Characterization of very low density lipoproteins and intermediate density lipoproteins of normo- and hyperlipidemic apolipoprotein E-2 homozygotes

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Abstract Triglyceride-rich lipoproteins derived from ten normo- and hyperlipidemic apoE-2 homozygotes were analyzed for their composition, β-VLDL content, and their ability to induce cholesteryl ester storage in macrophages. In six of these probands apoE sequence analysis revealed that the cysteine residues were at positions 112 and 158 of the amino acid sequence (Rall et al. 1983. *J. Clin. Invest.* **71:** 1023–1031). ApoE-2 of these six and the other four patients was further analyzed by SDS electrophoresis to exclude the presence of apoE-2* (Rall et al. 1982. *Proc. Natl. Acad. Sci. USA.* **79:** 4696–4700). The relative serum concentrations of free and esterified cholesterol transported in the d < 1.006 g/ml and d 1.006–1.019 g/ml lipoproteins of the apoE-2 homozygotes was significantly higher as compared to controls. Compositional analysis of these lipoproteins revealed a relative reduction of triglycerides and a relative increase of cholesteryl esters as compared to controls. In most patients, with increasing serum triglyceride levels the cholesteryl ester concentration increased in d < 1.006 g/ml and d 1.006–1.019 g/ml lipoproteins. However, in three patients with a low content of β-VLDL, the increase in the d < 1.006 g/ml fraction cholesterol was mostly due to free cholesterol and not due to cholesteryl esters. The degree of the macrophage cholesteryl ester accumulation induced by d < 1.006 g/ml lipoproteins was mostly dependent on the concentration of the β-migrating fraction (β-VLDL). The amount of β-VLDL and pre-β-VLDL contained in the d < 1.006 g/ml fraction was determined densitometrically after electrophoretic separation. It could be demonstrated that the β-VLDL content in the d < 1.006 g/ml fraction of the apoE-2 homozygous patients was largely independent of serum triglyceride and serum cholesterol levels. When macrophages were incubated with the IDL fraction (d 1.006–1.019 g/ml) from the apoE-2 patients, no significant increase in cellular cholesteryl esters above control levels was observed. Studies with purified lipoprotein lipase (LPL) and hepatic triglyceride lipase (HTGL) clearly revealed that both enzymes interacted with apoE-2 VLDL (binding, hydrolysis) to a lesser degree compared to control preparations. However, the apoE-2 VLDL preparations containing a low content of β-VLDL were better substrates for LPL and HTGL than those containing a high β-VLDL content. It is concluded from our studies that the plasma β-VLDL content in apoE-2 homozygotes is a major determinant for cholesteryl ester accumulation in macrophages. The plasma concentration of β-VLDL in apoE-2 homozygosity is not solely dependent on impaired interaction of apoE-containing lipoproteins with hepatic apoE- and/or apoB,E-receptors but may also be influenced by the degree to which these lipoproteins interact with LPL and HTGL. – **Schmitz, G., G. Assmann, J. Augustin, A. Dirkes-Kersting, B. Brennhaüsen, and C. Karoff.** Characterization of very low density lipoproteins and intermediate density lipoproteins of normo- and hyperlipidemic apolipoprotein E-2 homozygotes. *J. Lipid Res.* 1985. **26:** 316–326.

Supplementary key words cholesteryl esters · macrophages · lipoprotein lipase · hepatic triglyceride lipase

The understanding of the role of apolipoprotein E (apoE) in the metabolism of triglyceride-rich lipoproteins has progressed rapidly during the last few years (1–4). Important evidence about the function of apoE has been derived from mutations of this protein (5–7). It is now generally accepted that the apoE polymorphism as deduced from isoelectric focusing reflects genetic polymorphism of this protein (8, 9) and is caused by single amino acid substitutions (10–12). Three common isoforms of apoE are recognized. Two of these, apoE-3 with cysteine at position 112 and apoE-4 with arginine at position 112 in the amino acid sequence (10, 13), have normal binding properties with the apoB,E receptor of normal human fibroblasts. The third, apoE-2, with an additional cysteine substitution at position 158, fails to interact (2% binding) with the apoB,E receptor (10, 13). Various other single amino acid substitutions have been described at positions 142, 145, and 146, all associated with more or

Abbreviations: FCS, fetal calf serum; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; PTA/$MgCl_2$, phosphotungstate acid/magnesium chloride; VLDL, very low density lipoproteins; IDL, intermediate density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; HPTLC, high performance thin-layer chromatography; SDS, sodium dodecylsulfate; FC, free cholesterol; CE, cholesteryl ester; PC, phosphatidylcholine; SPM, sphingomyelin; LPL, lipoprotein lipase; HTGL, hepatic triglyceride lipase.

less reduced receptor binding properties (10, 14). From these studies the concept was developed that "critical regions" exist within the amino acid sequence of apoE, resulting in a total loss or a reduction of the receptor binding properties of the whole lipoprotein particle. It has been demonstrated that most patients with type III hyperlipoproteinemia are homozygous for apoE-2 (cysteine at positions 112 and 158) (5, 6). Considering the clinical expression of type III hyperlipoproteinemia, only one of fifty apoE-2 homozygotes develops the clinical signs of the disease (hyperlipidemia, peripheral and coronary artery disease, tubero-eruptive xanthoma) (15, 16). It has been demonstrated that the sera of all patients with type III hyperlipoproteinemia contain β-VLDL, an abnormal remnant particle, which stimulates cholesteryl ester accumulation in macrophages via a high affinity receptor-mediated uptake (17–20). Recently, a few cases of type III hyperlipoproteinemia have been described where the cysteine substitutions occurred at positions 112 and 145; the isoform was designated apoE-2* (10). It has been concluded from these observations that other factors in addition to the apoE-2 protein defect must be involved in the expression of type III hyperlipoproteinemia.

