

Familial combined hyperlipidemia plasma stimulates protein secretion by HepG2 cells: identification of fibronectin in the differential secretion proteome

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Abstract The aim of this study was to evaluate whether soluble factors in plasma of familial combined hyperlipidemia (FCHL) patients affect hepatic protein secretion. Cultured human hepatocytes, i.e., HepG2 cells, were incubated with fasting plasma (20%, v/v, in DMEM) from untreated FCHL patients or normolipidemic controls. Overall protein secretion was 10–15% higher after incubation with FCHL plasma. This was specifically caused by an increase in four secreted proteins, with estimated sizes of 240, 180, 120, and <40 kD ($P < 0.001$, $P < 0.006$, $P < 0.002$, $P < 0.02$, respectively). The 240 kD protein in the secretion proteome was identified as fibronectin by mass spectrometry. Plasma fibronectin concentrations were elevated in FCHL patients, confirming biological relevance of these data. Overall protein secretion by HepG2 cells correlated with concentrations of triglycerides ($r = 0.61$, $P < 0.001$) in the applied plasma samples. VLDL+IDL isolated from FCHL patients, induced a higher protein secretion than lipoproteins isolated from controls ($P < 0.001$). Remarkably, secretion of apoB, the structural protein of VLDL, was stimulated to a similar extent by FCHL and control plasma. FCHL plasma did not induce excess secretion of apoB by HepG2 cells compared with control plasma. FCHL plasma did stimulate secretion of several distinct hepatic proteins, among which fibronectin was identified.—van Greevenbroek, M. M. J., V. M. M-J. Vermeulen, and T. W. A. de Bruin. **Familial combined hyperlipidemia plasma stimulates protein secretion by HepG2 cells: identification of fibronectin in the differential secretion proteome.** *J. Lipid Res.* 2002. 43: 1846–1854.

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Familial combined hyperlipidemia (FCHL) is a common dyslipidemia with a strong genetic component. The prevalence of FCHL in the population is 1–2% and FCHL is estimated to cause 10–20% of premature coronary heart disease (CHD) (1). Metabolic disturbances in FCHL include overproduction of atherogenic apolipoprotein B

(apoB)-containing lipoproteins (i.e., VLDL), delayed clearance of lipoproteins, increased plasma apoB concentrations, increased free fatty acid (FFA) fluxes, and insulin resistance. The molecular mechanism(s) that underlie these metabolic abnormalities are not yet known. It has been suggested that increased plasma FFA fluxes, potentially induced by insulin resistance, may increase hepatic secretion of apoB containing lipoproteins. However, available data on the direct effects of increased FFA fluxes on hepatic apoB production in normal human subjects are limited and apparently conflicting (2, 3). These data do not yet allow definitive conclusions on the hepatic abnormality in FCHL.

The liver plays a central role in the development of atherosclerosis (4). It is an important source of vaso-active compounds as well as atherogenic, apoB containing lipoproteins. Current knowledge on intrahepatic assembly of apoB containing lipoproteins mainly derives from studies in laboratory animals and cells in culture (5). Following intracellular association of lipid with apoB, the precursor lipoproteins fuse with luminal triglyceride droplets to form mature VLDL that is secreted from the cells (6). The scarce availability of hepatocytes from human FCHL patients renders it not feasible to directly study the role of the liver-specific pathways in FCHL in vitro. It is presently unresolved whether the hepatic hypersecretion of lipoproteins that is observed in FCHL is a process inherent to metabolic abnormalities in the liver, or may be driven by soluble factors (for instance fatty acids or cytokines) in FCHL plasma, or both. In HepG2 cells, a human hepatoma cell line, VLDL production is usually measured as secretion of apoB. HepG2 cells secrete mainly IDL sized apoB containing lipoproteins, because of a hampered capacity to recruit intracellular triglycerides (7), possibly due to the absence of microsomal triglyceride hydrolase

Abbreviations: CHD, coronary heart disease; CRE, cAMP response elements; FCHL, familial combined hyperlipidemia; FFA, free fatty acid.

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(8), yet the major pathways that regulate intracellular lipid metabolism and lipoprotein production are evidently present in HepG2 cells. Various aspects of hepatic cholesterol and bile acid synthesis have been studied in HepG2 cells (9, 10), as well as hepatic lipoprotein turnover (11, 12) and intrahepatic lipoprotein assembly (13–15). ApoB secretion by HepG2 cells can be altered by numerous substances that are also found in human plasma, e.g. certain species of fatty acids (16), insulin (17), lysophosphatidylcholine (18), and interleukins (19). Such substances are thought to regulate the human VLDL secretion in vivo as well. The HepG2 cell line therefore is a useful in vitro model to evaluate potential effects that extrahepatic factors in the plasma of FCHL patients, for instance originating from adipose tissue, can exert on hepatic metabolism and (lipo)protein secretion.

It was the aim of the present study to evaluate whether plasma factors from FCHL patients can affect secretion of proteins or (apo)lipoproteins that can play a role in the development of FCHL, or its serious cardiovascular consequences, using the human cell line HepG2 as an in vitro model of the human hepatocyte.

METHODS

Cell culture

HepG2 cells (ATCC) were cultured according to suppliers instructions. For experiments, cells were plated in 12 well culture plates (Costar, Cambridge, MA) with 1 ml of DMEM/10% FCS and cultured for 2–3 days. One day prior to the experiments, medium was changed to serum free DMEM. Experiments were done when HepG2 monolayers were 80% confluent.

