Defective Metabolism of Hypertriglyceridemic Low Density Lipoprotein in Cultured Human Skin Fibroblasts

Normalization with Bezafibrate Therapy

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Abstract

The metabolism of hypertriglyceridemic low density lipoprotein (HTG-LDL) was investigated in upregulated cultured human skin fibroblasts. Low density lipoprotein (LDL) was isolated by zonal centrifugation from the plasma of seven HTG subjects, before and 2 wk after the initiation of bezafibrate (BZ) therapy. HTG-LDL is a cholesterol-poor, triglyceride-rich lipoprotein of smaller diameter than BZ-LDL or normal LDL (N-LDL). Binding, cell association, and proteolytic degradation of HTG-LDL were compared with that of BZ-LDL and N-LDL and were found to be significantly lower by a paired t test analysis ($P < 0.001$). After 6 h preincubation with unlabeled HTG-LDL, the incorporation of [^{14}C]acetate to sterols was significantly higher than with BZ-LDL or N-LDL (577±43.7; 330±41.5; 262±47, mean±SE, picomoles sterols per milligram cell protein per 2 h, respectively; $P < 0.001$ by paired t test).

To determine the effectiveness of HTG-LDL and BZ-LDL on the down-regulation of LDL receptor activity, up-regulated cells were incubated for 48 h with HTG-LDL and BZ-LDL. LDL receptor activity was significantly higher after preincubation with HTG-LDL compared with BZ-LDL, and the rates of sterol synthesis were similarly increased. These results demonstrate that HTG-LDL does not down-regulate the LDL receptor activity as efficiently as BZ-LDL and that its cholesterol content is not enough to adequately suppress cellular sterol synthesis.

Significant correlation between LDL composition and cholesterol synthesis by cultured cells was found with all LDL preparations over a wide range of cholesteryl ester to protein ratio (0.8–2.2). This correlation indicates that the compositional and structural abnormalities of HTG-LDL, and especially the low cholesterol content of the lipoprotein, alter LDL metabolism and cellular cholesterol formation.

Introduction

Several abnormalities have been consistently observed in lipoproteins isolated from the plasma of hypertriglyceridemic (HTG)[1] human subjects. Very low density lipoprotein (VLDL) is cholesteryl ester-rich and free cholesterol-rich and protein-poor (1, 2); low density lipoprotein (LDL) appears as multiple populations (1, 3), is protein-rich and triglyceride-rich, and contains considerably less free and esterified cholesterol than normal LDL (1, 2, 4–6). HTG-LDL is also smaller and denser than normal LDL (1, 2). HTG-high density lipoprotein (HDL) belongs predominantly to the HDL_3 subpopulation (1, 7), and like LDL, is relatively enriched with protein and triglyceride and contains less cholesteryl ester molecules than normal HDL_3 (1, 6, 8, 9). These compositional changes are strongly related to the plasma triglyceride levels and many if not all revert toward normal when hypolipidemic therapy is instituted (1).

It has been suggested that most of the abnormalities in HTG lipoproteins reflect an enhanced activity of the lipid-transfer reaction (1, 6, 8, 9), mediated by specific proteins present in plasma of human and some animal species (10–16). According to this view, the presence of a high concentration of triglyceride-rich lipoproteins in the plasma induces accelerated transfer of cholesteryl ester from LDL and HDL to VLDL and chylomicrons and of triglyceride in the opposite direction. Thus, cholesteryl ester and triglyceride redistribution seem to be an integral feature of the lipoprotein system in hypertriglyceridemia.

Lipoprotein cholesterol is an important regulator of cellular metabolic events (17, 18). Therefore, we considered the possibility that the altered composition of LDL found in HTG states may change the interaction between this lipoprotein and peripheral cells. We would expect also that correction of the compositional change of LDL would result in normalization of lipoprotein cell interaction. To test this hypothesis, we have studied the receptor mediated metabolism of LDL by cultured human skin fibroblasts exposed to HTG-LDL isolated before and 2 wk after initiation of bezafibrate (BZ) therapy. The results obtained demonstrate that HTG-LDL is less effective in down-regulation of the LDL receptor activity and inhibition of sterol synthesis by the cells. Normalization of this defect was found with therapy.

Methods

Patients. Seven male patients with primary endogenous hypertriglyceridemia (type IV) as defined by Fredrickson et al. (19) and six healthy male normolipidemic volunteers were studied. The hyperlipidemic patients were attending the Lipid Clinic at the Hadassah University Hospital, Jerusalem. Informed consent was obtained in all cases. All patients were normoglycemic and the hepatic, renal, and thyroid functions were normal. None was receiving drug treatment for other conditions.

Experimental protocol. Fasting plasma samples were obtained from an antecubital vein in the morning hours after a 14–16 h fast. In three patients, an HTG plasma sample was drawn, and on that day BZ therapy (0.2 g three times a day) was instituted. A second plasma sample was obtained after 2 wk of therapy (BZ sample). Blood was obtained from four additional patients who were on BZ therapy with

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1. *Abbreviations used in this paper:* apo, apoprotein; BZ, bezafibrate; FCS, fetal calf serum; HTG, hypertriglyceridemic; LPDS, lipoprotein deficient serum; N-LDL, normal low density lipoprotein.

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the following protocol: the first sample, A, was drawn while the subjects received BZ; on that day, therapy was discontinued, and 2 wk later, a second sample, B, was drawn and BZ was reinstituted. 2 wk later, a third sample, C, was obtained. LDL was isolated, characterized, and assayed in normal human skin fibroblasts for binding, cell association, proteolytic degradation, and [^{14}C]acetate incorporation into sterol. In each experiment, HTG and BZ-LDL were examined together with a control normal LDL (N-LDL) isolated from the normotriglyceridemic subjects.

