴について検討を進めていきたい。

Ｅ．結論

位相差トラッキング法を用いることで、ＦＨの動脈硬化形成の特徴が評価可能であり、ＦＨの予後向上に寄与できる可能性があると考えられた。

Ｇ．研究発表

なし

Ｈ．知的財産権の出願・登録状況

なし

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
該当なし							

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ORIGINAL ARTICLE

Ezetimibe improves postprandial hyperlipidaemia in patients with type IIb hyperlipidaemia

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ABSTRACT

Background Postprandial hyperlipidaemia is known to be a high-risk factor for atherosclerotic disease because of rapid and lasting accumulations of triglyceride-rich lipoproteins and remnants. The Niemann-Pick C1-Like 1 (NPC1L1) protein acts as an intestinal cholesterol transporter and ezetimibe, which inhibits NPC1L1, has been used in patients with hypercholesterolaemia. We investigated effects of ezetimibe on fasting lipid and lipoprotein profiles and postprandial hyperlipidaemia in patients with type IIb hyperlipidaemia.

Materials and methods Ezetimibe 10 mg per day was administered in ten patients with type IIb hyperlipidaemia for 2 months, and lipid and lipoprotein profiles were examined during fasting and after an oral fat loading (OFL) test.

Results In the fasting state, ezetimibe significantly decreased not only total cholesterol, low density lipoprotein (LDL)-cholesterol and apolipoproteinB-100 (apoB-100) levels but triglycerides (TG), apoB-48 and remnant lipoprotein cholesterol (RemL-C) levels. High performance liquid chromatography analysis showed that ezetimibe decreased cholesterol and TG levels in the very low density lipoprotein (VLDL) and LDL size ranges as well as apoB-100 levels, suggesting a decrease in numbers of VLDL and LDL particles. After OFL, ezetimibe decreased the area under the curve for TG, apoB-48 and RemL-C. Ezetimibe decreased postprandial elevations of cholesterol and TG levels in the chylomicrons (CM) size range, suggesting that the postprandial production of CM particles was suppressed by ezetimibe.

Conclusions These findings suggest that ezetimibe improves fasting lipoprotein profiles and postprandial hyperlipidaemia by suppressing intestinal CM production in patients with type IIb hyperlipidaemia and such treatment may prove to be effective in reducing atherosclerosis.

Keywords Apolipoprotein B-48, atherosclerosis, ezetimibe, postprandial hyperlipidaemia, remnants, triglycerides-rich lipoproteins.

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Introduction

Plasma triglycerides (TG) are mainly found in triglyceride-rich lipoproteins (TRL) consisting of chylomicrons (CM) and very low density lipoproteins (VLDL). TRL constitute a population of particles of heterogeneous size, origin and apolipoprotein (apo) and lipid content. CM assemble dietary cholesterol, TG and apoB-48 in enterocytes and VLDL assemble endogenous hepatic TG, cholesterol and apoB-100 in hepatocytes. These lipoprotein particles undergo partial hydrolysis predominantly by lipoprotein lipase (LPL) into smaller and more dense particles know as remnants, which are believed to be more atherogenic than the larger TRL. CM are produced in enterocytes, primarily through the use of exogenous lipid sources and apoB-48 recruitment and are secreted into thoracic lymph, from which they flow into the systemic circulation. LPL hydrolyses CM-TG to free fatty acids (FFA), and residual particles become CM remnants (CM-R) which are taken up by the liver via remnant receptors. VLDL assemble endogenous hepatic TG, cholesterol and apoB-100 in hepatocytes, which are secreted directly into the blood stream. There, LPL hydrolyses VLDL-TG to FFA, and residual particles become VLDL remnants. The liver takes up VLDL remnants and further hydrolysed particles, and the low density lipoproteins (LDL) are taken up via LDL receptors while these particles are supplying energy and lipids to peripheral tissues. In the postprandial state, blood levels of

CM and CM-R quickly rise to reflect the increased exogenous lipid supply. This subsequently activates endogenous lipid synthesis in the liver by increasing the hepatic lipid inflow, leading to augmented hepatic VLDL production. Postprandial hypertriglyceridaemia is caused by overproduction and/or impaired clearance of TRL and TRL remnants, leading to rapid accumulation and sustained blood levels after dietary intake. Both fasting and postprandial hypertriglyceridaemia are known to be risk factors for coronary heart disease [1,2].