Patient identification and plasma collection

We used plasma from FCHL patients and control subjects who were collected in our laboratory in the framework of genetic studies on hyperlipidemia (20). Control subjects were normolipidemic spouses of the FCHL patients. We used plasma from 54 FCHL patients and 33 controls in three separate series of incubations of HepG2 cells. We also used 41 FCHL patients and 34 controls to determine plasma fibronectin concentrations. In this latter group, 12 FCHL patients and 16 controls were newly studied, the remaining subjects (29 FCHL, 18 controls) had been studied in the HepG2 cell incubations. Venous blood was drawn after an overnight fast (12–14 h) in sodium citrate containing tubes (Greiner Labortechnik GmbH, Kremsmunster, Austria). Plasma samples were prepared by immediate centrifugation (21). Plasma concentrations of apoB, cholesterol, triglycerides, free fatty acids (FFA), and insulin were determined as described elsewhere (20, 22). The study protocol was approved by the Human Investigation Review Committee of the Academic Hospital Maastricht and performed according to the Helsinki declaration. All subjects gave written informed consent.

Protein labeling

All incubations were performed in duplicate wells. HepG2 monolayers were washed twice with serum free and methionine free DMEM, and pre-incubated for 60 min with 300 μ l DMEM containing 20% human plasma (vol/vol), 1% non-essential amino acids, 100 U/ml penicillin, and 100 μ g/ml streptomycin and 150 U/ml streptokinase as anticoagulant (Sigma Co, St Louis, MO) (23). To this medium, 60 μ Ci of L-[³⁵S]methionine

(Amersham-Pharmacia, Uppsala Sweden) in 24 μ l medium was added and cells were labeled for 60 min. Cells were chased for 3 h with 300 μ l of the same medium as used for pre-incubation. At the end of the incubation, media were collected on ice and cell debris was removed by short centrifugation at 4°C. The cells were immediately rinsed twice with 0.5 ml ice-cold phosphate buffered saline (PBS), and lysed and scraped in ice-cold PBS containing 0.5% triton X100. The wells were rinsed with an additional volume of PBS/triton X100 that was combined with the scraped cells. Final volume of the cell lysate was 570 μ l.

Processing of media

Fifty microliters of conditioned medium were used to quantify radioactivity in TCA precipitable protein (24). Secreted proteins were separated on 5% or 7.5% SDS polyacrylamide gel electrophoresis using the BioRad Mini Protean II configuration (BioRad, Hercules CA). Samples were applied in duplicate on the gel and purified apoB and pre-stained molecular weight markers were used as standards. Radioactivity in the protein bands was determined with a phosphor imager and the data were processed with the Quantity One software (BioRad).

Processing of cell lysates

One hundred microliters of lysate were used to quantify radioactivity in TCA precipitable protein.

Isolation of lipoprotein fractions

0.85 microliters KBr solution (58.4 g in 100 ml H₂O) was carefully mixed with 1 ml fresh plasma and 0.52 ml of 1 mM EDTA in saline, overlaid with 2.5 ml of 1 mM EDTA, and centrifuged for 1 h at 80,000 rpm in a NVT 90 rotor (Beckman, Inc Palo Alto, CA). The upper 0.8 ml contained VLDL+IDL (VLDL-IDL fraction), the middle 1.6 ml contained LDL (LDL fraction), and the bottom fraction contained HDL+lipoprotein depleted plasma (HDL/lpd fraction). For cell incubations, the isolated and desalted lipoproteins were diluted in DMEM with 1% non-essential amino acids, 100 U/ml penicillin, and 100 μ g/ml streptomycin and 150 U/ml streptokinase, so to correspond with the amount of lipoprotein present in 20% plasma. Incubations and analyses were done as described for the 20% plasma incubations.

Identification of 240 kD protein

Proteins secreted by HepG2 cells in a serum free DMEM incubation were separated on 5% gel and stained with Coomassie Brilliant Blue. The single 240 kD band was cut out, destained with 100 μ l of a mixture of 50% acetonitrile in 25 mM NH₄HCO₃ and dried. Gel pieces were incubated with 10 mM dithiothreitol (DTT) in 25 mM NH₄HCO₃ for 1 h at 56°C. Subsequently, DTT solution was replaced with the same volume of 55 mM iodoacetamide in 25 mM NH₄HCO₃ and vortexed for 45 min in the dark. Gel pieces were washed and dehydrated with 50% acetonitrile in 25 mM NH₄HCO₃, dried and digested with 0.1 μ g/ μ l trypsin at 37°C for 4 h. Fragments were extracted with 50% acetonitrile and 5% trifluoroacetic acid in H₂O and analysed on an electron spray ionization mass spectrometer (ESI-MS).

Plasma fibronectin concentrations

Fasting plasma concentrations of fibronectin were determined in citrate plasma of 41 FCHL patients and 34 spouse controls, using a commercially available nephelometric method (Dade Behring, Walton, UK).

Statistical analyses

Data are expressed as mean \pm SD. Data for triglyceride and insulin were analyzed after log transformations, because their non-transformed distributions were skewed. When applicable, data

were age and BMI corrected using multiple linear regression. Independent Student's *t*-tests were done to compare groups. Pearson's product moment correlation coefficients (Pearson's *r*) were calculated. All statistical analyses were conducted using SPSS 9.0.