Isolation and characterization of LDL. Blood was drawn into tubes containing EDTA (1 mg/ml). Zonal ultracentrifugation was employed to isolate LDL from the hyperlipidemic subjects according to the method published by Patsch et al. (20). This procedure was suitable to separate LDL of abnormal composition, size, and density (1). Zonal ultracentrifugation was performed in a Ti-14 zonal rotor. A NaBr gradient of 1.0–1.3 g/ml was established with the help of a high capacity gradient pump (141; Beckman Instruments, Inc., Fullerton, CA). The rotor was loaded with a plasma fraction of d 1.006–1.085 g/ml (isolated from 80–140 ml plasma) and the LDL was eluted after 140 min centrifugation at 42,000 rpm. The eluate was monitored continuously at 280 nm by a multiwavelength absorbance monitor (Model UA-5; Isco, Inc., Lincoln, NE). The main LDL fraction (1) was isolated and used in tissue culture experiments. N-LDL was isolated by preparative ultracentrifugation at d = 1.019–1.063 g/ml, and lipoprotein deficient plasma was isolated from normal subjects at $d > 1.25$ g/ml in fixed angle rotors (50Ti, 60Ti) using an ultracentrifuge (L5-50B; Beckman Instruments, Inc.). The LDL was dialyzed against 0.15 M NaCl, 1 mM EDTA solution. The zonal LDL was concentrated by vacuum ultrafiltration. All preparations were sterilized by passage through a 0.45-μm millipore filter. Electron microscopy was used to determine the size of lipoproteins. Electron micrographs were obtained using an electron microscope (300; Philips Electronic Instruments, Inc., Mahwah, NJ) as described previously (1). Triglycerides were determined according to the U. S. Lipid Research Clinics protocol using an AutoAnalyzer AAII (Technicon Corp., Tarrytown, NY) (21). Cholesteryl ester and free cholesterol content in lipoproteins were determined enzymatically (22) with a commercial kit (Boehringer Mannheim GmbH Diagnostics, Mannheim, West Germany). Radioactive sterols were isolated by thin layer chromatography on silica gel plates using the solvent system petroleum ether/diethyl ether/acetic acid, 80:20:1 (vol/vol/vol) (23). Phospholipids were determined by the Bartlett procedure (24). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of apolipoproteins on 10% gels was performed after the methods described by Weber and Osborn (25). LDL protein was measured by the method of Lowry et al. (26) using bovine serum albumin (BSA) as a standard.

Iodination of LDL. ^{125}I-labeled LDL was prepared by the iodine monochloride method of MacFarlane (27) as modified by Bilheimer et al. (28). The iodinated preparations were dialyzed extensively against 0.15 M NaCl, 0.001 M EDTA, pH = 7.4. The final specific activity varied between 50 and 500 cpm/ng protein. In all preparations, >95% of the radioactivity was precipitated by trichloroacetic acid and <5% was extractable by chloroform-methanol. All the iodinated and noniodinated LDL preparations were used within 2 wk and sterilized by passage through a 0.45-μm millipore filter. They were kept sterile and tightly closed at 4°C.

Cultures of human skin fibroblasts. Biopsies were obtained from the medial part of the forearm of normal male adult donors. The cells were cultured in plastic flasks (Falcon Labware, Div. of Becton-Dickinson & Co., Oxnard, CA) in modified Dulbecco-Vogt medium (29) containing 10% fetal calf serum (FCS), and maintained in a humidified incubator (5% CO_2) at 37°C. Fibroblasts from third to fifteenth subcultures were trypsinized and 3.5×10^4 cells were plated (day 0), in 35-mm dishes (Falcon Labware), containing 2 ml of medium with 10% FCS. The medium was changed on days 3 and 5 while the cells were not yet confluent. On the fifth day, monolayers were washed with Dulbecco-phosphate-buffered saline (PBS) buffer and 2 ml of fresh medium containing human lipoprotein deficient serum (LPDS) at a final protein concentration of 5 mg/ml were added. The cells were incubated for another 48 h. All experiments were initiated on day 7.

Assays of binding, cell association, and proteolytic degradation of ^{125}I-LDL. On the day of experiment, the serum containing medium was removed, the cell layer was washed with PBS, and the cells were incubated with ^{125}I-LDL, 15 μg protein/ml, in lipoprotein deficient medium at 37°C. After 6 h of incubation, the medium was examined for noniodide ^{125}I-LDL degradation products according to Bierman et al. (30). The results were expressed as nanograms of ^{125}I-LDL protein degraded per milligram cell protein. Dishes with ^{125}I-LDL but without cells were processed simultaneously and the results were subtracted from the degradation values in dishes with cells. After removal of medium, cell monolayers were cooled for 30 min at 4°C and washed extensively with ice-cold PBS + 0.2% BSA. Binding of ^{125}I-LDL was determined by the release of radioactivity after incubation with heparin (10 mg/ml) for 1 h at 4°C, according to Goldstein et al. (31). The cells were then washed and dissolved in 1 ml of 0.5 N NaOH. Cell associated LDL was determined as ^{125}I-LDL radioactivity in the dissolved cells; cell protein was determined subsequently by the method of Lowry et al. (26). Cell associated LDL represents the radioactivity recovered in the cells after the incubation with heparin.

Incorporation of [^{14}C]acetate to sterols. To determine the ability of LDL to regulate sterol synthesis, fibroblasts were incubated with unlabeled HTG-LDL or BZ-LDL, 15 μg protein in 1 ml LPDS medium. After 6 h, the medium was removed and the cells were washed extensively with PBS + 0.2% BSA. 1 ml of serum free medium containing 10 μCi of 2[^{14}C]acetate was added and incubation continued at 37°C for 2 h. The incorporation of labeled acetate into sterols was measured according to the method of Oram et al. (32). At the end of incubation, the medium was removed and the cells were washed three times with PBS. The lipids were extracted by the addition of hexane/isopropanol (3:2, vol/vol), for 1 h at room temperature, followed by two rapid similar extractions. The pooled solvents were evaporated to dryness, the cholesteryl ester was hydrolyzed with 1 N KOH in 95% ethanol for 1 h at 85°C, and the sterols were re-extracted into 2 ml hexane. A 0.5-ml hexane aliquot was counted in a beta scintillation spectrometer. The [^{14}C]sterol fraction was determined by thin layer chromatography on silica gel G plates, using petroleum ether/diethyl ether/acetic acid (80:20:1, vol/vol/vol) as the developing solvent. Usually, >80% of the hexane extracted radioactivity was found in the sterol fraction. Incorporation of [^{14}C]acetate to sterols is expressed as picomoles of acetate found in the sterol fraction (calculated from the specific activity of the acetate (55 mCi/mmol)).

Effect of a prolonged exposure to unlabeled LDL. Upregulated cells were preincubated for 48 h with HTG-LDL and BZ-LDL, at concentrations of 15 μg protein/ml. After the preincubation period, the medium was removed and ^{125}I-LDL obtained from normal donors (N-LDL; 15 μg protein/ml) in 1 ml medium was added and incubated with the cells for 3 h. The binding, cell association, and proteolytic degradation of ^{125}I-N-LDL and the incorporation of [^{14}C]acetate to sterols was determined as described above.

Materials. 2[^{14}C]acetate and ^{125}I-Na were purchased from the Radiochemical Centre, Amersham, England. Culture flasks and dishes were purchased from Falcon Labware. Medium and FCS were obtained from Gibco Laboratories, Grand Island, NY. All other chemicals and reagents were of analytical grade.