Recently Niemann-Pick C1 Like 1 (NPC1L1) protein has been reported to play a central role in cholesterol absorption in enterocytes [3,4]. Genetic inactivation of NPC1L1 protein decreases cholesterol levels and atherosclerotic lesions in hyperlipidaemic apoE knockout mice fed a western diet [5,6]. Ezetimibe, a novel lipid-lowering compound, selectively inhibits intestinal cholesterol absorption by binding to NPC1L1 protein, reducing total cholesterol (TC) and TG levels and also reducing the development of atherosclerosis in apoE knockout mice [7,8]. Clinically, it has already been shown that administration of ezetimibe diminished fasting levels of total and LDL-cholesterol in patients with primary hypercholesterolemia in Japan and the United States [9,10]. Due to the nature of its medicinal properties, the investigation into the pharmacological effects of ezetimibe has focused primarily on the metabolism of sterols, including cholesterol, rather than on TG or TRL. However, ezetimibe has been reported to decrease fasting TG levels significantly in patients with combined hyperlipidaemia [10], and its underlying mechanism of action has not yet been elucidated. As fasting and postprandial TG levels are closely related, it is essential to understand the effects of ezetimibe in combined hyperlipidaemic patients with reference to postprandial TRL and remnant metabolism. In this study, we administered ezetimibe 10 mg day^{-1} orally to 10 patients with type IIb hyperlipidaemia who have both hypercholesterolaemia and hypertriglyceridaemia, and used oral fat loading (OFL) tests to evaluate changes in fasting and postprandial lipid and lipoprotein profiles.

Materials and methods

Subjects

Ten Japanese patients (two female, eight male) were enrolled in this study. All patients had been diagnosed with type IIb hyperlipidaemia according to the Japanese criteria (fasting TC level ≥220 mg dL^{-1} and fasting TG level ≥150 mg dL^{-1}). Ezetimibe (Bayer Yakuhin Ltd. (Tokyo, Japan) and Schering-Plough K.K. (Tokyo, Japan)) 10 mg was administered once daily to all patients for 2 months. None of the patients took any other drugs that might affect lipid or lipoprotein metabolism. Every medication other than ezetimibe was continued unchanged throughout the study period. Total calorie intake and composition of the diet were kept constant for each patient. All subjects gave written informed consent before participating in this study, and the ethics committee of the Osaka University Hospital approved the study design.

Measurement of serum samples

Fasting blood samples were drawn from each of the 10 enrolled patients before the start and after the conclusion of ezetimibe administration. Serum was separated by low-speed centrifugation (1200 g, 15 min, at 4°C) and stored at 4°C until measurement within a week. All samples were treated in accordance with the Helsinki Declaration. Concentrations of TC, TG and FFA were measured using the enzymatic method. Concentrations of LDL-cholesterol (LDL-C) and high density lipoprotein cholesterol were measured using the direct method. Concentrations of apoAI, AII, B, CII, CIII, and E were measured using the immunoturbidity method. Concentrations of high sensitivity C-reactive protein were measured using the immunonephelometric assay (Sekisui Medical Co., Ltd., Tokyo, Japan). Haemoglobin A1c levels were measured using high performance liquid chromatography (HPLC) method. Fasting plasma glucose levels were measured using a hexokinase UV method. Concentrations of fasting plasma insulin were measured using a chemiluminescent enzyme immunoassay (CLEIA) method (SRL Inc., Tokyo, Japan). HOMA-IR (homeostasis model assessment of insulin resistance) index was calculated as [fasting plasma insulin (μIU mL^{-1}) × fasting plasma glucose (mg dL^{-1})]/405. Concentrations of apoB-48 were measured using a sandwich CLEIA (Fuji Rebio Inc., Tokyo, Japan) [11]. Remnant lipoprotein cholesterol (RemL-C) levels were measured using a RemL-C homogenous assay, RemL-C (Kyowa Medex, Tokyo, Japan), which enabled separation of CM-R and VLDL remnants from other lipoproteins with higher specificity than the remnant like particle-cholesterol method [12,13]. Before ezetimibe administration, RemL-C and apoB-48 levels were higher in enrolled patients than in normolipidaemic subjects, in conjunction with higher levels of TC, TG, apoB and LDL-C (patients vs. normolipidemic subjects shown in the previous studies: RemL-C 18·7 ± 10·5 vs. 3·5 ± 1·2 mg dL^{-1} in [13]; apoB-48 6·8 ± 4·3 vs. 5·2 ± 3·8 μg mL^{-1} in [11]).

Oral fat loading test

The OFL test was performed before and after the administration of ezetimibe. After an overnight fast for 12 h, oral fat tolerance test (OFTT) cream which was prepared from milk and adjusted to contain 35% fat without sugar (JOMO Foods, Gunma, Japan) was loaded to each patient sufficient to provide a fat load of 30 g fat m^{-2} body surface area. Blood samples were drawn before and 1, 2, 3, 4, 6 and 8 h after OFL and concentrations of TC, TG, apo B-48, FFA, RemL-C and apoB-100 were measured. To compare the net postprandial change in

these parameters, areas under the curve (AUC) for TC, TG, apo B-48, FFA, RemL-C and apoB-100 were calculated using the trapezoidal method and incremental AUC (ΔAUC) values by ignoring area beneath the fasting level.