RESULTS

Three independent series of incubations of HepG2 cells with plasma were done. The first set of fasting plasma samples was obtained from 16 FCHL patients and 11 spouse controls. The second set was obtained from 20 FCHL patients and 10 spouse controls. In the third set of incubations, the effects of separate lipoprotein fractions were compared using plasma from 18 FCHL patients and 12 spouse controls. There was no overlap in individuals between the three groups and, in total, plasma samples from 87 individuals (54 FCHL patients and 33 controls) were used in incubations with HepG2 cells. The characteristics of the subjects group used in one of the incubations are shown in **Table 1**. There were no differences between the groups of subjects used in the three series of incubations.

Effects of FCHL plasma on protein secretion by HepG2 cells

In the first series of experiments, the overall amount of newly synthesized protein that was secreted into the medium [expressed as (cpm in TCA precipitable protein in medium / cpm in TCA precipitable protein in cells + medium) × 100%] was higher in cells incubated with FCHL plasma ($9.5 \pm 1.0\%$) than with controls ($8.6 \pm 1.1\%$, $P < 0.05$). The amount of labeled protein in the cells was lower and the amount secreted into the medium was higher, resulting in the calculated higher proportion of newly synthesized protein that was secreted following incubation with FCHL plasma. These data were confirmed in a second series of incubations. Again, secretion of TCA precipitable protein was higher with FCHL plasma than in control incubations ($13.4 \pm 1.3\%$ vs. $11.6 \pm 1.3\%$, respectively, $P < 0.005$). Secretion of newly synthesized proteins correlated strongly with the concentration of log[Triglycerides] ($r = 0.49$, $P < 0.001$) and log[insulin] ($r = 0.41$, $P = 0.002$) in the plasma samples used (**Fig. 1A, B**). When the FCHL subjects were analyzed separately, the correlation between protein secretion and plasma log[triglycerides] was maintained. No correlations were found with any other measured plasma parameter, such as cholesterol, apoB, or FFA.

TABLE 1. Plasma parameters of FCHL and control subjects (mean ± SD)

	Control	FCHL
Cholesterol (mmol/l)	4.83 ± 0.53	7.34 ± 1.21^a
Triglycerides (mmol/l)	0.98 ± 0.28	2.53 ± 2.09^a
ApoB (g/l)	0.93 ± 0.14	1.60 ± 0.30^a
Free fatty acids (mmol/l)	0.37 ± 0.18	0.47 ± 0.25
Insulin (mU/l)	5.33 ± 3.62	10.15 ± 6.2^a

The control group included 10 subjects. The FCHL group included 20 subjects. Data are representative for the three groups of subjects used in the experiments.

^a $P < 0.001$ control versus FCHL (Student's *t*-test).

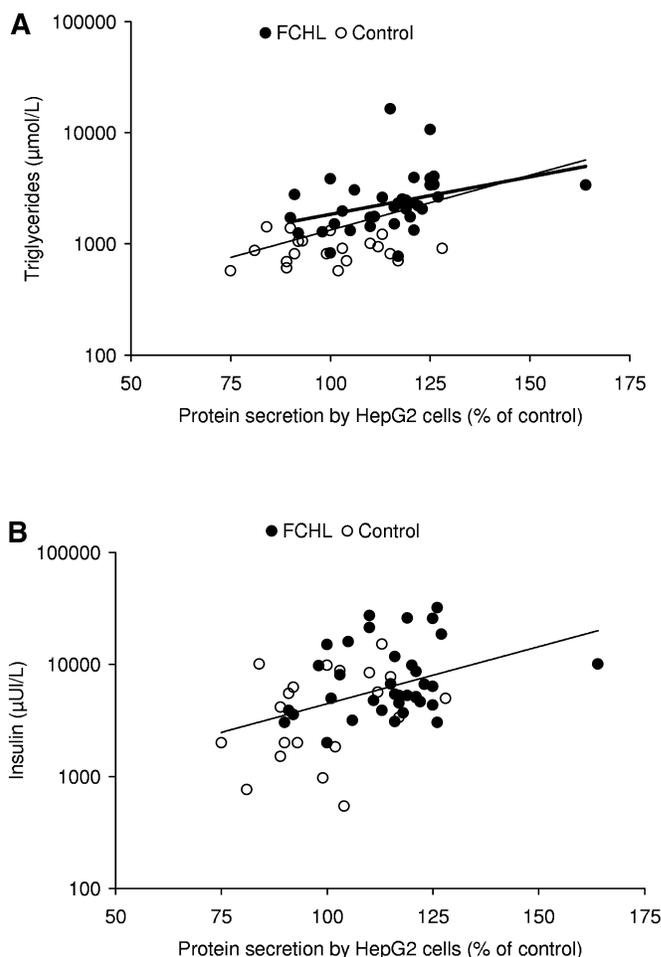


Fig. 1. Relationship between plasma triglycerides and insulin concentrations and the amount of newly synthesized protein that was secreted by the HepG2 cells. The two independent series of incubations were analyzed together as follows: in each series, individual values for protein secretion were normalized to the average value of the controls. A: Correlation between plasma triglycerides and protein secretion. In the entire group, $r = 0.49$, $P < 0.001$ (thin trendline); in the control group, $r = -0.19$ ns; in the FCHL group, $r = 0.35$, $P < 0.05$ (bold trendline). B: Correlation between plasma insulin and protein secretion. In the entire group, $r = 0.41$, $P = 0.002$ (thin trendline); in the control group, $r = 0.34$ ns; in the FCHL group, $r = 0.25$ ns.