We have recently studied the apoE polymorphism in 1200 patients who underwent coronary angiography, and approximately 1000 company employees during periodic checkups (21). Ten of the apoE-2 homozygotes detected were systematically examined by clinical and chemical studies. The data concerning the structural defects and the apoE receptor binding studies of six of these patients are already published (14). The sequence analysis revealed that the cysteine residues of their apoE-2 were at positions 112 and 158; the receptor binding activities of apoE isolated from these patients were reduced to 2% of the level found in controls. ApoE-2 of all patients was further analyzed by SDS electrophoresis. There was no abnormal apoE-2 band corresponding to apoE-2*, indicating that all patients have the same molecular defect (10). In this report we present data concerning the chemical composition and the heterogeneity of d $<$ 1.006 g/ml and d 1.006–1.019 g/ml lipoproteins. Further, the cholesteryl ester accumulation induced by these lipoproteins in mouse peritoneal macrophages and the interaction of the apoE-2 homozygote VLDL with purified lipoprotein lipase (LPL) and hepatic triglyceride lipase (HTGL) were investigated.

MATERIALS AND METHODS

Materials

Cells were harvested from male NMRI-SPF mice (25–35 g). FCS was obtained from Gibco Bio-Cult Ltd. (Cat. no.: 629). DMEM and PBS were purchased from Flow Laboratories (Cat. no.: 10-331-25 and 18-610-24, respectively). Cholesterol, cholesteryl esters, phosphatidylcholine, and sphingomyelin for densitometric standards, as well as penicillin (Cat. no.: PEN-NA) and streptomycin (Cat. no.: S-6501), were purchased from Sigma Chemical Co. Tissue culture equipment was obtained from Falcon. Separation of lipids for densitometric analysis was performed on HPTLC plates, 10 × 20 cm from Merck (Cat. no.: 5642). All solvents (analytical grade) were supplied by Merck. Heparin was purchased from Kettelhack Riker Pharma GmbH (Cat. no.: H 1473-3).

Mouse macrophage monolayers

Murine peritoneal macrophages were obtained from unstimulated mice by peritoneal lavage in phosphate-buffered saline (PBS) containing 0.5 U heparin/ml. The fluid from 50–70 mice was pooled and the cells were centrifuged (400 *g*, 10 min, at room temperature) (22), washed once with 30 ml of DMEM, and resuspended in 12 ml of DMEM containing 10% FCS, penicillin (100 U/ml), and streptomycin (100 μg/ml). Aliquots of the cell suspension (0.5 ml) obtained from three mice were dispensed into 60 × 10 mm plastic petri dishes with 3 ml of DMEM containing the additions mentioned above and then incubated in a humidified incubator at 37°C (5% CO_2). After 4–6 hr incubation each dish was extensively washed with 2 ml of DMEM without serum until there were no non-adherent cells visible under the microscope. Each dish contained approximately 200–300 μg of total cell protein. Aliquots of VLDL and IDL (in 3 ml of DMEM) adjusted to identical triglyceride concentrations (150 μg/dish) were then incubated with the cells for 16 hr at 37°C.

Lipid extraction of cells

At the end of the incubation period, medium was removed from the dishes and the cells were washed three times with 2 ml of DMEM and once with PBS. Cells were harvested with a rubber policeman in 500 μl of PBS and transferred to a conical glass tube. The cells of each collection were pooled (three dishes = 1.5 ml) and sonicated with a Branson sonifier three times for 20 sec in ice-water at an intensity of 30 watts (23). After protein determination, an aliquot (0.7–1.2 ml) containing 200 μg of cell protein was delipidated by a modified Folch procedure (23). The organic solvents were evaporated under nitrogen and the samples were stored overnight in a desiccator.

Chromatographic separation and quantitation of lipids

The evaporated samples were redissolved in 50 μl of chloroform. Then 0.2 or 0.5 μl was applied to HPTLC plates using a Camag Nanomat (Muttenz, Switzerland). Separation of neutral lipids was performed in n-hexane–

n-heptane–diethylether–acetic acid 63:18.5:18.5:1. Cholesteryl esters were separated in n-heptane–chloroform 60:40; phospholipids in methylacetate–n-propanol–chloroform–methanol–aqueous potassium chloride (2.5 g/l H_2O) 25:25:25:10:9 as previously described (23, 24). Cholesteryl formate was used as internal standard. Chromatography was performed in a linear development chamber (Camag, Muttenz, Switzerland). Spots were detected using a manganese chloride/sulfuric acid reagent. Quantification was performed by densitometry with a Camag TLC-Scanner combined with a Spectra-Physics SP 4100 Basic integrator equipped with a Kerr minifile 4100 D for data storage (23, 24).

Measurement of [^{14}C]oleate incorporation into cellular lipids

The cells were incubated for 24 hr with DMEM containing 10% human lipoprotein-deficient serum to increase the sensitivity of the assay system (22). [^{14}C]Oleic acid in hexane (NEN, sp act 59 mCi/mmol) was evaporated and resuspended in a solution containing 12.7 mM nonradioactive sodium oleate (Sigma 0-0750) in complex with 12% bovine albumin (Sigma fraction V Cat. no.: A-8022) in normal saline as described by van Harken, Dixon, and Heimberg (25). Three ml of DMEM containing the albumin complex at a concentration of 0.2 mM was incubated together with aliquots of the patients' lipoprotein fractions (as indicated in the figure legends) for 16 hr at 37°C (26). The cells were then harvested and de lipidated as described above. [^{14}C]Oleate incorporation into cholesteryl esters and triglycerides was determined after separation of the neutral lipids on HPTLC plates with the solvent system petroleum ether–diethyl ether–acetic acid 90:10:1. The radioactivity on the HPTLC plate was scanned directly with a Berthold Automatic-TLC-Linear-Analyzer LB 2832 (Labor Berthold, Wildbad, Germany).

Lipoproteins and lipid analysis

Lipoproteins (VLDL, d < 1.006 g/ml; IDL, d 1.006–1.019 g/ml; LDL, d 1.019–1.063 g/ml; HDL_2, d 1.063–1.125 g/ml; HDL_3, d 1.125–1.21 g/ml) were isolated from the fasting sera of apoE-2 homozygotes and a few normolipidemic apoE-3 volunteers by sequential ultracentrifugation in a Beckman L8-70 ultracentrifuge using a 50.3 Ti rotor at 49,000 rpm and 4°C. The lipoprotein fractions were exhaustively dialyzed against 0.05 M Tris-HCl, 0.15 M NaCl, 0.005 M EDTA buffer, pH 7.4, at 4°C.

