Cholesteryl ester storage disease: complex molecular effects of chronic lovastatin therapy

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Abstract To better characterize the in vivo effects of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibition on human lipid metabolism, an adolescent male with cholesteryl ester storage disease (CESD) was treated chronically with lovastatin. Therapy was associated with decreased liver-spleen size, improved but not normal serum lipids, a 26% decrease in hepatic cholesteryl ester, a 12% decrease in unesterified hepatic cholesterol, and a fourfold increase in hepatic low density lipoprotein (LDL) receptor protein. Hepatic mRNA levels for the LDL receptor and apolipoprotein (apo) B standardized to levels of hepatic gamma actin mRNA were unchanged with therapy. Kinetic studies revealed no change in the LDL fractional catabolic rate and a decrease in the LDL production rate. Size exclusion chromatography showed striking reductions in plasma very low density lipoprotein (VLDL) cholesterol and intermediate density lipoprotein (IDL) cholesterol but not LDL cholesterol with therapy. Mean LDL particle size and the LDL particle size range were increased by treatment. However, there was no difference in the ability of pretreatment or treatment LDL to bind to the LDL receptor on cultured cells consistent with previous studies in animals, indicating that lovastatin may alter LDL particles to impair interaction with the LDL receptor in vivo but not in vitro. Lovastatin therapy in CESD appears to be clinically beneficial and has complex effects on lipid metabolism that may include a dominant inhibitory effect on hepatic lipoprotein production, posttranscriptionally mediated induction of the LDL receptor, and alterations of LDL particles that interfere with their clearance by the LDL receptor in vivo.
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Supplementary key words liver size • very low density lipoprotein • low density lipoprotein • LDL receptor • hyperlipidemia

Cholesteryl ester storage disease (CESD) is an autosomal recessive disorder caused by a deficiency in the enzyme responsible for the lysosomal hydrolysis of triglycerides and cholesteryl esters, lysosomal acid lipase (1). A more severe deficiency of lysosomal acid lipase activity results in Wolman's disease, characterized by massive hepatosplenomegaly, failure to thrive, and death usually at age 3–6 months. Individuals with CESD often present in childhood with hepatomegaly which tends to be progressive. Generalizations about the clinical course of patients with CESD are difficult to make given the extremely small number of cases in the literature. Long-term survival can apparently occur but chronic liver disease and atherosclerosis have been identified as causes of premature death in some patients.

Atherosclerosis in CESD may be related in part to the striking hypercholesterolemia which also characterizes the disorder. Deficiency of acid lipase in CESD impairs the normal lysosomal catabolism of lipoproteins. Lipoprotein-derived cholesteryl ester is not efficiently hydrolyzed to free cholesterol, the sterol responsible for suppression of endogenous cholesterol synthesis through transcriptional and posttranscriptional control of the rate-limiting enzyme in cholesterol synthesis, HMG-CoA reductase (2). Thus, endogenous hepatic cholesterol synthesis as well as production of apolipoprotein B-containing lipoproteins are elevated in CESD (3) resulting in hypercholesterolemia. Overproduction of apoB-containing lipoproteins is also characteristic of a common genetic disorder, familial combined hyperlipidemia, and may be frequent among patients with hypercholesterolemia in the general population (4–6). Since LDL receptor function is normal in CESD (7) and in the vast majority of individuals in the general population with elevated cholesterol levels, CESD may be a suitable paradigm for the study of hypercholesterolemia due to overproduction.

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LDL, low density lipoprotein; apo, apolipoprotein; VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; CESD, cholesteryl ester storage disease; GGE, gradient gel electrophoresis; GGT, gamma glutamyl transpeptidase; FCR, fractional catabolic rate.

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Lovastatin, one of several HMG-CoA reductase inhibitors, is widely used for the treatment of hypercholesterolemia in humans. Animal studies indicate that the drug induces hepatic LDL receptor activity which is thought to result in increased hepatic uptake of LDL and ultimately excretion of cholesterol as bile acids. If this mechanism were dominant in CESD, one might predict that the hepatic dysfunction seen in this disorder would progress since the accelerated clearance of LDL via the LDL receptor would result in increased nonhydrolyzed cholesteryl ester in lysosomes. However, if instead the dominant effect of lovastatin were to reduce endogenous cholesterol synthesis and/or total transport of apoB-containing lipoproteins, hepatic dysfunction might be predicted to improve. In fact, two recent reports have noted favorable clinical responses to lovastatin in CESD (3, 8). In hopes of achieving a similar response and to gain insight into the clinical, biochemical, and molecular effects of lovastatin therapy in humans, we studied a boy with CESD treated chronically with lovastatin.