In the second series of incubations, we additionally analyzed the secretion proteome of the HepG2 cells on one-dimensional SDS electrophoresis. We identified four protein bands in the conditioned media with estimated sizes of 240, 180, 120, and <40 kD, respectively, that were significantly more secreted with FCHL plasma (**Table 2**). The 240 kD protein band was secreted 1.4-fold higher with FCHL plasma than with control plasma ($P < 0.0005$). This 240 kD protein was secreted in relatively large amounts by the HepG2 cells, i.e., 8–9% of total secreted radiolabeled proteins. The 180 and 120 kD proteins were secreted 1.2- and 1.3-fold higher with FCHL plasma ($P < 0.01$ and $P < 0.005$, respectively). These proteins were less abundant, up to 3% and 1%, respectively, of total secreted protein. The contribution of the smallest protein band

TABLE 2. Newly synthesized proteins secreted by HepG2 cells

	Control	FCHL
Total protein (au × 10 ³)	257.9 ± 36.0 (100%)	303.7 ± 64.3 ^a (118%)
240 kD protein band (au × 10 ³)	20.3 ± 4.3 (100%)	27.7 ± 42.9 ^d (137%)
80 kD protein band (au × 10 ³)	8.5 ± 1.0 (100%)	10.3 ± 1.7 ^b (121%)
120 kD protein band (au × 10 ³)	3.2 ± 0.4 (100%)	4.0 ± 1.0 ^c (127%)
40 kD protein band (au × 10 ³)	15.1 ± 2.6 (100%)	20.0 ± 4.2 ^a (132%)
ApoB (au × 10 ³)	19.3 ± 4.9 (100%)	20.5 ± 3.6 (106%)

The control group included 10 subjects. The FCHL group included 20 subjects. HepG2 cells were incubated in duplicate for 3 h with 20% plasma from control subjects or FCHL patients as described in the experimental procedures. Secreted proteins were separated in duplicate on gelelectrophoresis and radioactivity in the protein bands was determined. Data are mean ± SD. Total protein is the total amount of radioactivity per lane. The data for total protein, 140, 180, 120, and <40 kD proteins are from a 20 h exposure, the apoB data are from a 3 day exposure of the phosphor imager screen.

^a $P < 0.05$, ^b $P < 0.01$, ^c $P < 0.005$, ^d $P < 0.001$ control versus FCHL (Student's *t*-test).

(<40 kD) was 5–6% of total, and its secretion was stimulated 1.3-fold with FCHL plasma ($P < 0.05$). In addition, we specifically examined the secretion of apoB-100 in the media. ApoB, detectable as a 550 kD protein on SDS gelelectrophoresis, was present in the secretion proteome of all incubations, but no difference was seen in the stimulation of apoB secretion between the incubations with FCHL and control plasma (Table 2).

Effects of VLDL-IDL lipoproteins on total protein secretion

Because of the observed correlation between plasma triglyceride concentration and total protein secretion by HepG2 cells, we incubated the cells with lipoproteins of variable triglyceride content, i.e., VLDL-IDL, LDL, and HDL/lpd isolated from plasma of FCHL patients and controls. VLDL-IDL from FCHL patients stimulated secretion of newly synthesized proteins by HepG2 cells 1.4-fold, compared to control VLDL-IDL ($P = 0.001$; Table 3). The amount of 240 kD in the medium was also higher, but this did not reach the threshold of statistical significance. The amount of triglycerides in FCHL-VLDL-IDL correlated significantly with the secretion of newly synthesized proteins ($r = 0.56$, $P < 0.02$; Fig. 2A) as well as with secretion of the 240 kD protein ($r = 0.54$, $P < 0.025$) (Fig. 2B). This correlation was specifically caused by FCHL-derived VLDL-IDL. Notably, there were no significant correlations with the triglyceride content of either LDL or HDL lipoprotein fraction isolated from FCHL patients or controls. Moreover, protein secretion did not differ between FCHL and controls, when LDL or HDL/lpd fractions were used (Table 3).

Identification of the 240 kD protein

The protein band with an apparent molecular weight of 240 kD protein was visible as a single thick ³⁵S methionine

TABLE 3. Lipoprotein fractions and protein secretion by HepG2 cells

	Total Protein Secretion		240 kD Protein Secretion	
	Control	FCHL	Control	FCHL
VLDL-IDL	7.29 ± 2.37 (100%)	9.97 ± 1.75 ^a (137%)	885 ± 164 (100%)	992 ± 219 (112%)
LDL	8.25 ± 0.73 (100%)	8.79 ± 0.89 (107%)	897 ± 74 (100%)	913 ± 351 (102%)
HDL/lpd	11.27 ± 1.47 (100%)	10.42 ± 2.44 (92%)	1065 ± 176 (100%)	1100 ± 351 (103%)

HepG2 cells were incubated in duplicate for 3 h with isolated lipoproteins from control subjects (n = 12) or FCHL patients (n = 18) as described in the experimental procedures. Total protein secretion is the secretion of newly synthesized TCA precipitable protein (expressed as cpm in medium/cpm in cells + medium × 100%) by HepG2 cells. Secreted 240 kD protein is the amount of radiolabeled 240 kD protein (expressed as au) that is present in the media at the end of the incubations.