Lipid analysis was performed in a Cobas-Bio Centrifugal Analyzer (Hoffmann-LaRoche) with enzymatic methods for triglycerides (Hoffmann-LaRoche, Cat. no.: 0710865) and esterified and free cholesterol (Boehringer, Mannheim Cat. no.: 236691). HDL cholesterol was determined after PTA/$MgCl_2$ precipitation (27). Phospholipids were measured by the densitometric method as previously described (28).

Agarose electrophoresis

The d < 1.006 g/ml fraction was analyzed for the relative content of pre-β- (pre-β-VLDL) and β-migrating (β-VLDL) lipoproteins with electrophoresis in 0.6% agarose according to Noble (29). The d < 1.006 g/ml lipoproteins were prestained for 30 min at 4°C with Sudan black-B dissolved in 1% ethylene glycol as described elsewhere (30). Electrophoresis was performed for 30 min with 9 V/cm. The gels were subsequently fixed for 1 hr in a 10% solution of trichloroacetic acid. The relative distribution of the pre-β- and β-VLDL was determined densitometrically with an LKB 2200-002 laser densitometer connected to a Spectra Physics 4270 Basic integrator. A linear correction factor (0.94–1.13), based on the cholesteryl ester content in the d < 1.006 g/ml fraction to be analyzed, was used to correct the integrated signals for differences in binding of the Sudan black B dye to the d < 1.006 g/ml lipoproteins (31, 32). The correction factor was derived from calibration curves of VLDL preparations with different cholesteryl ester content. In addition, the relative cholesteryl ester content of the separated β- and pre-β-migrating lipoproteins was analyzed according to the method of Kupke (33). As tested with this method, the separated bands revealed a similar cholesteryl ester content within each individual apoE-2 homozygote patient.

Determination of lipoprotein lipase (LPL) and hepatic triglyceride lipase (HTGL)

Enzyme source. LPL and HTGL were purified as described elsewhere (34). Purified LPL and HTGL released 18,000 and 15,000 mmol of free fatty acids per mg per hr from a triolein-gum arabic suspension. Before use, the enzyme solutions were stored at −80°C.

LPL assay. The assay was performed as previously described (35, 36). One milliliter of the final incubation mixture contained 0.1 M Tris-HCl (pH 8.2), 0.15 M NaCl, 2.3 μmol of triolein (sp act 5 mCi of ^{14}C/μmol), 4 mg of gum arabic, 5 mg of albumin, and 20 μg of apoC-II (or 20 μl of human serum). After 10 min preincubation at 28°C in a water bath, 10 μl of the enzyme source was added to 200 μl of the incubation mixture and incubated for the time intervals as indicated under binding and hydrolysis studies. The reaction was stopped by addition of 1.6 ml of chloroform–heptane–methanol 75:60:84 and 0.5 M NaOH to 100 μl of the enzyme substrate mixture. After centrifugation of the sample for 20 min at 3000 rpm and 4°C, 0.6 ml of the upper phase was counted (liquid scintillation counter).

HTGL assay. HTGL activity was determined as described under LPL assay, but with a different composition of the incubation mixture: 0.1 M Tris-HCL buffer (pH 8.8), 0.75 M NaCl, 2.3 μmol of triolein (sp act 5 mCi of ^{14}C/μmol), 4 mg of gum arabic, 5 mg of albumin, and 20 μg of apoC-II (or 20 μl of human serum).

Enzyme binding studies

Eight different VLDL concentrations (VLDL-triglycerides, 0-1500 μmol) from each individual patient were analyzed for their enzyme binding properties. One hundred forty μl of each VLDL suspension was added to Beckman Airfuge cellulose propionate tubes. Forty μl of the enzyme source (containing 300 μg of enzyme protein) was added to the tubes at 4°C and the mixtures were centrifuged in the Beckman Airfuge for 15 min at 90,000 rpm. This procedure resulted in the complete flotation of all lipoproteins as determined by lipoprotein electrophoresis and triglyceride determination. After centrifugation a needle was placed in the bottom of each tube and 120 μl of the solution was carefully aspirated; this represented the bottom fraction with the unbound enzyme protein. The remaining 60 μl of the top fraction was vortexed in the centrifuge tube for resolubilization. LPL and HTGL activities of each top and bottom fraction were determined as described above.

VLDL-hydrolysis studies

In vitro hydrolysis of VLDL by HTGL and LPL was performed with optimized conditions for each enzyme. For HTGL, 300 μl of the lipoprotein substrate contained 0.1 M Tris-HCl (pH 8.8), 0.75 M NaCl, 0.01 M $CaCl_2$, and 4% albumin. LPL assays were conducted with a substrate containing 0.1 M Tris (pH 8.2), 0.15 M NaCl, 0.01 M $CaCl_2$, and 2% albumin. After preincubation for 10 min, 60 μl of the enzyme solution, previously adjusted to a release of 5 μmol of free fatty acid per hr per ml enzyme solution from a triolein-gum arabic solution, was added to these substrates. Final volume in each tube was 360 μl. Incubations were carried out at 37°C for 40 min. The reaction was stopped by addition of 250 μl of Dole's solution. Free fatty acid release was measured by the method of Novák (37).

Other methods

The protein content of lipoprotein fractions and cells was determined by a modified Lowry method (38) using bovine serum albumin as a standard. Standard and samples were delipidated by addition of 100 μl of a 2.5% Triton X-100 solution prior to photometric measurement.

Patients

All ten patients listed were not related by kin.

I: 50-yr-old male; 105% of normal body weight; BP: 125/80; no xanthelasma or xanthoma; no arcus corneae; retina arterioles: age appropriate; ECG (rest and after exercise): no abnormal findings; oscillography and Doppler sonography: normal; OGTT: normal; family history: inconspicuous; non-smoker.