METHODS

Human studies approvals

These studies were approved by our subject and his parents and by institutional human studies committees at Washington University and Rush-Presbyterian-St. Luke's.

Lipid and lipoprotein determinations

Unesterified cholesterol was measured in choloroform–methanol 2:1 extracts of liver biopsy tissue by gas–liquid chromatography using 5α-cholestane as an internal standard as described (9). Esterified cholesterol was determined by calculating the difference between total cholesterol, measured by gas–liquid chromatography after saponification of cholesteryl esters as described by Sokoloff and Rothblat (10), and unesterified cholesterol. Total cholesterol, triglycerides, and lipoproteins were measured by standard Lipid Research Clinic techniques.

LDL receptor immunoblotting

Human liver biopsy samples stored at −80°C were removed to a −20°C cold room on dry ice, and fragments of different regions of the biopsy samples were obtained. Liver fragments were homogenized in 1.6% Triton X-100, 5 M urea, 0.3 mM leupeptin, 1.5 mM phenylmethanesulfonylfluoride and centrifuged as described (11) to yield tissue extracts. Immunoblotting was performed exactly as previously described (12) using rabbit IgG raised against the bovine LDL receptor (a kind gift from Drs. Michael Brown and Joseph Goldstein, Dallas) as primary antibody and radiolabeled goat anti-rabbit IgG as secondary antibody. Immunoreactive bands were quantitated by densitometric scanning of autoradiograms.

Construction of plasmids for in vitro transcription

Messenger RNA levels for apoB, the LDL receptor, and gamma actin were determined by solution hybridization/RNase protection using radiolabeled RNA probes generated by in vitro transcription using cDNA fragments as templates. A 215 nucleotide (nt) PstI-EcoRI fragment of the human apoB cDNA (a kind gift from Dr. Lawrence Chan, Houston) corresponding to nt positions 1146–1360 (inclusive) as numbered in reference 13 was subcloned into pGEM-3Z. A 359 nt EcoRI-BamHI fragment of the human LDL receptor cDNA (purchased from the American Type Culture Collection-ATCC #57004) corresponding to nt positions 719–1077 (inclusive) as numbered by Yamamoto et al. (14) was subcloned into pGEM-3Z. A 267 nt HindIII-Xbal fragment of the human gamma actin cDNA (15) corresponding to nt positions 1333–1599 (inclusive) as numbered in GenBank (accession #M24241) was subcloned into pGEM-3Z. Separate aliquots of each plasmid were then linearized with both enzymes used to generate the cDNA insert. The linearized plasmids were gel-purified and used to generate nonradioactive sense RNA and ^{32}P-labeled antisense RNA using SP6 and T7 RNA polymerases. Transcripts were separated from unincorporated nucleotides by push-column (Stratagene, La Jolla) chromatography; nonradioactive sense RNA was stored in aliquots of sterile water at −70°C and radioactive RNA probes were used within a few hours of preparation.

The authenticity of the apoB, LDL receptor, and gamma actin inserts was confirmed by direct double-stranded sequencing of the recombinant plasmids using Sequenase version 2.0. Labeled antisense riboprobes recognized apoB, LDL receptor, and gamma actin mRNA species and did not react with tRNA on Northern blots of total RNA prepared from the subject's second liver biopsy.

Quantitation of hepatic mRNA levels

Total RNA was prepared from liver biopsies as described by Chirgwin et al. (16). The solution hybridization assay was performed by a modification of the procedure reported by Azrolan and Breslow (17). Aliquots of liver total RNA stored as ethanol precipitates at −70°C were centrifuged, air-dried briefly then reconstituted in diethylpyrocarbonate-treated sterile water. Aliquots of solubilized RNA were removed to determine nucleic acid concentration and the remainder of the solution was vacuum-dried followed by re-solubilization of the RNA in 75 μl of hybridization buffer prepared exactly as described in reference 17. Hybridization buffer containing RNA was aliquoted to three separate tubes and RNA probe (500,000 dpm in 1 μl) was added to each tube. Each assay included separate tubes containing tRNA at a concentration equal to or greater than the concentration of liver

RNA being assayed. The solutions were overlaid with 30 μl of mineral oil, heated at 85°C for 10 min then hybridized at 50–65°C. After 12–16 h, 300 μl of ice-cold RNase solution (40 μg/ml RNase A, 250 U/ml RNase T1, 10 mM Tris-pH 7.4, 5 mM EDTA, 300 mM NaCl) was added to each tube and each was incubated at 37°C for 40 min.

In experiments to determine that an RNA fragment of the correct size was protected, 20 μl of 10% SDS was added to each tube and vortexed; the solutions were extracted once with phenol–chloroform then precipitated in the presence of tRNA carrier. The pellets were washed with 70% ethanol, solubilized, heated to 95°C for 3 min, then electrophoresed in 6% polyacrylamide–8 M urea sequencing gels with unhybridized probe and end-labeled HaeIII-digested phiX174.