Statistical analysis. Tests for significant differences between HTG-LDL and BZ-LDL were performed by paired t test evaluation. Linear regression analysis was used to determine coefficients of correlation between cholesteryl ester/protein ratios to sterol synthesis and rates of degradation of HTG-LDL to BZ-LDL. Significance levels were obtained by t and r tables (33).

Results

Plasma and lipoprotein lipids. The patients' plasma lipid levels before and after 2 wk of BZ therapy are shown in Table I.

Table I. Plasma and Lipoprotein Lipid Concentrations of HTG Patients Before and 2 Wk After Institution of BZ Therapy

	Values before BZ therapy					Values during BZ therapy				
	TG	Cholesterol				TG	Cholesterol			
Patient		Plasma	VLDL	LDL	HDL		Plasma	VLDL	LDL	HDL
	mg/dl plasma	*mg/dl*	*mg/dl*	*mg/dl*	*mg/dl*	*mg/dl plasma*	*mg/dl*	*mg/dl*	*mg/dl*	*mg/dl*
B.S.	901	247	177	50	20	346	183	53	117	23
E.K.	620	283	113	141	29	186	221	27	162	32
Y.S.	770	330	212	82	26	243	254	61	158	35
B.E.	882	362	141	197	24	194	288	56	192	30
A.K.	620	266	157	87	22	323	207	53	128	26
C.O.	3,010	480	419	42	19	665	271	145	104	22
M.A.	840	242	180	46	16	420	160	71	67	22
Mean	1,092	316	200	92.1	22.3	340	226	65.6	133	27.1
SEM	±323*	±32‡	±38*	±21.8‡	±1.7*	±63	±18	±14.0	±15.8	±2.0

Differences between HTG and BZ samples are significant at levels of $P < 0.001$* and $P < 0.005$‡ by paired t test. TG, triglyceride.

The mean plasma TG decreased after 2 wk of BZ therapy by 70.9±3.86% (mean±SEM, $P < 0.001$). The same trend was also found for cholesterol, which decreased by 30.4±6.05% (mean±SEM, $P < 0.001$).

HTG-LDL and BZ-LDL were separated on a zonal ultracentrifugation system (1). As judged by the zonal profile, HTG-LDL eluted later than BZ-LDL (peak elution volumes of 273±8 ml and 250±5 ml [mean±SEM], respectively), indicating an increased density of the lipoprotein. The chemical composition and size of LDL particles before and after BZ therapy is shown in Table II. The HTG-LDL contained more triglyceride than BZ-LDL, less cholesterol (free and esterified), and the particles were smaller and relatively enriched with protein. Sodium dodecyl sulfate polyacrylamide gel electrophoresis revealed that apoprotein (apo) B was the only protein found in all preparations (by visual estimation, more than 95%). Comparing BZ-LDL with HTG-LDL, the cholesteryl ester/protein ratio increased by 54±8.8% (mean±SEM) and the triglyceride/protein ratio decreased by 32.5±7.5% (mean±SEM).

Binding, cell association, and proteolytic degradation. Fig. 1 presents the mean±SEM values of binding, cell association, and proteolytic degradation of HTG-LDL, BZ-LDL, and N-LDL assayed by human skin fibroblasts. The binding of ^{125}I-LDL was determined in nine studies on six subjects. The amount of HTG-LDL bound to the cells (nanograms LDL protein/milligrams cell protein) was 60.1±8.4 (mean±SEM), while that of BZ-LDL was 76.6±6.5 (paired t test, $P < 0.005$). The binding of N-LDL included in the same experiments was 80.0±4.5. A similar trend was found for cell associated LDL (Fig. 1 *B*). Degradation of LDL after 6 h of incubation was determined in 11 studies carried out in seven patients. More LDL was degraded after 2 wk of BZ treatment (1,561±178) as compared with HTG-LDL (1,261±193) (Fig. 1 *C*). The differences were highly significant by paired t test ($P < 0.001$). Degradation of N-LDL was 1,639±200. The individual changes of the above parameters are shown in Fig. 2 *A*, *B*, and *C*. Despite variations between studies, in all except one, the change in LDL binding, cell association, and degradation occurred in the same direction.

To determine whether the defective metabolism of HTG-LDL represents an affinity change for specific LDL binding sites, a comparison of the ability of HTG-LDL and N-LDL to compete with ^{125}I-N-LDL for binding and degradation by the cells was carried out. The competition assay was carried out with 15 μg protein/ml of ^{125}I-labeled N-LDL and a wide range of unlabeled HTG-LDL or N-LDL (0–500 μg protein/ml). As shown in Fig. 3, HTG-LDL was far less efficient than N-LDL in the competition assay. This observation indicated that the defective binding of HTG-LDL to the cells is indeed due to lower affinity of the lipoprotein to the receptor.

Effect of unlabeled LDL on the incorporation of [^{14}C]acetate

Table II. Chemical Composition and Diameter of HTG-LDL, BZ-LDL, and N-LDL

Source of LDL	Protein	CE	TG	FC	PL	CE/PR	TG/PR	Diameter A
	mg/100 mg lipoprotein					*mg/mg*	*mg/mg*	
HTG	31.4±0.9	32.1±1.5	12.6±1.4	5.4±0.6	18.4±0.7	1.03±0.07	0.40±0.04	191±2.8
BZ	24.5±0.6*	38.7±0.6*	6.5±0.5*	8.4±0.3*	21.9±0.6‡	1.59±0.04*	0.27±0.02*	210±4.7*
N	22.7±1.1	40.2±0.8	4.9±0.6	9.4±0.6	23.0±1.4	1.78±0.08	0.22±0.03	213±4.2

Data are mean±SEM of the seven HTG patients and six normolipidemic subjects who participated in the study. Differences between HTG-LDL and BZ-LDL are significant at levels of $P < 0.005$* or $P < 0.01$‡ by paired t test. CE, cholesteryl ester; FC, free cholesterol; PL, phospholipid; PR, protein; TG, triglyceride.