II: 48-yr-old male; 103% of normal body weight; BP: 120/70; no xanthelasma or xanthoma; no arcus corneae; retina arterioles: atherosclerotic changes; angina pectoris; ECG: disturbance in the ventricular repolarization, after exercise: no further disturbance; 24-hr ECG: ventricular extrasystoly, stage Lown III; oscillography and Doppler sonography: no abnormal findings; OGTT: normal; family history: 53-yr-old brother suffering from coronary heart disease; non-smoker.

III: 51-yr-old female; 120% of normal body weight; BP: 130/80; no xanthelasma or xanthoma; no arcus corneae; retina arterioles: age appropriate changes; ECG (rest and after exercise): no abnormal findings; oscillography and Doppler sonography: normal; OGTT: normal; cholecystectomy at 39 yrs because of cholelithiasis; family history: inconspicuous; non-smoker.

IV: 40-yr-old male; 110% of normal body weight; BP: 130/80; no xanthelasma or xanthoma; no arcus corneae; retina arterioles: atherosclerotic changes; ECG: slight disturbance in the ventricular repolarization; after exercise: no further disturbance; oscillography and Doppler sonography: normal; OGTT: normal; family history: inconspicuous; smoker: 20 cigarettes/day.

V: 20-yr-old female; 110% of normal body weight; BP: 170/70; no xanthelasma or xanthoma; no arcus corneae; retina arterioles: age appropriate changes; ECG (rest and after exercise): normal; oscillography and Doppler sonography: normal; OGTT: normal; family history: 45-yr-old mother suffering from hypertension and hypercholesterolemia; smoker: 15–20 cigarettes/day.

VI: 60-yr-old male; 110% of normal body weight; BP: 135/75; no xanthelasma or xanthoma; no arcus corneae; retina arterioles: age appropriate changes; ECG (rest and after exercise): normal; oscillography and Doppler sonography: normal; OGTT: normal; family history: inconspicuous; smoker: 5 cigars/day.

VII: 45-yr-old male; 115% of normal body weight; BP: 130/75; no xanthelasma and xanthoma; no arcus corneae; retina arterioles: age appropriate changes; ECG (rest and after exercise): normal; oscillography and Doppler sonography: normal; OGTT: normal; family history: inconspicuous; non-smoker.

VIII: 40-yr-old male; type III hyperlipoproteinemia; 125% of normal body weight; BP: 180/95; palmar and planar xanthoma, xanthelasma of the eye lids, tuberoeruptive xanthoma; retina arterioles: atherosclerotic changes; stenocardia after exercise; ECG (rest): normal; after exercise: ST-reduction; oscillography and Doppler sonography: pathologic; the patient is able to walk 120 steps per min over 80 m without pain; OGTT: normal; family history: mother and brother are apoE-2 homozygotes, but they have no clinical signs of type III hyperlipoproteinemia; smoker: 40 cigarettes/day.

IX: 54-yr-old female; condition following bilateral ovariectomy at the age of 50 yr. after which hyperlipidemia developed; type III hyperlipidemia; 135% of normal body weight; BP: 130/80; palmar and planar

xanthoma, xanthelasma, tubero-eruptive xanthoma at the typical sites of arms and legs; arcus corneae; retina arterioles: age appropriate changes; ECG (rest): disturbances of ventricular repolarization, after exercise: no further disturbances; oscillography and Doppler sonography: signs of peripheral atherosclerosis stage I; OGTT: normal; family history: a brother is apoE-2 homozygote, but has no clinical signs of type III hyperlipoproteinemia; non-smoker.

X: 36-yr-old male; 170% of normal body weight; BP: 120/70; no xanthelasma; no arcus corneae; retina arterioles: atherosclerotic changes; ECG (rest and after exercise): normal; oscillography and Doppler sonography: normal; OGTT: diabetes; family history: unknown; non-smoker.

Controls: the sera derived from ten different normolipidemic apoE-3 homozygote students were used as control samples for lipoprotein analyses, tissue culture, and lipase incubation experiments.

RESULTS

Classification of subjects

The serum lipid, glucose, and uric acid concentrations of the ten apoE-2 homozygote patients are shown in **Table 1.** The patients were listed in this table (as well as Tables 2 and 3 and Figs. 1–5) according to increasing serum triglyceride levels. The HDL-cholesterol levels of patients I, III, and VI were normal, whereas all other patients had significantly reduced HDL-cholesterol levels. Fasting glucose and uric acid were elevated in patient X. Patients VII and X had an impaired glucose tolerance. Patient X, at repeated visits, occasionally had triglyceride concentrations between 1000 and 1600 mg/dl. Patients VII and IX had clinical signs of type III hyperlipoproteinemia. Patient IX developed type III hyperlipoproteinemia within the last 3 years following total ovariectomy in 1980.

TABLE 1. Various serum parameters of the ten apoE-2 homozygote patients

Patient	Sex	Triglyceride	Cholesterol	HDL-Cholesterol	Glucose	Uric Acid
			mg/dl			
I	M	35	121	56	93	6.0
II	M	99	122	23	89	6.4
III	F	124	171	50	96	2.8
IV	M	127	190	33	85	4.0
V	F	186	156	42	94	4.0
VI	M	196	241	55	123	4.1
VII	M	244	190	35	113	5.6
VIII	M	375	328	33	77	2.0
IX	F	528	468	30	91	5.3
X	M	725	284	18	161	7.1

Patients are listed according to increasing serum triglyceride levels.

Characterization of lipoproteins

Fig. 1 demonstrates the relative serum concentrations of free and esterified cholesterol among the d < 1.006 and d 1.006–1.019 g/ml lipoprotein fractions of the patients and controls. With increasing serum triglyceride levels, the relative serum cholesteryl ester concentration increases in both the d < 1.006 and d 1.006–1.019 g/ml lipoproteins. The relative composition of the d < 1.006 g/ml lipoprotein lipids (calculated as mol %) is presented in **Table 2.** There is a relative increase of both free and esterified cholesterol in d < 1.006 g/ml fractions obtained from apoE-2 homozygotes as compared to control VLDL. The increase in d < 1.006 g/ml lipoprotein total cholesterol in patients I, III, and VI is mostly due to an increase in free cholesterol. This is also reflected by the highest FC/CE ratios (1.6, 0.94, and 1.3). The content of phosphatidylcholine as well as sphingomyelin in the d < 1.006 g/ml lipoproteins was either normal or reduced in the apoE-2 homozygotes as compared to the controls.