Lipoprotein profiles assessed by HPLC

The effect of ezetimibe on lipoprotein profile during fasting and 4 h after OFL was evaluated using the HPLC method. Samples of 200 microlitres of serum (fasting state and 4 h after OFL before and after administration of ezetimibe) were analysed at Skylight Biotech Inc. (Akita, Japan) and dissolved with the loading buffer (0·05 mol L^{-1} Tris-buffered acetate, pH 8·0). These samples were loaded into two tandem connected TSK-gel Lipopropak XL columns and concentrations of TC and TG in the flow-through of each sample were measured continuously and simultaneously [14]. The flow-through of dissolved serum (n = 10) which was drawn 4 h after OFL was collected serially every 1 min into collection tubes (tube No. 1-20) both before and after administration of ezetimibe. The apoB-48 levels of tube No. 1-11 which were supposed to contain lipoproteins in the size range of CM (tube No.1-2), VLDL (tube No. 3-7) and LDL (tube No. 8-10) were measured using the method as mentioned above. The beginning and ending time of the collection of the flow-through was shown in the chromatographic pattern using grey bars in Fig. 3a. We calculated cholesterol and TG concentrations of lipoprotein fractions in the size categories of CM, VLDL, LDL and HDL, based on findings from a prior investigation that confirmed the correspondence of lipoprotein fractions in CM, VLDL, LDL, and HDL-sizes and the elution time, by comparing the HPLC pattern of each lipoprotein separated using ultracentrifugation [15]. Those categories were as follows: CM-size, estimated particle size > 80 nm, elution time 15–17 min; VLDL size 30–80 nm, 17–22 min; LDL size, 16–30 nm, 22–25·5 min; HDL-size, 8–16 nm, 25·5–28·5 min.

Statistical analyses

The results were expressed as mean ± SD. The Student's paired t-test was used for pairwise comparisons between values before and after administration of ezetimibe. A value of $P < 0·05$ was considered to be statistically significant.

Results

Effect of ezetimibe on fasting serum levels of lipid biomarkers in patients with type IIb hyperlipidaemia

Table 1 shows fasting serum levels of lipid biomarkers before and after administration of ezetimibe for 2 months. Ezetimibe effectively reduced serum levels of TC, TG, apoB and LDL-C in the fasting state as we expected. LDL-C reducing response varied between 9·8% (reducing from 151 to 136 mg dL^{-1}) and 33·2% (from 152 to 101 mg dL^{-1}). However, the mean rate of reduction in TG was larger than previously reported for ezetimibe treatment in patients with primary hypercholesterolaemia (mean reduction rates: TC -16·5%, TG -24·5%, apoB -15·7, LDL-C -20·3%). It is especially striking that fasting levels of apoB-48, and RemL-C were also significantly decreased after the administration of ezetimibe (mean reduction rates: RemL-C -22%, apoB-48 -31%) in type IIb hyperlipidaemic patients. These results suggest that ezetimibe may affect not only VLDL and LDL particles containing apoB-100, but also CM and CM-R particles containing apoB-48. There was no difference in body weight and waist circumference through the treatment. Ezetimibe treatment did not alter serum levels of HDL cholesterol, apoAI, apoAII, apoCIII, apoE, FFA and diabetic parameters, fasting plasma glucose, plasma insulin or haemoglobin A1c

Table 1 Fasting levels of lipid biomarkers before and after administration of ezetimibe

		Ezetimibe(−)	Ezetimibe(+)	*P*-value
TC	(mg dL^{-1})	231 ± 43	194 ± 26	0·001
TG	(mg dL^{-1})	218 ± 83	178 ± 85	0·031
LDL-C	(mg dL^{-1})	145 ± 42	120 ± 25	0·005
HDL-C	(mg dL^{-1})	53 ± 14	52 ± 13	0·394
FFA	(μEq L^{-1})	508 ± 187	483 ± 184	0·270
RemL-C	(mg dL^{-1})	18·7 ± 10·5	12·0 ± 6·3	0·006
apoAI	(mg dL^{-1})	144 ± 29	142 ± 31	0·130
apoAII	(mg dL^{-1})	32·2 ± 8·0	30·8 ± 7·6	0·071
apoB-100	(mg dL^{-1})	116 ± 22	101 ± 13	0·004
apoB-48	(μg mL^{-1})	6·8 ± 4·3	4·7 ± 2·3	0·019
apoCII	(mg dL^{-1})	5·3 ± 2·8	4·3 ± 2·1	0·043
apoCIII	(mg dL^{-1})	11·7 ± 4·3	10·5 ± 3·8	0·082
apoE	(mg dL^{-1})	6·2 ± 1·3	5·6 ± 1·4	0·054
Glucose	(mg dL^{-1})	107 ± 21	104 ± 19	0·165
Insulin	(μIU mL^{-1})	12·1 ± 5·5	14·5 ± 5·5	0·231
HOMA-IR		3·2 ± 1·6	3·7 ± 3·6	0·165
HbA1c	(%)	5·6 ± 0·4	5·5 ± 0·4	0·165
hs-CRP	mg dL^{-1}	0·11 ± 0·08	0·16 ± 0·15	0·17

TC, total cholesterol; TG, triglyceride; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; FFA, free fatty acid; RemL-C; remnant lipoprotein cholesterol; apo, apolipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; HbA1c, haemoglobin A1c; hs-CRP; high sensitivity C reactive protein.
HOMA-IR index was calculated as [fasting plasma insulin (μ IU mL^{-1}) × fasting plasma glucose (mg dL^{-1})]/405.
Data were shown as mean ± SD and statistical significance was calculated using paired t-test.

levels as well as HOMA-IR index (Table 1). In this study, there was no significant change in the levels of hs-CRP which is an independent marker for the development of atherosclerotic cardiovascular diseases by ezetimibe treatment.