^a $P = 0.001$ control versus FCHL (Student's *t*-test).

labeled band on 5% SDS gelelectrophoresis. The radiolabeled samples could not be directly used for protein analysis since they contained 20% human plasma. To determine the identity of the protein of approximate molecular weight of 240 kD, HepG2 cells were cultured for 18 h in serum free DMEM and secreted proteins were separated on 5% SDS PAGE and Coomassie stained. Again, a single thick band was seen at ~240 kD. This band was excised, analyzed on ESI-MS, and identified as human fibronectin precursor (NCBI access No GI [279675]). Ten trypsin digestion fragments were identified at positions 939–953, 1198–1207, 1252–1274, 1255–1274, 1435–1452, 1525–1539, 1533–1539, 1540–1561, 1892–1910, and total coverage of the protein was 5.7% by amino acid count. The identification was confirmed by immunoblotting with a specific polyclonal antibody against human plasma fibronectin (Sigma-Aldrich) (Fig. 3).

Plasma fibronectin concentrations in FCHL subjects

We evaluated whether FCHL-plasma-induced increases in fibronectin synthesis and secretion in vitro could be confirmed in the in vivo situation. Indeed, plasma fibronectin concentrations were significantly higher in FCHL patients (541 ± 147 mg/l; n = 41) than in controls (329 ± 89 mg/l; n = 33, $P < 0.001$ after correction for age and BMI). Plasma fibronectin concentration correlated significantly with plasma triglyceride concentrations ($r = 0.73$, $P < 0.001$) (Fig. 4). In addition, associations were seen with several parameters of hyperlipidemia, e.g., cholesterol, apoB, LDL-cholesterol (positively), and HDL-cholesterol (negatively). However, most of these apparent correlations resulted from the fact that both fibronectin and the parameter studied were higher in the FCHL patients than in controls. The single correlation which persisted when the FCHL and control groups were analyzed separately, was found with plasma triglyceride concentrations (FCHL; $r = 0.45$, $P < 0.005$, Controls $r = 0.60$, $P < 0.001$). This finding is in agreement with the in vitro data.

FCHL patients can present with different phenotypes of hyperlipidemia. To determine whether different plasma

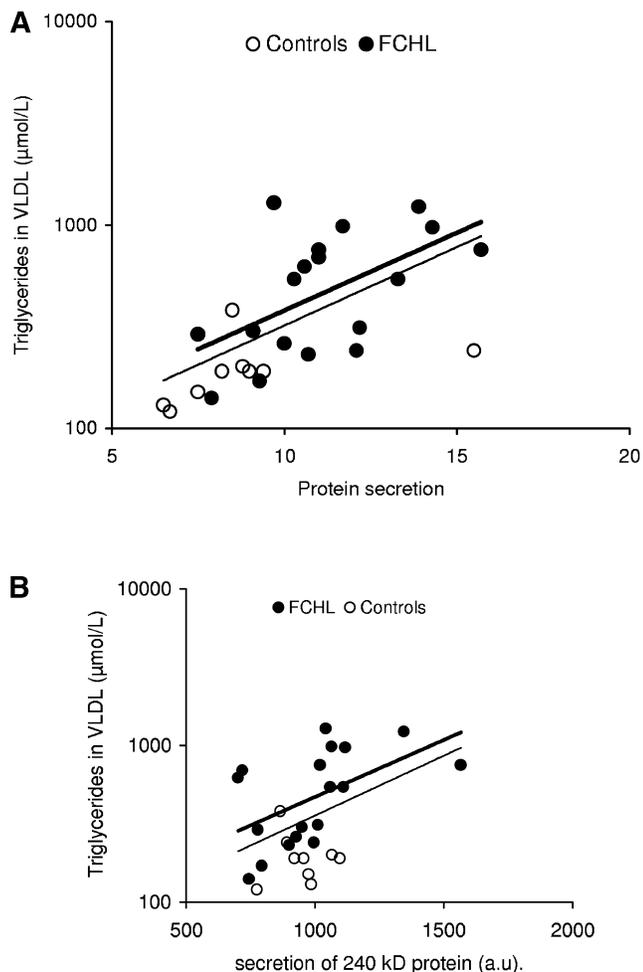


Fig. 2. Relationship between VLDL-triglyceride concentration and amount of newly synthesized protein or the amount of 240 kD protein secreted by the HepG2 cells. **A:** Correlation between VLDL-triglycerides and total protein secretion. In the entire group, $r = 0.62$, $P < 0.001$ (thin trendline); in the control group, $r = 0.490$ ns; in the FCHL group, $r = 0.56$, $P < 0.02$ (bold trendline). **B:** Correlation between VLDL-triglycerides and 240 kD protein secretion. In the entire group, $r = 0.45$, $P < 0.02$ (thin trendline); in the control group, $r = -0.02$ ns; in the FCHL group, $r = 0.54$, $P < 0.025$ (bold trendline).