The relative composition of the d 1.006–1.019 g/ml lipoprotein lipids (calculated as mol %) is shown in **Table 3.** In all patients, there is a relative reduction of triglycerides and a relative increase of total cholesterol as compared to controls. The content of phosphatidylcholine was elevated in patients II, III, IV, IX, and X, normal in patients VII and VIII, and reduced in patient I. The relative content of sphingomyelin was found elevated only in patient IV. All other apoE-2 homozygotes revealed a normal sphingomyelin concentration in the d 1.006–1.019 g/ml fraction.

The ratio of pre-β- and β-migrating lipoproteins contained in the d < 1.006 g/ml fraction was determined densitometrically following agarose electrophoresis. As demonstrated in **Table 4,** patients I, III, and VI had significantly lower β-VLDL concentrations (<15% of total d < 1.006 g/ml fraction) than the other patients (>55% of the total d < 1.006 g/ml fraction).

Macrophage incubation experiments

Incubation of d < 1.006 g/ml lipoproteins from most apoE-2 homozygote patients (except I, III, and VI) resulted in a significant increase of total cellular cholesterol and cellular cholesteryl esters (**Fig. 2** and Fig. 3). The concentration of esterified cholesterol (Fig. 2, middle panel) measured by densitometric analysis, was five- to tenfold elevated above control levels in patients II, IV, V, and VII–X. The d < 1.006 g/ml lipoproteins of these patients are characterized by a high β-VLDL concentration (>55%). By contrast, the d < 1.006 g/ml lipoproteins of the three apoE-2 patients (I, III, and VI) with lower β-VLDL content (<15%) did not increase macrophage cholesteryl esters above control levels as obtained with normal triglyceride-rich VLDL. The concentration

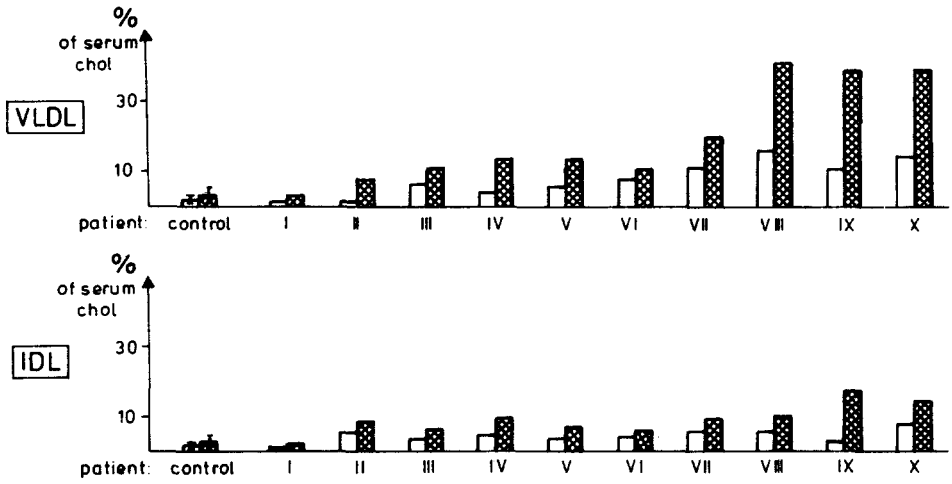

Fig. 1 Percent of serum free cholesterol □ and serum cholesteryl esters ■ transported in the d < 1.006 g/ml and d 1.006–1.019 g/ml lipoproteins. Lipoproteins were isolated by ultracentrifugation at d < 1.006 g/ml and d 1.006–1.019 g/ml in the apoE-2 homozygote patients and apoE-3 homozygote controls and cholesterol was determined in the supernatant and infranatant fractions. Values are calculated as mean values derived from two centrifugation tubes and double determination of lipids (enzymatic analysis).

of cellular free cholesterol was quite different from esterified cholesterol and varied between half and twice the control levels.

The ability of the d < 1.006 g/ml lipoproteins to stimulate [^{14}C]cholesteryl-oleate formation in macrophages is demonstrated in **Fig. 3.** These data, except for patient V, largely correspond to the densitometric mass analysis of cellular cholesterol. In patient V the increase of cellular cholesteryl esters, as observed by the densitometric analysis, was almost exclusively due to the accumulation of cholesteryl linoleate (not shown). In all other patients the incubation with the d < 1.006 g/ml lipoproteins led to cholesteryl oleate accumulation.

In contrast to the results obtained with d < 1.006 g/ml lipoproteins, the d 1.006–1.019 g/ml lipoproteins from the apoE-2 homozygote patients did not affect cholesteryl ester metabolism in macrophages. Neither densitometric analysis of cellular lipids nor incubation of cells with [^{14}C]oleate revealed an increase in cellular cholesteryl esters (**Fig. 4**).

VLDL-binding studies to purified LPL and HTGL

The binding capacity of VLDL (derived from nine of the ten apoE-2 homozygote patients and from ten apoE-3 homozygote controls) to purified human LPL and HTGL was determined (**Fig. 5**). The binding capacity of HTGL (upper panel) and LPL (lower panel) to individual VLDL preparations (expressed as ng of enzyme protein bound per mmol of VLDL-TG) was highest for preparations derived from patients I, III, and VI, and almost comparable to control VLDL. In general, those VLDL fractions that contained the highest β-VLDL concentrations exhibited significantly lower binding to both of these lipolytic enzymes (Fig. 5).