Effect of ezetimibe on fasting lipoprotein profiles in patients with type IIb hyperlipidaemia

To evaluate the effect of ezetimibe on fasting lipoprotein profiles, serum samples were analysed by HPLC, and cholesterol and TG levels were measured. Representative chromatographic patterns of cholesterol and TG before and after ezetimibe treatment are shown in Fig. 1a. For each patient cholesterol and TG levels in the indicated pooled fractions corresponding to CM-, VLDL-, LDL- or HDL-sized particles were summed and averages were calculated. The levels of cholesterol and TG decreased in the VLDL and LDL fractions after ezetimibe treatment, and the LDL peak in cholesterol tended to shift slightly to the left (lower elution time, greater apparent size), which may represent large LDL particles (before vs. after administration of ezetimibe: VLDL-C 46 ± 13 vs. 32 ± 12 mg dL^{-1}, $P = 0{\cdot}0016$; LDL-C 150 ± 33 vs. 120 ± 27, $P = 0{\cdot}0018$; VLDL-TG 176 ± 67 vs. 116 ± 54, $P = 0{\cdot}0027$; LDL-TG 49 ± 12 vs. 41 ± 7, $P = 0{\cdot}034$). However, this shift was not observed in all specimens. Findings for cholesterol and TG content in CM- and HDL-size particles after ezetimibe treatment were similar to the treatment baseline.

OFL test before and after ezetimibe administration

As shown in Fig. 2, postprandial changes in lipid profiles were determined by OFL testing with OFTT cream before and after administration of ezetimibe in 10 patients with type IIb hyperlipidaemia. Initial values for serum TC and apoB-100 after