phenotypes have specific effects on fibronectin production, we subdivided our patient groups into three phenotypic categories i.e., 1) Hypercholesterolemic individuals (HC): FCH relatives with cholesterol >6.5 mmol/l (7.5 ± 1.1 mmol/l) and normal triglycerides (1.5 ± 0.3 mmol/l; $n = 11$); 2) hypertriglyceridemic individuals (HTG): FCH relatives with triglyceride >2.0 mmol/l (3.5 ± 1.3 mmol/l) and normal cholesterol (5.6 ± 0.7 mmol/l; $n = 9$); and 3) combined hyperlipidemic individuals (CHL): FCH relatives with a combined phenotype of triglycerides >2.0 mmol/l (3.4 ± 3.1 mmol/l) and cholesterol >6.5 mmol/l (7.8 ± 0.6 mmol/l; $n = 21$). In the normolipidemic control group (control), plasma concentrations of triglycerides and cholesterol were 1.0 ± 0.4 and 4.9 ± 0.7 mmol/l, respectively ($n = 34$). Plasma concentrations of fibronectin were increased in all three categories of FCH patients versus controls (Fig. 5A, ANOVA $P < 0.001$). A similar pat-

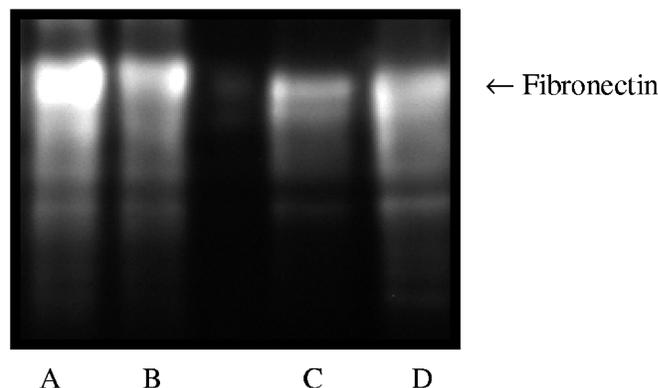


Fig. 3. Immunodetection of fibronectin (240 kD) in medium of HepG2 cells with polyclonal rabbit-anti-human fibronectin (Sigma-Aldrich Inc). The total secreted protein fraction of HepG2 cells was separated on lanes A and B (containing ~ 1.0 μ g and 0.5 μ g of the 240kD protein, respectively). Lane C and D contain 0.1 and 1.0 μ g of fibronectin isolated from human plasma.

tern was observed in the fibronectin secretion by the HepG2 cells incubated with plasma from each of these categories (Fig. 5B, ANOVA $P < 0.001$). The HTG subjects showed the highest plasma fibronectin concentrations, and most fibronectin secretion, followed in descending order by CHL $>$ HC $>$ normolipidemic controls. These data support the biological plausibility that the triglyceride content of FCHL-VLDL or a factor that is associated with these lipoproteins determines the secretion of fibronectin by HepG2 cells in vitro, as well as the production of fibronectin, in vivo.

DISCUSSION

The aim of the present study was to determine whether plasma from FCHL patients differed from control plasma in its capability to increase hepatic protein or lipoprotein secretion. The human cell line HepG2 was used as an in vitro model of the human hepatocyte. The data demonstrated that FCHL plasma stimulated secretion of newly synthesized proteins from HepG2 cells to a significantly higher extent than control plasma. This stimulation was restricted to a number of secreted proteins with estimated sizes of 240, 180, 120, and <40 kD, respectively. The 240 kD protein was identified as fibronectin (discussed below). Overall cellular protein secretion correlated with triglyceride and insulin concentrations in the plasmas that were applied in the incubations. It was a striking observation that cholesterol concentrations in the plasma samples did not correlate with the protein synthesis parameters that were measured in the HepG2 cells and medium. The fact that both triglyceride and insulin correlated with secreted protein may be inherent to the fact that these plasma parameters are independent regulators of protein secretion, or to the fact that they are correlated in the plasma samples that we used for HepG2 cell incubations ($r = 0.35$ for $\log[\text{insulin}]$ with $\log[\text{triglycerides}]$ $P =$

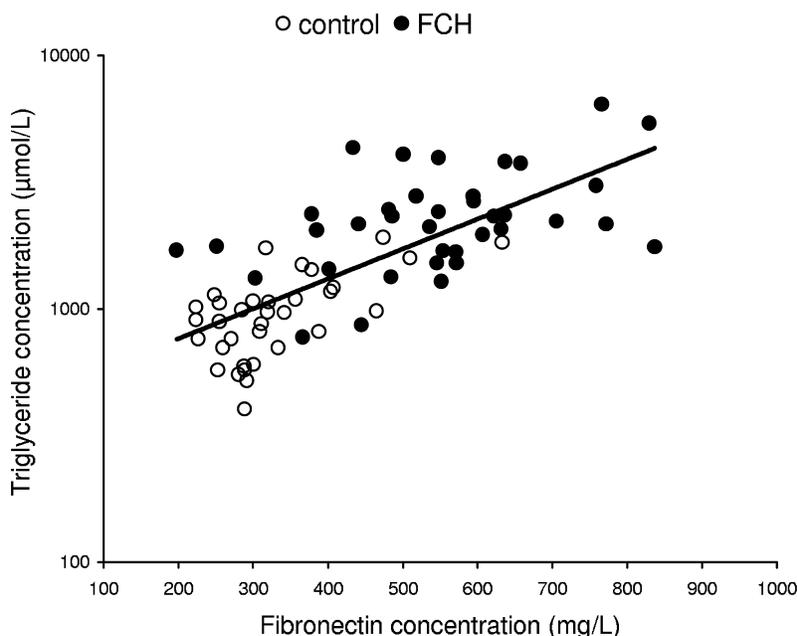


Fig. 4. Relationship between plasma concentrations of fibronectin and triglycerides. In the entire group, $r = 0.73$, $P < 0.001$; in the control group, $r = 0.60$, $P < 0.001$; in the FCHL group, $r = 0.45$, $P < 0.005$; trendline is given for the FCHL and control groups combined.