TABLE 2. Relative composition of d < 1.006 g/ml lipoprotein fractions from ten apoE-2 homozygote patients and ten apoE-3 homozygote controls

	I	II	III	IV	V	VI	VII	VIII	IX	X	Control (n = 10)
						mol %					
TG	68	64	55	50	56	60	58	50	46	60	63 ± 8
CE	10	20	18	20	22	13	19	31	34	27	14 ± 3
FC	16	5	17	14	10	17	17	17	14	10	8 ± 2
PC	4	7	7	11	9	7	4	1	5	2	10 ± 3
SPM	2	4	3	5	3	3	2	1	1	1	5 ± 2
FC/CE	1.6	0.25	0.94	0.7	0.45	1.3	0.89	0.55	0.41	0.37	0.57

Values for each patient are given as the mean of duplicate lipoprotein preparations. Triglycerides, free, and esterified cholesterol were measured enzymatically; PC and SPM were measured by the densitometric method (28).

TABLE 3. Relative composition of the d 1.006–1.019 g/ml lipoprotein lipids

	I	II	III	IV	V	VI	VII	VIII	IX	X	Control (n = 10)
						mol %					
TG	50	37	30	20	39	32	30	25	16	30	55 ± 10
CE	32	30	30	28	28	34	32	40	43	32	18 ± 5
FC	16	23	30	18	26	26	32	32	33	29	22 ± 6
PC	1	7	8	24	5	6	4	2	6	7	3 ± 1
SPM	1	3	2	10	2	2	2	1	2	2	2 ± 1
FC/CE	0.48	0.77	1.0	0.64	0.93	0.76	1.0	0.8	0.77	0.91	1.22

See table 2 for patient values and analyses.

VLDL-triglyceride hydrolysis studies with purified LPL and HTGL

The Lineweaver-Burk plots of the VLDL-triglyceride hydrolysis (investigating VLDL fractions derived from nine of the ten apoE-2 homozygote patients) are demonstrated in **Fig. 6.** The hydrolysis kinetics for HTGL are demonstrated in the upper panel (Fig. 6) and the kinetics for LPL in lower panel (Fig. 6). It could be demonstrated in these experiments, that for both enzymes the reaction kinetics (1/V) and the enzyme affinities (1/S) were lower for all apoE-2 VLDL preparations as compared to VLDL from apoE-3 homozygote controls. However, within the group of apoE-2 homozygotes, the VLDL fractions from patients III and VI revealed for both enzymes significant better enzyme kinetics compared to the other apoE-2 homozygotes.

DISCUSSION

The group of apoE-2 homozygote patients reported in this study has been well characterized concerning the structural and functional properties of apoE (14) and is derived in part from our recently described epidemiological study (21).

It could be demonstrated that in the apoE-2 patients the percent amount of free cholesterol and cholesteryl esters transported in the d < 1.006 g/ml lipoproteins and d 1.006–1.019 g/ml lipoproteins increased with the degree of hypertriglyceridemia (Fig. 1) and was found to be three- to tenfold higher as compared to controls. In addition, the composition of these lipoproteins changed with the degree of hyperlipoproteinemia and, in the most hypertriglyceridemic patients, total cholesterol at the expense of triglycerides became a major lipid constituent (Tables 2 and 3).

It is interesting to note that the mass ratio of β-VLDL to pre-β-VLDL (% β-VLDL/% pre-β-VLDL) differed widely and was 8%, 14%, and 12% in patients I, III, and VI, respectively; between 58% and 78% in patients II, IV, V, and VII; and above 80% in patients VIII, IX, and X. This ratio did not parallel the degree of hypertriglyceridemia which is evident upon comparing patient II (normotriglyceridemic, high β-VLDL content) and patient VI (normotriglyceridemic, low β-VLDL content).

The d < 1.006 g/ml lipoproteins obtained from patients I, III, and VI (lowest β-VLDL content) did not cause cellular cholesteryl ester accumulation in macrophages. By contrast, cellular cholesteryl ester concentrations reached five- to tenfold concentrations above control levels when macrophages were incubated with d < 1.006 g/ml lipoproteins obtained from all other apoE-2 patients (β-VLDL content above 55%). Therefore, it appears that the capacity of the d < 1.006 g/ml lipoproteins to induce the macrophage cholesteryl ester storage is related to the relative proportion of β-VLDL in this density class. In contrast to the d < 1.006 g/ml lipoproteins, d 1.006–1.019

TABLE 4. Relative distribution of d < 1.006 g/ml lipoprotein subfractions with pre-β- and β-mobility in agarose electrophoresis (total lipoprotein fraction = 100%)

Mobility	I	II	III	IV	V	VI	VII	VIII	IX	X
						%				
Pre-β	92	39	86	22	25	88	42	19	15	15
β	8	61	14	78	75	12	58	81	85	85

Electrophoresis was carried out for 30 min at 9 V/cm with Sudan black B-prestained d < 1.006 g/ml lipoproteins. After fixing the gel, the relative distribution of the pre-β- and β-band was determined with an LKB 2200-002 laser densitometer connected to a Spectra Physics 4270 Basic Integrator.