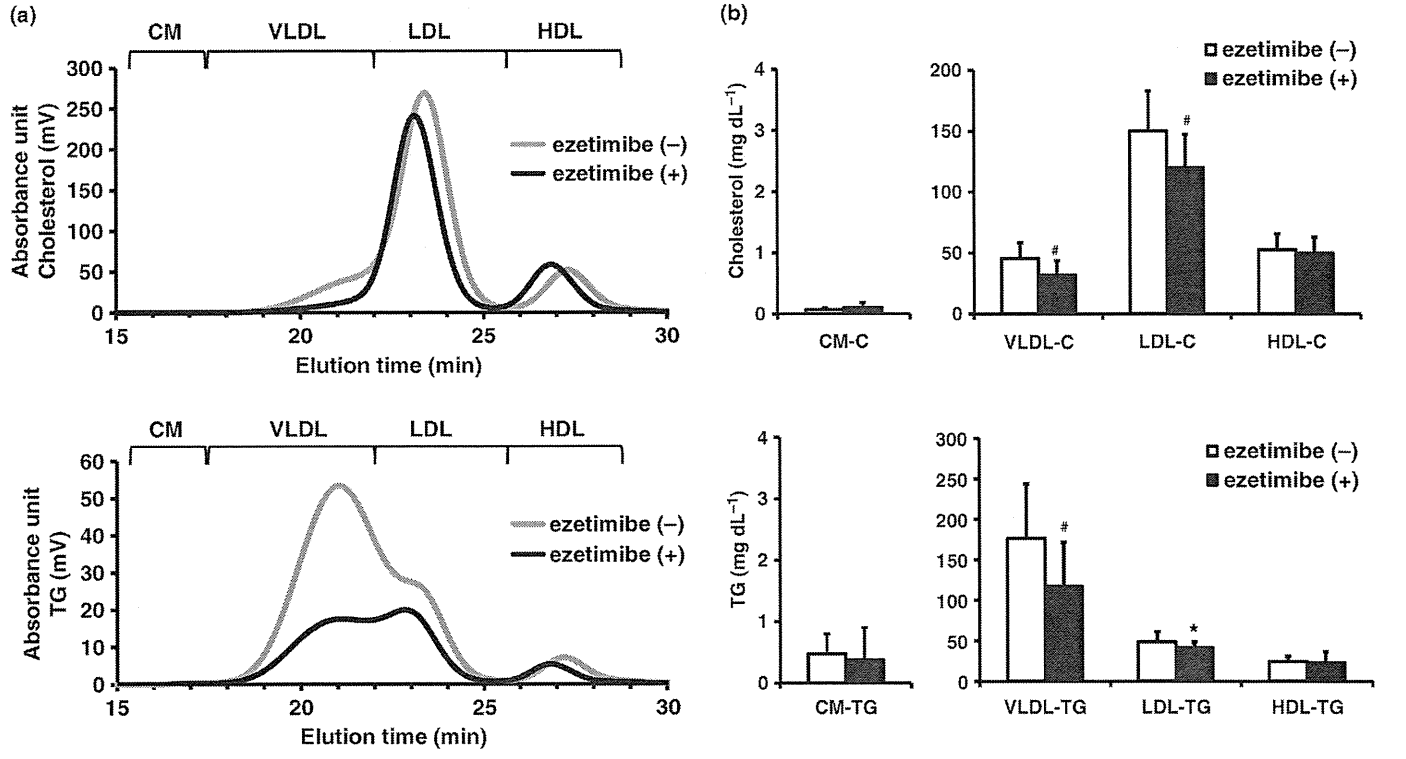

Figure 1 Lipoprotein profiles in the fasting state before and after administration of ezetimibe. Ezetimibe 10 mg was administered in patients with type IIb hyperlipidaemia (n = 10, two females and eight males) for 2 months. Two hundred microlitres of serum were separated from blood drawn in the fasting state before and after administration of ezetimibe. Lipoprotein profiles were analysed by high performance liquid chromatography. The concentrations of cholesterol and triglyceride (TG) in the flow-through of each sample were measured continuously and simultaneously. (a) Representative chromatograms of cholesterol and TG of fasting serum before (grey line) and after (black line) administration of ezetimibe were shown with approximate elution times of chylomicrons (CM), very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high density lipoprotein (HDL). (b) For each patient cholesterol and TG levels in the indicated pooled fractions corresponding to CM-, VLDL-, LDL- or HDL-size particles were summed and averages were calculated before (open squares) and after (closed squares) administration of ezetimibe. *$P < 0{\cdot}05$, #$P < 0{\cdot}005$.