0.001, $n = 83$). To discriminate between the separate effects of insulin and triglycerides, HepG2 cells were incubated with VLDL-IDL, LDL, and HDL/lpd fractions isolated from the FCHL and control samples. Plasma triglycerides are primarily present in the VLDL-IDL fraction, whereas insulin is present in the HDL/lpd fraction. VLDL-IDL from FCHL plasma samples stimulated protein secretion by HepG2 cells [137% of incubation compared to VLDL-IDL from control plasma (100%)], whereas the HDL/lpd fraction from FCHL was not stimulatory (92% of incubation with HDL/lpd from control plasma). Analogous to the effects on total protein secretion, FCHL-VLDL-IDL also increased the amount of radiolabeled 240 kD protein, i.e., fibronectin, in the medium (ns). Significant correlations were observed between secretion of protein as well as secretion of fibronectin and triglyceride concentrations in FCHL-VLDL-IDL but not control VLDL-IDL. These correlations were absent with the triglyceride content of LDL and HDL/lpd fractions from both FCHL patients and controls. We conclude that VLDL-IDL triglycerides stimulate the secretion of newly synthesized proteins by HepG2 cells and that the correlation between HepG2 protein secretion and insulin is secondary to the correlation between insulin and triglycerides in human plasma. In theory, this effect of VLDL-IDL may also be caused by a plasma factor, e.g., a cytokine that is physically associated with the lipoproteins in the VLDL-IDL fraction.

It has been reported that VLDL isolated from normal plasma can stimulate apoB secretion by HepG2 cells compared to serum free incubations (25) and that such a stimulation of apoB secretion from HepG2 cells may be limited (7). A consistent result in the present study was that

FCHL plasma did indeed induce secretion of apoB by HepG2 cells, but this was not in excess of control plasma. In the interpretation of these data we cannot exclude the possibility that the maximum secretion capacity of HepG2 cells for apoB had already been reached when cells were incubated with 20% control plasma, thus precluding further stimulation when FCHL plasma was used. It could also be argued that stimulation of apoB secretion could not be different because plasma concentrations of FFA were not significantly different between FCHL and controls in these HepG2 cell incubations. However, there is no unequivocal evidence that plasma FFA directly induce apoB secretion by the human liver (2, 3). In our view, an alternative, more plausible explanation for the fact that FCHL plasma did not induce excess secretion of apoB compared to control subjects is that, also in vivo, FCHL plasma will not directly induce hepatic apoB secretion to a higher extent than control plasma does. This latter interpretation would imply that biochemical defect(s) involved in the overproduction of hepatic apoB-containing lipoproteins in FCHL, reside within the FCHL hepatocytes. Indeed, in human subjects with FCHL, fatty liver is frequently present (T. W. A. de Bruin, unpublished observations) in agreement with metabolic abnormalities at the level of hepatic triglyceride metabolism in FCHL. In the present study, HepG2 cells had similar intracellular triglyceride content before incubation with control and FCHL plasma samples. The potential relationship between fatty liver and excess apoB secretion is under study in our laboratory.

The increased fibronectin secretion by HepG2 cells in response to stimulation with FCHL plasma prompted us to determine the plasma concentration of this protein in