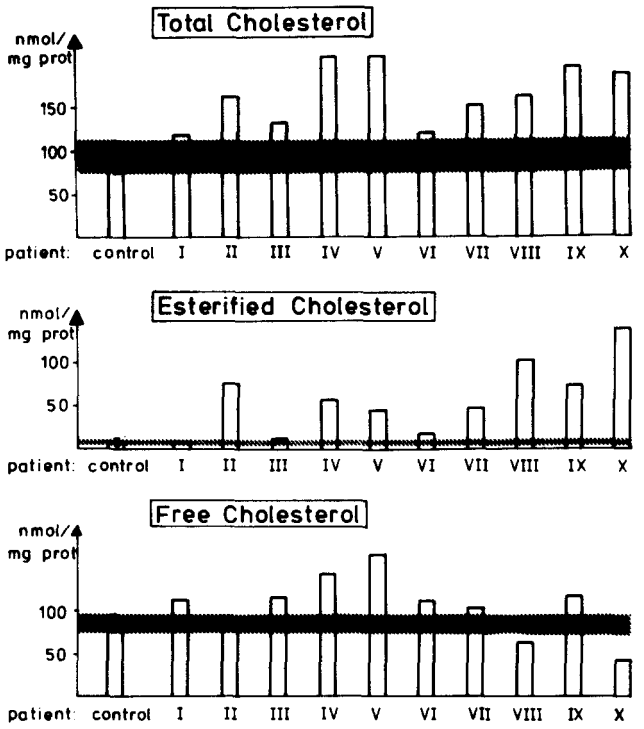

Fig. 2 Incubation of the d < 1.006 g/ml lipoprotein with cultured mouse peritoneal macrophages. Murine peritoneal cells (8–10 × 10^6) were dispensed into replicate 60-mm dishes as described in Materials and Methods. After the adherence step and subsequent washes, each dish received 3 ml of DMEM containing the d < 1.006 g/ml lipoproteins, added at identical triglyceride concentrations (150 μg of triglycerides/dish). After 16 hr incubation, the monolayers were washed and the cells were harvested, and their lipid content was measured by densitometric analysis. Each value is the average of duplicate incubations. The hatched beam indicates the control level.

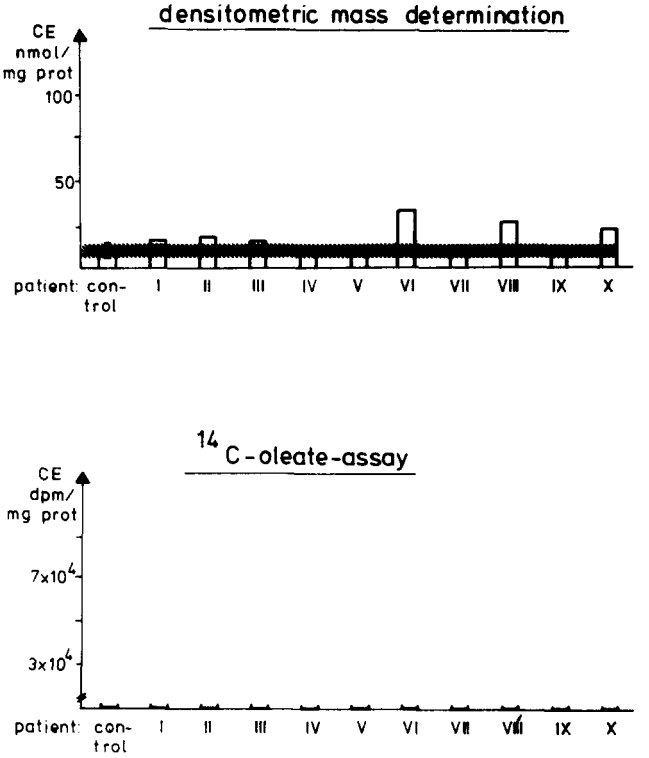

Fig. 4 Incubation of the d 1.006–1.019 g/ml fraction of apoE-2 homozygotes with mouse peritoneal macrophages. Cells were cultured under the same conditions as described in Figs. 2 and 3. The d 1.006–1.019 g/ml fraction was added at a concentration of 150 μg of triglycerides/dish. After incubation the cellular cholesteryl ester content (upper panel) was measured by densitometric analysis and the formation of [^{14}C]cholesteryl oleate (lower panel) was determined by the [^{14}C]cholesteryl ester oleate assay (see Methods).

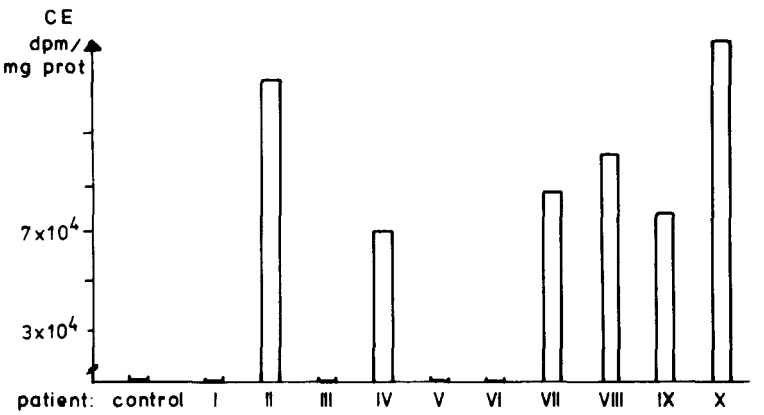

Fig. 3 Ability of the d < 1.006 g/ml lipoproteins to stimulate cholesteryl ester formation in macrophages. Murine peritoneal cells (8–10 × 10^6) were dispensed into replicate 60-mm dishes. After the adherance step and subsequent washes, each dish received 3 ml of DMEM containing 10% human lipoprotein-deficient serum and was then incubated for 24 hr. The medium was replaced with 3 ml of DMEM containing 0.2 mM [^{14}C]oleate (5 × 10^6 cpm/dish) and the d < 1.006 g/ml fraction at identical triglyceride concentrations (150 μg of triglycerides/dish). After 16 hr incubation, the monolayers were washed and the cells were harvested and their content of cholesteryl [^{14}C]oleate was then measured as described in Methods. Each value is the average of duplicate incubations.