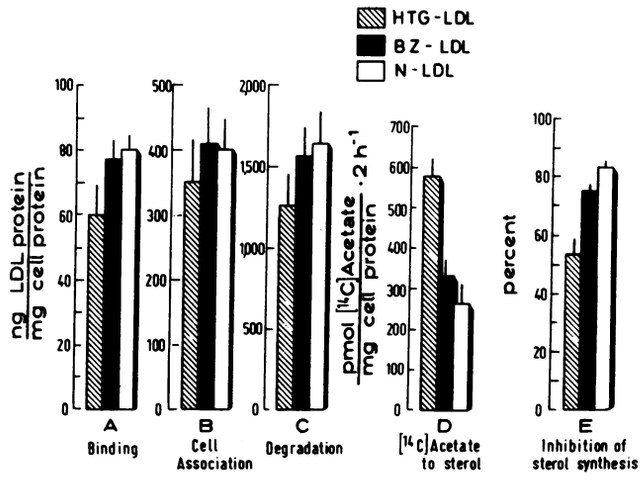

Figure 1. Binding, cell association, proteolytic degradation, incorporation of [^{14}C]acetate to sterols, and inhibition of [^{14}C]sterol synthesis by HTG-LDL, BZ-LDL, and N-LDL in cultured human skin fibroblasts. Data are mean±SEM of 11 studies on seven patients and six studies in six normotriglyceridemic subjects.

into sterols. Incorporation of [^{14}C]acetate into sterols was measured in 10 studies (seven patients) after 6 h of preincubation of the cells with either HTG-LDL or BZ-LDL. The incorporation of [^{14}C]acetate (picomoles/milligram cell protein per 2 h) was higher after preincubation with HTG-LDL as compared with BZ-LDL in all studies (Fig. 1 *D*). The mean±SEM of the incorporation data were 577±43.7 and 330±41.5, respectively, $P < 0.001$ by paired t test. Incorporation into sterols after preincubation with N-LDL in the same experiment was 262±47. Inhibition of sterol synthesis (defined as percent [^{14}C]acetate incorporation into sterols in plates with different LDL preparations compared with plates without LDL) is shown in Fig. 1 *E*. HTG-LDL inhibited the incorporation of [^{14}C]acetate into sterols consistently and considerably less than BZ-LDL; 53.2±5.6% and 73.8±2.96%, respectively ($P < 0.001$ by paired t test). The inhibition by N-LDL was 82.3±1.94%. The individual values are shown in Fig. 2 *D* and *E*.

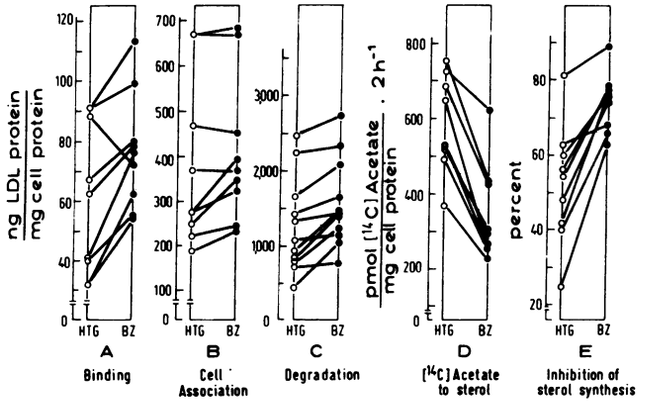

Figure 2. Individual variation of receptor mediated LDL metabolism of HTG-LDL and BZ-LDL in cultured human skin fibroblasts. Open circles represent results with HTG-LDL and closed circles represent results with BZ-LDL. Data obtained in paired studies (HTG-LDL and BZ-LDL investigated simultaneously) are shown by the lines.

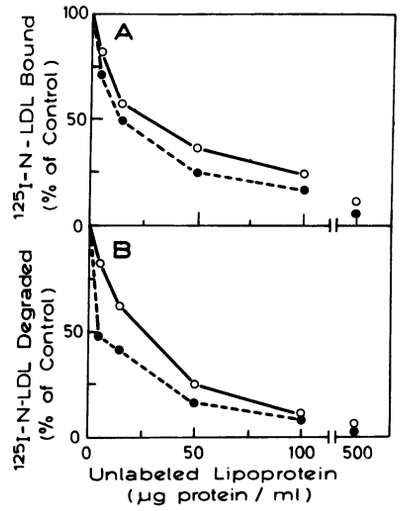

Figure 3. Comparison of the ability of HTG-LDL (—○—) and N-LDL (- - -●- - -) to compete with ^{125}I-labeled N-LDL for binding (*A*) and degradation (*B*) in human skin fibroblast. Each 35-mm dish of cultured fibroblasts received 1 ml of Eagle's minimal essential medium containing 5 mg/ml LPDS, 15 μg/ml of ^{125}I-N-LDL, and the indicated concentrations of unlabeled HTG-LDL or N-LDL. After incubation for 6 h at 37°C, the amounts of ^{125}I-LDL bound to the cells and of noniodide ^{125}I-N-LDL degradation products was determined as described in Methods.

Effect of increasing concentrations of HTG-LDL and N-LDL on the relationships between proteolytic degradation, cholesterol influx, and incorporation of [^{14}C]acetate to sterols. To assess the role of LDL composition on sterol synthesis, fibroblasts were incubated for 6 h with increasing amounts of ^{125}I-LDL (2.5–30 μg protein). Proteolytic degradation of the ^{125}I-LDL and incorporation of [^{14}C]acetate to sterols were determined as described in Methods. Cholesterol influx was calculated from the proteolytic degradation data and the total cholesterol to protein ratios of LDL. A reciprocal plot of the data is shown in Fig. 4. At all LDL concentrations, more [^{14}C]acetate was incorporated to sterols with HTG-LDL when the data were correlated to proteolytic degradation (Fig. 4 *A*). However, when [^{14}C]acetate incorporation to sterol was related to the amount of cholesterol that entered the cells, no differences were found between HTG-LDL and N-LDL (Fig. 4 *B*). Thus, it seems that with equal influx of cholesterol into the cells, [^{14}C]acetate incorporation to sterols is affected to the same extent irrespective of the type of LDL used.

Effect of cessation and readministration of BZ on LDL interaction with cultured fibroblasts. In all experiments, one of the paired samples (HTG-LDL or BZ-LDL) was kept sterilized at 4°C for 2 wk. To evaluate the effect of storage on LDL metabolism, the following procedure was used. In four subjects, LDL was obtained on three occasions: sample A was obtained during BZ treatment; sample B, 2 wk after cessation of therapy; and sample C, 2 wk after reinstitution of therapy (begun on the day that sample B was collected). For each patient, binding, cell association, proteolytic degradation of LDL, and [^{14}C]-acetate incorporation into sterol were determined on two occasions: first, sample A (stored for 2 wk) and sample B (fresh); and 2 wk later, sample B (stored for 2 wk) and sample C (fresh). Table III presents the data expressed as ratios of values between sample B (HTG-LDL) and samples A or C