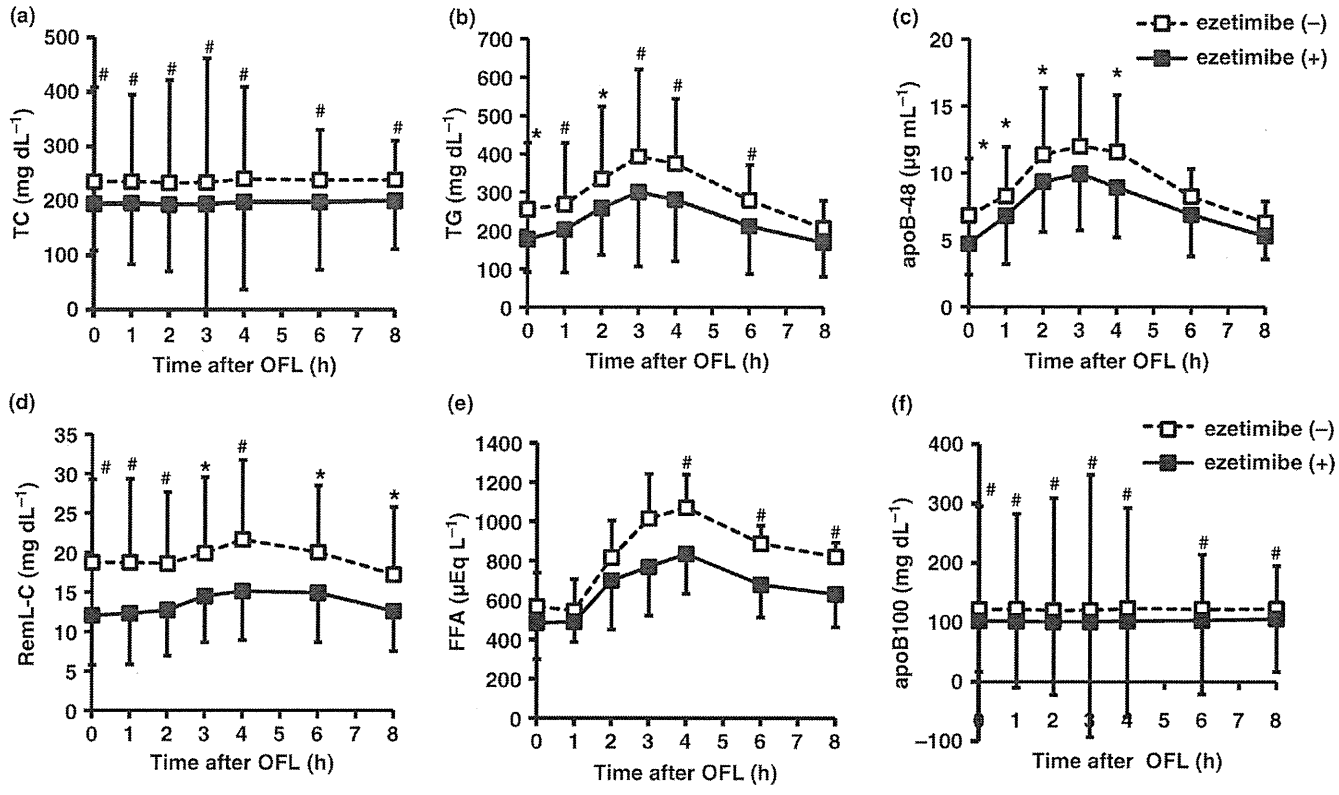

Figure 2 Oral fat loading (OFL) test before and after administration of ezetimibe. Patients with type IIb hyperlipidaemia (n = 10, two females and eight males) were given OFTT cream (containing 35% fat without sugar, 30 g fat m^{-2} body surface area) after overnight fasting before (open squares) and after (closed squares) administration of ezetimibe. Blood samples were drawn during fasting and 1, 2, 3, 4, 6 and 8 h after OFL, and serum and plasma were separated immediately. Concentrations of (a) total cholesterol (TC), (b) triglyceride (TG), (c) apolipoprotein B-48(apoB-48), (d) remnant lipoprotein cholesterol (RemL-C), (e) free fatty acids (FFA) and (f) apoB-100 were measured as described in Materials and methods. *P < 0·05, #P < 0·01.

ezetimibe treatment were significantly lower than before the treatment. Serum TC and apoB-100 levels remained constant throughout the 8-h OFL test. TG, apoB-48 and RemL-C levels rose for the first 3 or 4 h, and returned to fasting levels 8 h after OFL. Ezetimibe significantly diminished fasting and peak levels for these parameters and for AUC, which reflects the postprandial integrated response (AUC-TC 1892 ± 350 vs. 1570 ± 204 mg dL^{-1} 8 h, P = 0·0001; AUC-apoB-100 2167 ± 649 vs. 1519 ± 488 mg dL^{-1} 8 h, P = 0·023; AUC-TG 2448 ± 1130 vs. 1863 ± 1012 mg dL^{-1} 8 h, P = 0·003; AUC-apoB-48 75 ± 23 vs. 61 ± 22 μg dL^{-1} 8 h, P = 0·044; AUC-RemL-C 156 ± 72 vs. 110 ± 46 mg dL^{-1} 8 h, P = 0·008). However, incremental AUCs (ΔAUCs), which are thought to describe postprandial integrated response more accurately, after ezetimibe administration were comparable to the corresponding values before ezetimibe administration for TG, apoB-48 and RemL-C (ΔAUC-TC 11 ± 98 vs. 15 ± 61 mg dL^{-1} 8 h, P = 0·448; ΔAUC-apoB-100 483 ± 334 vs. 236 ± 318 mg dL^{-1} 8 h, P = 0·168; ΔAUC-TG 405 ± 442 vs. 443 ± 553 mg dL^{-1} 8 h, P = 0·442; ΔAUC-apoB-48 21 ± 33 vs. 23 ± 17 μg dL^{-1} 8 h, P = 0·394; ΔAUC-RemL-C 6·5 ± 22 vs. 14 ± 14 mg dL^{-1} 8 h, P = 0·432). Ezetimibe intervention reduced peak level, AUC and ΔAUC for FFA after OFL (AUC-FFA 6856 ± 1362 vs. 5433 ± 1231 mg dL^{-1} 8 h, P = 0·004; ΔAUC-FFA, 2329 ± 1159 vs. 1564 ± 1249 mg dL^{-1} 8 h, P = 0·017), indicating a possible decrease in FFA production and/or increase in FFA clearance. There were no changes in serum levels for other apolipoproteins (apoAI, AII, CII, CIII, and E) throughout the OFL test, either before or after ezetimibe treatment (data not shown).

HPLC analysis of postprandial lipoprotein profiles

To further elaborate on postprandial lipid changes, HPLC analysis was conducted 4 h after the OFL test to compare cholesterol and TG concentrations of lipoprotein fractions in the CM, VLDL, LDL and HDL-size ranges before and after administration of ezetimibe. Chromatographic patterns of