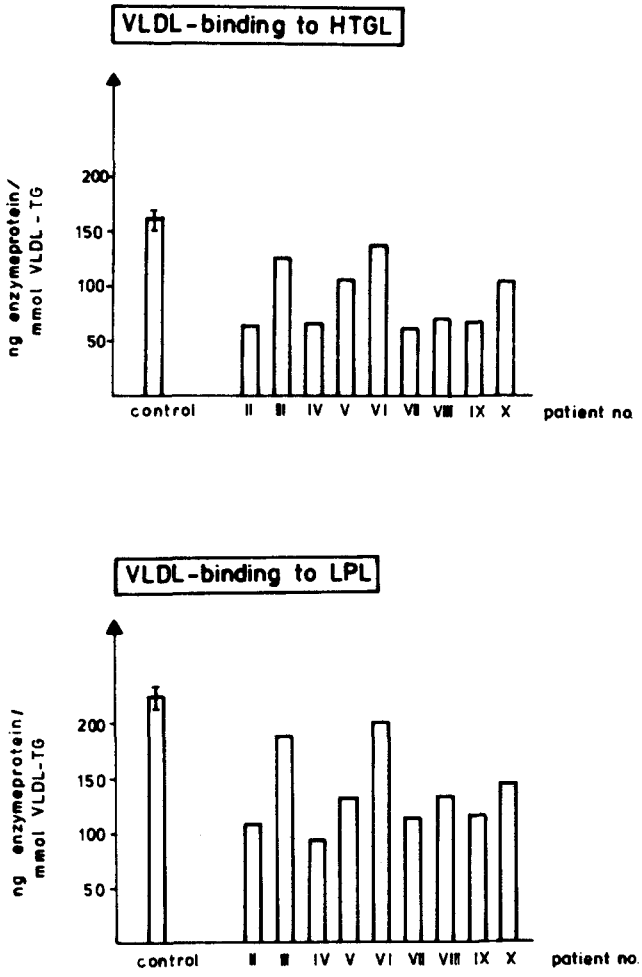

Fig. 5 The maximal binding activities for HTGL (upper panel) and LPL (lower panel) were determined with eight different VLDL-triglyceride concentrations (0–1500 μmol of VLDL triglycerides) from each individual patient (for details see Materials and Methods). Binding activities (ng of enzyme protein/mmol of VLDL triglycerides) were calculated from the differences of enzyme activities (measured as μmol of free fatty acids liberated per l per hr) in the top and bottom fractions and represent mean values of double determinations. Patient I could not be investigated because of his unwillingness to participate in this study.

g/ml lipoproteins isolated from apoE-2 homozygotes failed to induce cholesteryl ester storage in macrophages (Fig. 4). This result may be due to the low LDL receptor activity on mouse peritoneal macrophages.

VLDL binding and hydrolysis studies with purified LPL and HTGL clearly indicated, similar to the data reported by Chung and Segrest (39), that there is a reduction in enzyme binding of both enzymes and the rate of hydrolysis in the presence of VLDL preparations derived from the apoE-2 homozygote patients. The VLDL fractions from patients with a low β-VLDL content (I, III, VI) were better substrates for these lipolytic enzymes compared to those with a high β-VLDL content. The origin of this finding is at present unclear. Isolation of β-VLDL and its subfractions (chylomicron remnants, VLDL remnants) will be helpful in further elucidating the nature of this impaired interaction.

It is well established that apoE-2 homozygosity and type III hyperlipoproteinemia are associated with the presence of β-VLDL (5, 6, 8, 9). There is growing evidence that β-VLDL is possibly related to the atherogenic process (2, 7, 40). However, apparently not all patients affected by apoE-2 homozygosity are at risk for premature atherosclerosis. In our previous studies in coronary angiography patients, we could demonstrate

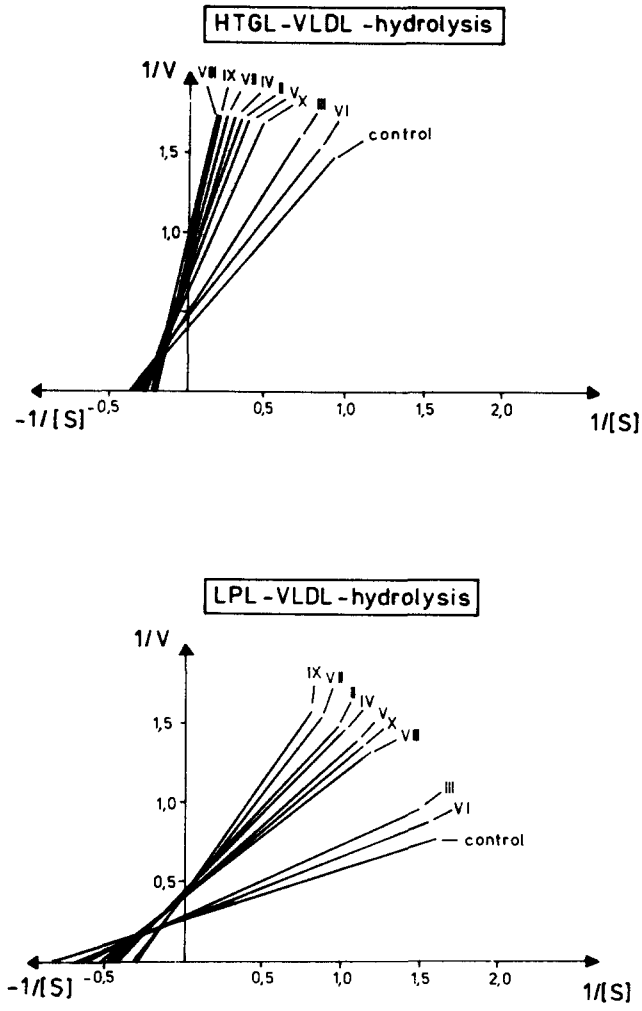

Fig. 6 Lineweaver-Burk plots for VLDL hydrolysis of nine apoE-2 homozygote patients and normolipidemic apoE-3 homozygote controls with purified HTGL (upper panel) and LPL (lower panel). The data were calculated from hydrolysis determinations with eight different VLDL concentrations (0–1500 μmol of VLDL-triglycerides) from each individual patient (for details see Materials and Methods). Enzyme activities were measured as μmol of free fatty acids liberated per l per hr. Patient I could not be investigated, because of his unwillingness to participate in this study.

that approximately 50% of these patients belong to the CAD$^+$ group (equal to or greater than 50% stenosis of one or more coronary vessels) (21). Theoretically, the other patients unaffected by CAD may be at average risk for atherosclerosis because of a more favorable concentration of LDL (low LDL-cholesterol) or β-VLDL (low concentration) or both. Because of the multiple factors influencing LDL and β-VLDL concentrations (VLDL synthesis, lipase interaction, receptor recognition, expression of receptors), reliable assessment of individual atherogenic risk cannot be achieved at present. However, it is not unlikely that the determination of the β-VLDL plasma concentrations, irrespective of normolipidemia or hyperlipidemia, may turn out to be an important prognostic parameter.

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