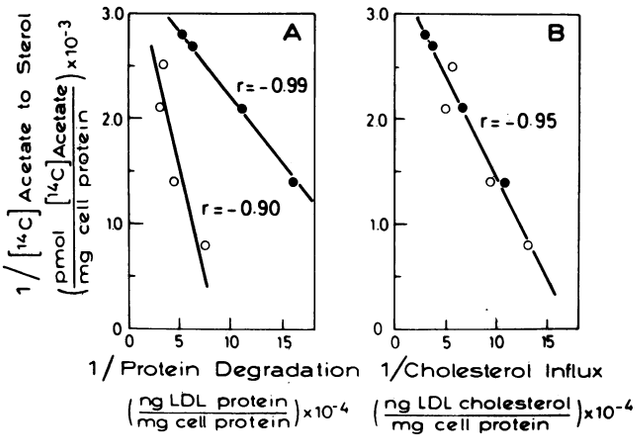

Figure 4. Effect of increasing amounts of HTG-LDL and N-LDL on the relationships between proteolytic degradation (*A*), cholesterol influx (*B*), and incorporation of [^{14}C]acetate to sterols. The ordinate represents reciprocal values of [^{14}C]acetate incorporation into sterols. The abscissa indicates the reciprocal values of proteolytic degradation (*A*) and cholesterol influx (*B*) to cells incubated with various amounts (2.5–30 μg LDL protein per milliliter of medium) of N-LDL (●) or HTG-LDL (○). Cholesterol influx was calculated by multiplying the proteolytic degradation values by the total cholesterol to protein weight ratio of the HTG-LDL or N-LDL.

(BZ-LDL). Comparing the data obtained with freshly prepared HTG sample (A/B ratio) with that obtained with freshly prepared BZ sample (C/B ratio), it is evident that binding, cell association, and proteolytic degradation of BZ-LDL is higher than HTG-LDL irrespective of the sequence of the study. The same is observed for incorporation of [^{14}C]acetate to sterols, which is higher with HTG-LDL. We therefore conclude that storage of LDL for 2 wk did not affect appreciably the metabolic behavior of the LDL and cannot explain the differences between HTG-LDL and BZ-LDL.

Effect of prolonged incubation of HTG-LDL and BZ-LDL on receptor activity. To test the long-term effects of HTG-LDL and BZ-LDL on fibroblasts, upregulated cells were preincubated for 48 h with 15 μg protein/ml, of unlabeled LDL (11 studies on seven patients). After washings (see Methods), N-^{125}I-LDL (15 μg protein/ml) was added and binding, cell association, and degradation of the LDL and incorporation of [^{14}C]acetate to sterols were determined. The results of these experiments are shown in Table IV. Binding, cell association, and proteolytic degradation were significantly higher after preincubation with HTG-LDL compared with BZ-LDL. Incorporation of [^{14}C]acetate to sterols was also significantly higher in cells exposed to HTG-LDL. These results demonstrate that HTG-LDL does not down-regulate the LDL receptor activity as efficiently as BZ-LDL. Moreover, the data indicate that even after 48 h of preincubation, sterol synthesis in cells exposed to HTG-LDL is higher compared with BZ-LDL.

Table III. Effect of Cessation and Readministration of BZ on LDL Metabolism by Cultured Fibroblasts

Parameter	A/B	C/B
Binding, ng LDL protein/mg cell protein	1.43±0.21	1.37±0.19
Cell association, ng LDL protein/mg cell protein	1.11±0.11	1.11±0.10
Proteolytic degradation, ng LDL protein/mg cell protein	1.54±0.29	1.33±0.16
[^{14}C]acetate incorporation into sterols, pmol acetate/mg cell protein/2 h	0.57±0.03	0.48±0.03

Human skin fibroblasts were cultured in medium supplemented with 10% FCS for 5 d and LDL receptor was upregulated by subsequent 48-h incubation in medium with LPDS. Binding, cell association, degradation, and incorporation of [^{14}C]acetate into sterol were performed as described in Methods. The LDL used in the four experiments was handled as described in Results. Values are mean±SEM of four paired experiments and are expressed as ratios of metabolism activities between samples A and B and samples C and B. Differences between A/B and C/B results are not significant. Sample A, on bezafibrate therapy; sample B, 2 wk after cessation of BZ; sample C, 2 wk after reinstitution of BZ.

Table IV. Effect of 48-h Exposure of Fibroblasts to HTG-LDL and BZ-LDL on N-LDL Metabolism and Sterol Synthesis

Parameter	HTG-LDL	BZ-LDL
Binding (*ng protein/mg cell protein*)	64.6±4.9	47.3±2.5
Cell association (*ng protein/mg cell protein*)	203±16.3	140±8.1
Proteolytic degradation (*ng protein/mg cell protein per 3 h*)	370±37.6	256±15.5
[^{14}C]acetate incorporation to sterols (*pmol acetate/mg cell protein per 2 h*)	818±73.0	527±42.0

Cells were cultured for 5 d and the LDL receptor was upregulated for 48 h. Thereafter, the cells were incubated for an additional 48 h in medium containing LPDS and 15 μg protein of either HTG-LDL or BZ-LDL. The medium was changed and binding, cell association, and degradation were determined using N-LDL as ligand. The results are expressed as mean±SEM of 11 studies carried out on LDL preparations obtained from seven patients. Differences between HTG-LDL and BZ-LDL are significant at levels of $P < 0.001$ for all four parameters by paired *t* test.

Relationships between LDL composition and incorporation of [^{14}C]acetate into sterols. All data on incorporation of [^{14}C]acetate to sterols were plotted against the ratio of cholesteryl

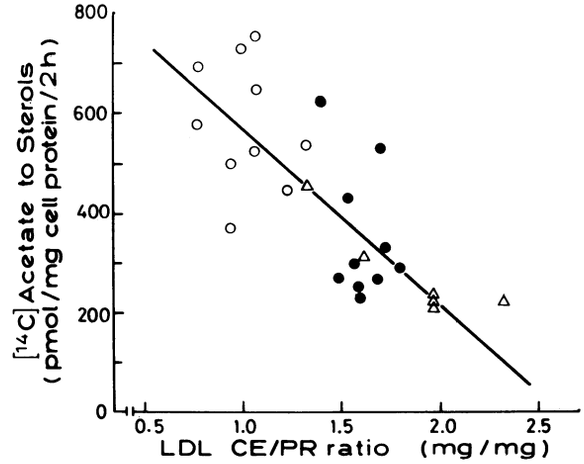

Figure 5. The relationship between incorporation of [^{14}C]acetate to sterols and LDL cholesteryl ester protein ratios. Data are from six preparations of N-LDL (△), and ten preparations of HTG-LDL (○) and BZ-LDL (●). Intercept, 920; slope, 355; $r = 0.806$; $P < 0.0001$.