serum 4 h after OFL revealed that three peaks were observed in the size range of CM, VLDL and LDL by the detection of cholesterol and TG levels. The VLDL peak by the detection of TG after OFL was shifted to the left (lower elution time, greater apparent size) compared with that in the fasting state, suggesting that any other lipoprotein particles which were contained in the size range of VLDL and larger than VLDL observed in the fasting state were produced after OFL. HPLC analysis of serum which was obtained 4 h after OFL before and after ezetimibe treatment showed that three peaks by the detection of TG in the size range of CM, VLDL and LDL tended to decrease after ezetimibe treatment (Fig. 3a). By the calculation of average cholesterol and TG levels in the size range of CM, VLDL, LDL and HDL, HPLC analysis 4 h after OFL revealed that the reduction in serum TC and TG after ezetimibe treatment was mainly due to cholesterol and TG changes in the size range of CM and VLDL, not due to those in the size range of LDL (CM-C 0·63 ± 0·26 vs. 0·31 ± 0·09 mg dL^{-1}, P = 0·0029; VLDL-C 50 ± 14 vs. 37 ± 11 mg dL^{-1}, P = 0·0022; LDL-C 138 ± 41 vs. 116 ± 2 mg dL^{-1}, P = 0·059; CM-TG 10·2 ± 5·4 vs. 4·7 ± 2·2 mg dL^{-1}, P = 0·014; VLDL-TG 251 ± 93 vs. 180 ± 88 mg dL^{-1}, P = 0·0009, LDL-TG 50 ± 13 vs. 43 ± 8 mg dL^{-1}, P = 0·056) (Fig. 3b). Furthermore, to evaluate whether CM-R were contained in the size range of VLDL and LDL 4 h after OFL and their contents were changed before and after ezetimibe treatment, we measured apoB-48 levels of serially collected flow-through of dissolved serum (n = 10) which was drawn 4 h after OFL, as shown in Materials and methods (Fig. 3a). Both before and after ezetimibe treatment, apoB-48 was detected in the fractioned flow-through which was suggested to contain lipoproteins in the size range of not only CM but also VLDL and LDL (Fig. 1a). Before ezetimibe treatment, we can see two peaks of apoB-48 levels at the position of tube No. 5 and No. 8, which was coincided with peaks by the detection of TG in the size range of VLDL and LDL 4 after OFL. These findings suggested that CM-R particles existed in various size ranges, from the size of CM to HDL, and the peak of the size of CM-R particles existed both in the size range of VLDL and LDL. After ezetimibe treatment, apoB-48 levels were decreased in all size ranges and the peak of apoB-48 levels in the size range of VLDL had disappeared. However, the decreases in apoB-48 levels by ezetimibe treatment were significant in tube No.1, No. 7 and No. 8, but not significant in other tubes. (No.1:before vs after treatment, 0·012 ± 0·008 vs. 0·003 ± 0·001 μg dL^{-1}, P = 0·020, No. 7; 0·031 ± 0·020 vs. 0·013 ± 0·003 μg dL^{-1}, P = 0·043, No.8; 0·044 ± 0·018 vs. 0·018 ± 0·006 μg dL^{-1}, P = 0·021). These results suggested that the decreases in particle numbers of CM and CM-R by the ezetimibe treatment occurred significantly in the size range of CM and small VLDL, but relatively in the size range of large VLDL particles. To address whether suppression of lipoprotein production resulted in any reduction of TG and cholesterol in the size range of CM and VLDL particles after ezetimibe administration, we calculated differences in cholesterol and TG levels in the size range of CM and VLDL particles between fasting and 4-h OFL, and compared these differences before and after ezetimibe treatment (Fig. 3c). Ezetimibe attenuated the increase in cholesterol level at the CM-size (0·56 ± 0·25 vs. 0·21 ± 0·11 mg dL^{-1}, P = 0·0008), which might reflect the inhibition of cholesterol absorption in the intestine in accordance with the mechanism of action of ezetimibe. In a particularly striking finding, the increase in CM-size TG was also attenuated after the administration of ezetimibe (9·7 ± 5·4 vs. 4·4 ± 2·3 mg dL^{-1}, P = 0·017) (Fig. 3c) along with the significant decrease in apoB-48 level in the size range of CM 4 h after OFL (Fig. 3a), which raised the possibility that the decreased intestinal cholesterol absorption associated with ezetimibe administration might also influence the intestinal production of CM. There were no significant differences before and after ezetimibe treatment in increased VLDL particle size levels for cholesterol or TG between fasting and 4-h OFL, even though ezetimibe decreased the fasting and postprandial (4 h after initiating OFL test) TG levels for the VLDL size range (Figs 1 and 3). These findings suggest that VLDL metabolism, at least during the 4-h OFL test, was unaffected by ezetimibe treatment.

Discussion

In this study, we elucidated the fasting and postprandial lipid and lipoprotein profiles of patients with type IIb hyperlipidaemia before and after ezetimibe administration. We clearly showed that ezetimibe treatment decreased the fasting apoB-48 and RemL-C levels as well as TC, TG, and apoB-100 levels. When we subtracted apoB-48 levels from the apoB levels, the resulting values also showed decreases in apoB-100 after ezetimibe administration. HPLC analysis showed reduced levels of cholesterol and TG in VLDL and LDL fractions at fasting after ezetimibe administration, suggesting that the levels of apoB-100-containing lipoproteins such as VLDL, VLDL remnants and LDL particles were reduced in conjunction with decreased serum apoB-100 levels. Telford *et al.* demonstrated, in a study of miniature pigs, that ezetimibe decreased the intrahepatic cholesterol pool through inhibition of intestinal cholesterol absorption, leading to the suppression of hepatic VLDL production and enhanced LDL clearance by upregulation of LDL receptor expression in hepatocytes [16]. As a consequence, serum levels of apoB-100-containing lipoproteins were reportedly reduced after ezetimibe administration in that experiment. Unlike rodents, humans express high levels of NPC1L1 protein in the liver as well as in the intestine. A study using liver-specific NPC1L1 transgenic mice has indicated that the function of liver NPC1L1 was to take up cholesterol from