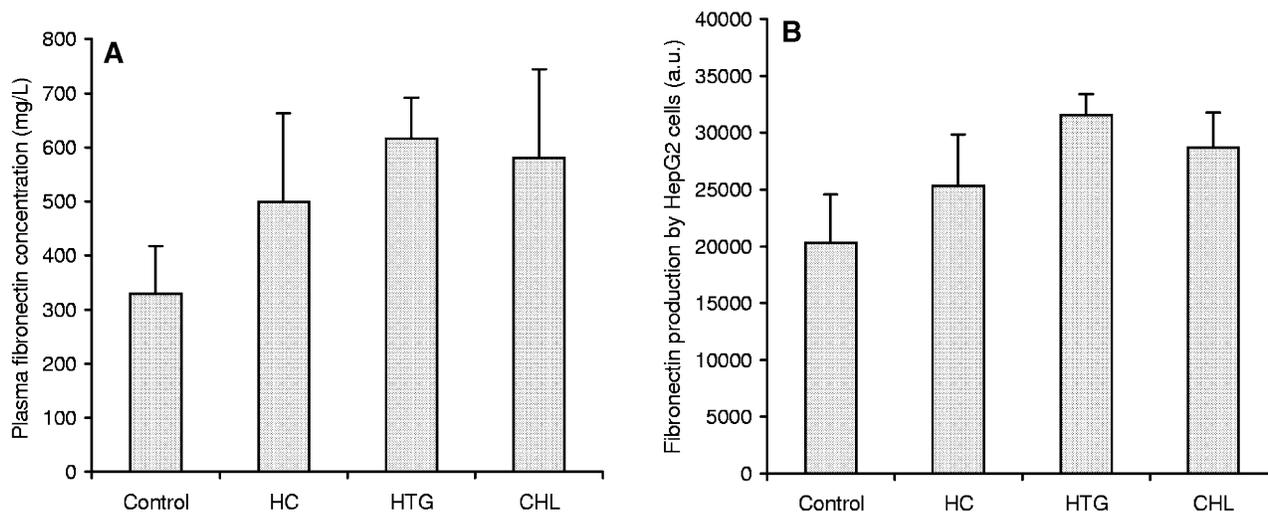


Fig. 5. The subjects used in the experiments were subdivided into four phenotype categories. Normolipidemic spouse controls (NL), FCH relatives with a phenotype of normal triglycerides and high cholesterol (>6.5 mmol/l) concentrations (CH), FCH relatives with a phenotype of normal cholesterol and high triglyceride (>2.0 mmol/l) concentrations (HTG), FCH relatives with a combined hyperlipidemic phenotype of high triglycerides (>2.0 mmol/l) and high cholesterol (>6.5 mmol/l) concentrations (CHL). A: The plasma concentration of fibronectin in the four phenotype categories. In each of the three FCH phenotypes fibronectin was higher than in controls (HC, HTG, CHL were $P < 0.005$, $P < 0.001$, $P < 0.001$ vs. Control, respectively, after Bonferroni correction). B: Shows the amount of radiolabeled fibronectin that was secreted by HepG2 cells in incubations with plasma from either of these four categories. Again, in all three FCH phenotypes, fibronectin secretion was, or tended to be, higher than in controls (HC, HTG, CHL were $P = 0.057$, $P < 0.001$, $P < 0.001$ vs. Control, respectively, after Bonferroni correction).

FCHL. A higher plasma fibronectin concentration was found in FCHL patients (541 ± 147 mg/l) than in controls (329 ± 89 mg/l). It was recently reported that fibronectin is elevated in ischemic heart disease and that hypertriglyceridemia especially is associated with plasma fibronectin concentrations (26). We observed remarkable similarities in the pattern of plasma fibronectin levels in four phenotype categories (i.e., NL, HC, HTG, or CHL) and the pattern of cellular fibronectin secretion in incubations with plasma from either of these categories. These data suggest that the same factors in FCHL plasma that induce higher secretion of fibronectin and other distinct proteins in vitro may be relevant for a higher production of fibronectin in vivo as well. The presently employed method of incubating hepatic cells with plasma or plasma fractions from patients and controls can also be implemented in future studies to identify the plasma factors involved in this stimulation.

The greater synthesis and secretion of proteins in response to FCHL plasma may be induced through activation of transcription factors. The fibronectin promoter contains, among others, cAMP response elements (CRE) (27), a vitamin D3 response element (28), a NF κ B binding motif (29), and a CCAAT box (30). The CRE element located at -170 bp in the fibronectin gene is serum-responsive (31). The identification of the three other proteins with stimulated secretion (180, 120, and <40 kD), besides fibronectin, will contribute significantly to the identification of pathways that are stimulated by FCHL plasma. The identity of these proteins is under current investigation in our laboratory. Comparison of the promoter structures and the pathways that induce their tran-

scription will yield valuable information on the specific intracellular signalling pathways that are activated by FCHL plasma. In this light, it is interesting that we have recently shown that TNF α gene expression is up-regulated in adipose tissue of FCHL patients (32) and that the TNF α type 2 receptor (TNFRSF1B) contributes to FCHL in linkage and association studies (33, 34). TNF α can induce NF κ B activation via TNF receptor-associated factor (TRAF) 2 (35) and may thus be involved in fibronectin secretion in FCHL.

In first-degree relatives of FCHL probands, the risk for non-fatal myocardial infarction is 5.1-fold increased over spouses (36). This high risk is difficult to understand from the relatively mild hyperlipidemia in FCHL given the concentrations of total cholesterol and LDL cholesterol. It is likely that other factors contribute to the high risk of cardiovascular disease. Such other risk factors may represent independent factors or factors resulting from adaptational mechanisms to the hyperlipidemia, such as an increase in fibronectin production. Serum and lipoproteins stimulate fibronectin matrix assembly by fibroblasts, and lysophosphatidic acid, which is abundant in serum and lipoproteins, has been shown to mediate this effect (37). Fibronectin is deposited in the vascular wall and can induce vascular changes that contribute to the process of early atherosclerosis (38). Increased intima-media thickness has recently been reported in FCHL subjects (39, 40). Data in the literature indicate that the possible link between fibronectin and development of atherosclerosis and hypertension is mediated via locally expressed fibronectin in the vessel wall (41–43), but our present data imply that liver-derived fibronectin could also contribute substan-

tially to these processes. Alternatively, the cellular mechanisms that induce fibronectin secretion by HepG2 cells may increase local production of fibronectin in the vessel wall.

In conclusion, we have shown that plasma from both FCHL patients and controls induces apoB secretion by HepG2 cells, but there was no evidence of excess apoB production with FCHL plasma. This potentially implies that biochemical defects involved in VLDL overproduction, a hallmark of FCHL, reside within the FCH hepatocytes. Additionally, we show that FCHL plasma specifically stimulated the secretion of four proteins by HepG2 cells. One of these was identified as fibronectin. Fibronectin can be relevant in the sequelae of FCHL, such as development of atherosclerosis, increased IMT, plaque formation, and cardiovascular events. The origin of the excess stimulation of fibronectin secretion by FCHL plasma needs to be elucidated and future identification of the other three upregulated proteins may lead to a better understanding of the cellular mechanisms underlying the induction of protein secretion by plasma from FCH patients. 

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