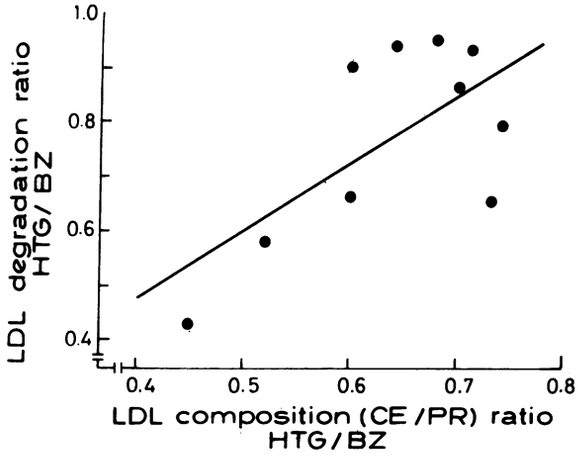

Figure 6. The relationship between ratios of degradation of HTG-LDL to BZ-LDL (from the same patients) and ratios of cholesteryl ester protein values of the two lipoprotein preparations. Data are from ten paired studies in six patients. Intercept, −0.02; slope, 1.24; $r = 0.66$; $P < 0.05$.

ester to protein of the different LDLs (six N-LDL, nine HTG-LDL, and nine BZ-LDL). A highly significant negative correlation was found (Fig. 5). The coefficient of correlation between the two parameters was $r = -0.806$, $P < 0.0001$. Similar correlations were obtained with other LDL compositional parameters (e.g., total cholesterol to protein ratio; triglyceride to protein or triglyceride to cholesterol ratios). LDL composition was also highly positively correlated with percent suppression of sterol synthesis.

The relationships between LDL compositional ratios and proteolytic degradation ratios. This relationship was determined by testing the ratios of proteolytic degradation of HTG-LDL to BZ-LDL against the cholesteryl ester to protein ratios of HTG-LDL to BZ-LDL. The analysis was carried out on 10 paired (HTG-LDL and BZ-LDL) studies. LDL degradation ratios were positively and significantly related ($r = 0.66$, $P < 0.05$) to the LDL compositional ratios (Fig. 6).

Discussion

The aim of this investigation was to determine whether LDL from HTG patients, which is known to be abnormal in structure and composition (1–8), will behave differently with regard to its metabolism in cultured human skin fibroblasts when compared with LDL obtained from normal donors. In view of the difficulties to determine absolute parameters when studying lipoprotein metabolism in cells in culture (31), several precautions were taken. First, HTG-LDL was tested simultaneously with N-LDL and LDL obtained from the same patient 2 wk after initiation (or discontinuation) of triglyceride lowering therapy. Second, the order of obtaining of HTG-LDL and BZ-LDL has been reversed in four patients. Third, identical experimental procedures were employed throughout the study. With these precautions, several metabolic abnormalities could be identified in the tissue culture system. HTG-LDL binding and proteolytic degradation were decreased, LDL receptor mediated activities were down-regulated less efficiently, and handling of N-LDL by the cells was different after prolonged exposure to HTG-LDL or BZ-LDL. These abnormalities reverted toward normal when plasma triglyceride levels were reduced by BZ treatment.

Reduced cellular binding of LDL isolated from uncontrolled type I diabetic patients (34) and from patients with diabetes with hypertriglyceridemia (35) has been reported. In a second study, binding of LDL isolated from diabetics with normal plasma triglyceride levels was found to be normal (35). In the present investigation, we observed decreased binding by the cells of LDL obtained from patients with hypertriglyceridemia but with no diabetes. We also found that LDL binding increased when plasma triglyceride levels were reduced by BZ therapy. The defective binding was due to lower affinity of HTG-LDL for binding by the LDL receptor and seemed to reflect the degree of triglyceridemia. At the end of 6 h incubation, when LDL receptor activity is not expected to be appreciably down-regulated (36), both cell association and proteolytic degradation of HTG-LDL were markedly reduced. These parameters also reverted to normal with BZ therapy. We suggest that the defective binding of HTG-LDL by the cells is responsible for decreased entry of the LDL to the cells and to its decreased proteolytic degradation. The reason for the reduced binding and degradation of HTG-LDL is not clear. In our study, several possibilities could be ruled out. The reversal of the binding defect with treatment indicates that the defect is not related to the genetic nature of the dyslipoproteinemic state. Apo C has been reported to inhibit degradation of chylomicron remnants by the perfused rat liver (37), while apo E present in HTG-VLDL modulates VLDL binding by the LDL cellular receptors (38, 39). These apoproteins were not detected in the LDL preparations used presently. The reversible compositional abnormalities of the LDL could provide one possible explanation for the defective binding. However, when the correlation between the change in cholesteryl ester/protein ratio of HTG-LDL and its reduced degradation by the cultured cells was sought, it was found to be relatively low ($r = 0.66$). We therefore favor another possibility, namely, that the smaller size of HTG-LDL induces conformational changes of apo B and decreases its binding to the receptors. That possibility is being investigated.

Decreased ability of LDL to suppress sterol synthesis in upregulated human skin fibroblasts has previously been shown in LDL depleted of cholesteryl esters either by reconstitution techniques or by the lipid transfer protein reaction in vitro (40, 41). The present study demonstrates a similar phenomenon with cholesteryl ester-poor triglyceride-rich LDL isolated from HTG human subjects. Furthermore, our data indicate that the ability of the cells to incorporate [^{14}C]acetate into sterols is strongly and significantly correlated with LDL composition over a wide range of LDL cholesteryl ester to protein ratios (between 0.8 to 2.2) commonly seen in humans. This phenomenon is manifested even when only normotriglyceridemic subjects are considered (see Fig. 5, normal LDL), but becomes pronounced with hypertriglyceridemia. When similar amounts of cholesterol are delivered to cells with either N-LDL or HTG-LDL, the two lipoproteins exert identical effects on sterol synthesis. In that experiment, increasing amounts of triglycerides were also delivered to the cells with HTG-LDL; that seemed to be without effect on sterol synthesis. It seems, therefore, that the reduced delivery of cholesterol to the cells by LDL of varying cholesterol content is the determinant that modulates intracellular sterol synthesis rates. Note that the degradation of HTG-LDL was reduced by 26% as compared with BZ-

LDL. Since the increase of [^{14}C]acetate incorporation to sterols was 83%, the decreased LDL degradation explains only a part of the increased sterol synthesis. The other part must reflect the decreased cholesterol content of the HTG-LDL. This suggestion is supported by the concept of Brown and Goldstein (42, 43) that cholesterol molecules regulate 3-hydroxy-3-methylglutaryl coenzyme A reductase activity.