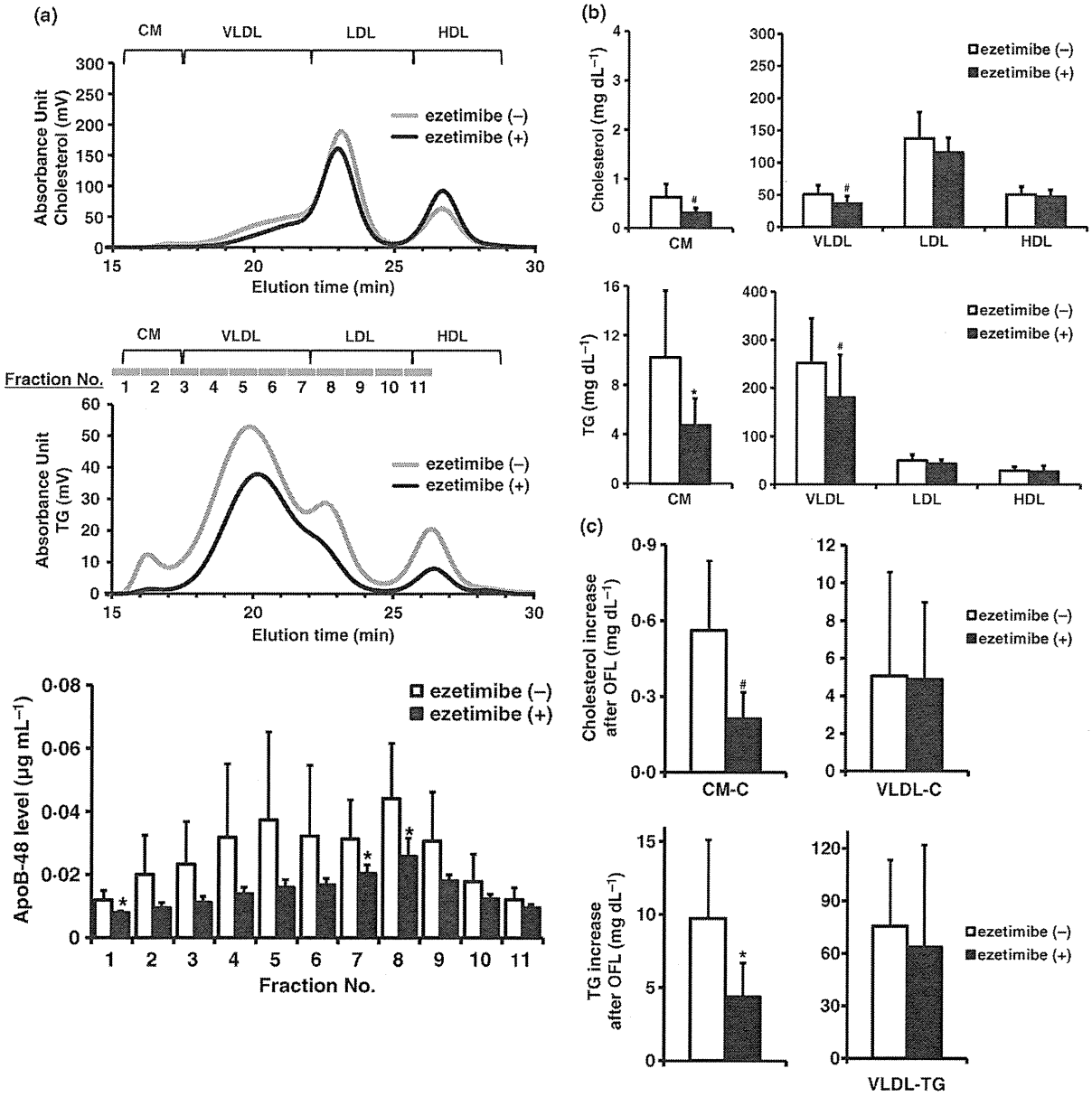

Figure 3 Lipoprotein profiles in postprandial state and incremental fasting/postprandial serum cholesterol and triglyceride (TG) levels before and after administration of ezetimibe. Two hundred microlitres of serum were separated from blood samples drawn 4 h after oral fat loading (OFL) before (open squares) and after (closed squares) administration of ezetimibe for 2 months in patients with type IIb hyperlipidaemia (n = 10, two females and eight males). (a) Representative chromatograms of cholesterol and TG of serum 4 h after the OFL before (grey line) and after (black line) administration of ezetimibe are shown with approximate elution times of chylomicrons (CM), very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high density lipoprotein (HDL). The flow-through of dissolved serum was collected serially every 1 min into collection tubes (tube No. 1-20), apoB-48 levels of tube No. 1-11 which were supposed to contain lipoprotein in the size range of CM, VLDL and LDL were measured using a chemiluminescent enzyme immunoassay method. Grey bars indicate the beginning and ending time of the collection of the flow-through. (b) For each patient (n = 10), cholesterol and TG concentrations of lipoprotein fractions in the size range of CM, VLDL, LDL and HDL were calculated before (open squares) and after (closed squares) administration of ezetimibe. (c) Incremental serum cholesterol and TG levels in the indicated pooled fractions corresponding to CM- or VLDL size particles between fasting and postprandial (4 h after OFL) states were calculated before (open squares) and after (closed squares) ezetimibe treatment. *P < 0·05, #P < 0·005.