The experiments in which cells were exposed to HTG-LDL and BZ-LDL for a prolonged period of time (48 h) simulate in part the in vivo HTG state. In these experiments higher LDL receptor activity was observed in cells pretreated with HTG-LDL, as shown by increased binding and degradation of LDL protein; there was also an increased incorporation of [^{14}C]acetate to sterols. The higher LDL receptor activity was apparently due to a lesser delivery of cholesterol to cells, and thus less effective down-regulation of receptor protein synthesis occurred (18). These results are relevant to lipoprotein and cholesterol metabolism in the HTG state. LDL-apo B turnover studies in human subjects with hypertriglyceridemia (in particular familial) reported accelerated degradation of the apo B moiety of the lipoprotein (44–46). In one study, the mean t½ of LDL-apo B in type IV patients was 3.4 d, compared with 4.26 d in normals, while in type V, it was 2.48 d (44). In a second study, the fractional catabolic rate of LDL-apo B in patients with familial hypertriglyceridemia was 0.47 pools/day compared with 0.31 in normals (46). In other studies, total body cholesterol synthesis was reported to be considerably higher in patients with hypertriglyceridemia as compared with normals and patients with hypercholesterolemia (47–50). The increased fractional catabolic rate of LDL-apo B could be related to the presently described finding that the HTG-LDL particle delivers less cholesterol to cells than N-LDL and therefore is less effective in the down regulation of the LDL receptor and in inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase. The resulting higher activity of this enzyme would account for the increase in cholesterol synthesis in hypertriglyceridemia. Our data predict that when plasma triglyceride levels are reduced, both abnormalities should be corrected and indeed reduction in cholesterol synthesis has been reported in HTG patients treated with clofibrate (48, 49).

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References

1. Eisenberg, S., D. Gavish, Y. Oschry, M. Fainaru, and R. J. Deckelbaum. 1984. Abnormalities in very low, low, and high density lipoproteins in hypertriglyceridemia. Reversal toward normal with bezafibrate treatment. *J. Clin. Invest.* 74:470–482.

2. Vakakis, N., T. G. Redgrave, D. M. Small, and W. P. Castelli. 1983. Cholesterol content of red blood cells and low density lipoproteins in hypertriglyceridemia. *Biochim. Biophys. Acta.* 751:280–285.

3. Nelson, C. A., and M. D. Morris. 1983. Human low density lipoprotein structure: correlations with serum lipoprotein concentrations. *Lipids.* 18:553–557.

4. Breckenridge, W. C., J. A. Little, G. Steiner, A. Chow, and M. Poapt. 1978. Hypertriglyceridemia associated with deficiency of apolipoprotein C-II. *N. Engl. J. Med.* 298:1265–1273.

5. Miller, N. E., S. N. Rao, R. Alaupovic, N. Noble, J. Slack, J. D. Brunzell, and B. Lewis. 1981. Familial apolipoprotein CII deficiency: plasma lipoproteins and apolipoproteins in heterozygous and homozygous subjects and the effects of plasma infusion. *Eur. J. Clin. Invest.* 11:69–76.

6. Deckelbaum, R. J., E. Granot, Y. Oschry, L. Rose, and S. Eisenberg. 1984. Plasma triglyceride determines structure-composition in low and high density lipoproteins. *Arteriosclerosis.* 4:224–231.

7. Patsch, J. R., and A. M. Gotto. 1979. Separation and analysis of HDL subclasses by zonal ultracentrifugation. *In* Report of the High Density Lipoprotein Methodology Workshop. Department of Health, Education, and Welfare, National Institutes of Health Publication No. 79-1661. U. S. Government Printing Office, Washington, DC. 310–324.

8. Myers, L. H., N. R. Phillips, and R. J. Havel. 1976. Mathematical evaluation of methods for estimation of the concentration of the major lipid components of human serum lipoproteins. *J. Lab. Clin. Med.* 88:491–505.

9. Phillips, N. R., R. J. Havel, and J. P. Kane. 1982. Serum apolipoprotein A-I levels: relationship to lipoprotein lipid levels and selected demographic variables. *Am. J. Epidemiol.* 116:302–313.

10. Zilversmit, D. B., L. B. Hughes, and J. Balmer. 1975. Stimulation of cholesterol ester exchange by lipoprotein-free rabbit plasma. *Biochim. Biophys. Acta.* 409:393–398.

11. Chajek, T., and C. J. Fielding. 1978. Isolation and characterization of a human serum cholesterol ester transfer protein. *Proc. Natl. Acad. Sci. USA.* 75:3445–3449.

12. Morton, R. E., and D. B. Zilversmit. 1982. Purification and characterization of lipid transfer protein(s) from human lipoprotein-deficient plasma. *J. Lipid Res.* 23:1058–1067.

13. Albers, J. J., J. H. Tollefson, C. H. Chen, and A. Steinmetz. 1984. Isolation and characterization of human plasma lipid transfer proteins. *Arteriosclerosis.* 4:49–58.

14. Hopkins, G. J., and P. J. Barter. 1980. Transfers of esterified cholesterol and triglyceride between high density and very low density lipoproteins: in vitro studies of rabbits and humans. *Metab. Clin. Exp.* 29:546–550.

15. Deckelbaum, R. J., S. Eisenberg, Y. Oschry, E. Butbul, I. Sharon, and T. Olivecrona. 1982. Reversible modification of human plasma low density lipoproteins toward triglyceride-rich precursors. *J. Biol. Chem.* 257:6509–6517.

16. Morton, R. E., and D. B. Zilversmit. 1983. Inter-relationship of lipids transferred by the lipid-transfer protein isolated from human lipoprotein-deficient plasma. *J. Biol. Chem.* 258:11751–11757.

17. Goldstein, J. L., and M. S. Brown. 1977. The low density lipoprotein pathway and its relation to atherosclerosis. *Annu. Rev. Biochem.* 46:897–930.

18. Brown, M. S., P. T. Kovanen, and J. L. Goldstein. 1981. Regulation of plasma cholesterol by lipoprotein receptors. *Science (Wash. DC).* 212:628–635.

19. Fredrickson, D. S., R. I. Levy, and R. S. Lees. 1967. Fat transport in lipoproteins—an integrated approach to mechanisms and disorders. *N. Engl. J. Med.* 276:34–44, 94–103, 148–156, 215–225, 273–281.

20. Patsch, J. R., S. Sailer, G. Kostner, F. Sandhofer, A. Holasek, and H. Braunsteiner. 1974. Separation of the main lipoprotein density classes from human plasma by rate-zonal centrifugation. *J. Lipid Res.* 15:356–366.

21. Lipid Research Clinics Program: Manual of Laboratory Operations. 1976. Vol. I. Lipid and lipoprotein analysis. Department of Health, Education and Welfare, National Institutes of Health Publication No. 76-628. U. S. Government Printing Office, Washington, D.C.

22. Patsch, W., S. Sailer, and H. Braunsteiner. 1976. An enzymatic method for the determination of the initial rate of cholesterol esterification in human plasma. *J. Lipid Res.* 17:182–185.

23. Chajek, T., and S. Eisenberg. 1978. Very low density lipoproteins. Metabolism of phospholipids, cholesterol, and apolipoprotein C in the isolated perfused rat heart. *J. Clin. Invest.* 61:1654–1665.

24. Bartlett, G. R. 1959. Phosphorous assay in column chromatography. *J. Biol. Chem.* 234:466–468.

25. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* 244:4406–4412.

26. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–275.

27. MacFarlane, A. S. 1958. Efficient trace labeling of proteins with iodine. *Nature (Lond.).* 182:53.

28. Bilheimer, D. W., S. Eisenberg, and R. I. Levy. 1972. The metabolism of very low density lipoprotein proteins. *Biochim. Biophys. Acta.* 260:212–221.

29. Stein, O., J. Vanderhoek, G. Friedman, and Y. Stein. 1976. Deposition and mobilization of cholesterol ester in cultured human skin fibroblasts. *Biochim. Biophys. Acta.* 450:367–378.

30. Bierman, E. L., O. Stein, and Y. Stein. 1974. Lipoprotein uptake and metabolism by rat aortic smooth muscle cells in tissue culture. *Circ. Res.* 35:136–150.

31. Goldstein, J. L., S. K. Basu, and M. S. Brown. 1983. Receptor-mediated endocytosis of low-density lipoprotein in cultured cells. *Methods Enzymol.* 98:241–260.

32. Oram, J. F., J. J. Albers, and E. L. Bierman. 1980. Rapid regulation of the activity of the low density lipoprotein receptor of cultured human fibroblasts. *J. Biol. Chem.* 255:475–485.

33. Hill, A. B. 1971. Principles of Medical Statistics. Lancet Ltd., London. Ninth ed. 143–151; 317; 376.

34. Lopes-Virella, M. F., G. K. Sherer, A. M. Lees, H. Wohltman, R. Mayfield, J. Sagel, E. C. Leroy, and J. A. Colwell. 1982. Surface binding, internalization and degradation by cultured human fibroblasts of low density lipoproteins isolated from type I (insulin dependent) diabetic patients: changes with metabolic control. *Diabetologia.* 22: 430–436.

35. Hiramatsu, K., A. Chait, and E. L. Bierman. 1983. Abnormal metabolism of low density lipoprotein derived from patients with diabetic hypertriglyceridemia. *Diabetes.* 32(Suppl. 1):15A(57).

36. Mahley, R. W., and T. L. Innerarity. 1983. Lipoprotein receptors and cholesterol homeostasis. *Biochim. Biophys. Acta.* 737: 197–222.

37. Windler, E., Y. S. Chao, and R. J. Havel. 1980. Regulation of the hepatic uptake of triglyceride-rich lipoproteins in the rat (opposing effects of homologous apolipoprotein E and individual C apoproteins). *J. Biol. Chem.* 255:8303–8307.

38. Schonfeld, G., W. Patsch, B. Pfleger, J. L. Witztum, and S. W. Weidman. 1979. Lipolysis produces changes in the immunoreactivity and cell reactivity of very low density lipoproteins. *J. Clin. Invest.* 64: 1288–1297.

39. Gianturco, S. H., A. M. Gotto, Jr., S. L. C. Hwang, J. B. Karlin, A. H. Y. Lin, S. C. Prasad, and W. A. Bradley. 1983. Apolipoprotein E mediates uptake of S_f 100–400 hypertriglyceridemic very low density lipoproteins by the low density lipoprotein receptor pathway in normal human fibroblasts. *J. Biol. Chem.* 258:4526–4533.

40. Krieger, M., M. S. Brown, J. R. Faust, and J. L. Goldstein. 1978. Replacement of endogenous cholesteryl esters of low density lipoprotein with exogenous cholesteryl linoleate. *J. Biol. Chem.* 253: 4093–4101.

41. Chait, A., S. Eisenberg, A. Steinmetz, J. Albers, and E. L. Bierman. 1984. Low density lipoprotein modified by cholesteryl ester transfer proteins have altered biological activity. *Biochim. Biophys. Acta.* 795:314–325.

42. Brown, M. S., and J. L. Goldstein. 1975. Regulation of the activity of the low density lipoprotein in human fibroblast. *Cell.* 6: 307–316.

43. Brown, M. S., S. E. Dana, and J. L. Goldstein. 1974. Regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in cultured human fibroblasts: comparison of cells from a normal subject and from a patient with homozygous familial hypercholesterolemia. *J. Biol. Chem.* 249:789–796.

44. Sigurdsson, G., A. Nicoll, and B. Lewis. 1976. The metabolism of low density lipoprotein in endogenous hypertriglyceridemia. *Eur. J. Clin. Invest.* 6:151–158.

45. Brook, J. G., H. Torsvik, R. S. Lees, M. A. McCluskey, and H. A. Feldman. 1979. Low density lipoprotein metabolism in type IV and type V hyperlipoproteinemia. *Metabolism.* 28:4–8.

46. Janus, E. D., A. M. Nicoll, P. R. Turner, P. Magill, and B. Lewis. 1980. Kinetic bases of the primary hyperlipidaemias: studies of apolipoprotein B turnover in genetically defined subjects. *Eur. J. Clin. Invest.* 10:161–172.

47. Reaven, G. M., D. B. Hill, R. C. Gross, and J. W. Farquhar. 1965. Kinetics of triglyceride turnover of very low density lipoproteins of human plasma. *J. Clin. Invest.* 44:1826–1833.

48. Sodhi, H. S., and B. J. Kudchodkar. 1973. Correlating metabolism of plasma and tissue cholesterol with that of plasma lipoproteins. *Lancet.* I:513–519.

49. Kudchodkar, B. J., and H. S. Sodhi. 1976. Turnover of plasma cholesteryl esters and its relationship to other parameters of lipid metabolism in man. *Eur. J. Clin. Invest.* 6:285–298.

50. Angelin, B., K. Einarsson, K. Hellstrom, and B. Leijd. 1978. Bile acid kinetics in relation to endogenous triglyceride metabolism in various types of hyperlipoproteinemia. *J. Lipid Res.* 19:1004–1016.