For quantitative filter assays, 150 μg of tRNA and 400 μl of ice-cold 20% trichloroacetic acid (TCA) solution were added to each tube after digestion with RNase solution. The solutions were incubated on ice for 15 min, then applied to Whatman GF/C filters by suction. The filters were washed extensively with cold 7% TCA, then air-dried and counted in a liquid scintillation counter after addition of scintillation cocktail. RNase-resistant counts in the tRNA blanks were consistently less than 0.3% of the input radioactivity. For these experiments, each assay included a series of hybridization tubes containing between 5 and 500 pg of in vitro-transcribed sense RNA from which a standard curve was generated. The RNA content of unknowns was determined by linear regression. The amount of messenger RNA in unknowns was calculated by correcting for the contribution of polylinker sequence to the mass of sense RNA used to generate standard curves and for the difference in size between the RNA probe and the mature message.

Kinetic studies of LDL metabolism

Turnover studies were performed on an outpatient basis with adherence to a prescribed diet assessed by 14-day food records initiated immediately prior to infusion of the label. Dietary energy intake and composition were calculated using the computer program Datadiet (IPC Datadiet, Camarillo, CA). Dietary composition before/during lovastatin therapy was: carbohydrate 51.7%/57.1%, fat 33.8%/28.8%, protein 14.5/14.1%, and polyunsaturated: saturated fat 0.26/0.26.

Autologous, freshly isolated LDL (1.019–1.063 g/ml) was sterilely isolated and radiolabeled with ^{131}I and infused as described previously (18). Over 98% of the label was associated with apoB. TCA-precipitable radioactivity in plasma was determined over 9 days and calculations were performed as described (18) using the computer program Simulation, Analysis and Modeling (SAAM) obtained from the Resource Facility for Kinetic Analysis at the University of Washington, Seattle. The statistical significance of the difference in fractional catabolic rates between studies was determined by solving both sets of data simultaneously as parallel but unconnected models.

Separation and characterization of lipoproteins

Lipoproteins were separated from 1.0 ml of plasma by FPLC using two Superose 6B columns in series as previously described (19). The cholesterol content of eluent fractions was determined enzymatically using commercially available kits (Wako Fine Chemicals, Richmond). LDL was subjected to gradient gel electrophoresis (GGE) using 2–16% polyacrylamide gradient gels essentially as described by Krauss and Burke (20). Particle diameter was calculated from a standard curve generated using the migration distances of apoferritin (12.2 nm diameter), thyroglobulin (17.0 nm), and carboxymethylated latex beads (38.0 nm). For these experiments control plasma was fresh. It was not possible to analyze fresh plasma from our subject by GGE since gel variability would have made accurate comparisons between LDL particles before and during therapy impossible. Therefore, plasma samples from our subject before and during lovastatin therapy were stored frozen and never thawed until just before analysis by GGE in the same gels.

LDL binding studies

The ability of LDL to compete for specific ^{125}I-labeled LDL binding to the LDL receptor of human fibroblasts and HepG2 cells cultured in lipoprotein-deficient serum was determined as previously described (21, 22). Because of potential variation in LDL binding assays performed at different times due to factors such as cell lines at different passages and different preparations of lipoprotein-deficient serum, assays comparing the competition of different unlabeled LDL particles for ^{125}I-labeled LDL binding were performed at the same time. Control LDL prepared by ultracentrifugation (density 1.019–1.063 g/ml) was fresh. Pre-treatment and lovastatin LDL particles from our subject were isolated by size exclusion chromatography from plasma samples that had been stored frozen and never thawed until just before use. The concentration of apoB in each LDL preparation was determined by enzyme-linked immunosorbent assay (ELISA) as described by Ordovas and colleagues (23) and identical concentrations of apoB were used in the binding assays comparing competition for ^{125}I-labeled LDL binding by different LDL samples.

RESULTS

The subject of the current study presented at age 13 with hepatosplenomegaly, mildly elevated liver function tests, hypercholesterolemia, and hypertriglyceridemia. At age 14, glucose and lactate levels were normal after intra-

venous administration of glucagon excluding Type I glycogen storage disease. The diagnosis of cholesteryl ester storage disease was confirmed by the measurement of acid lipase activity in peripheral leukocytes as described by Koster, Vaandrager, and Van Berkel (24): subject = 0.96 nm substrate hydrolyzed/min per mg protein, normal control = 15.65 nm substrate hydrolyzed/min per mg protein. The subject has one brother who is healthy; another brother died at the age of one day of uncertain causes. Both parents are of Eastern European descent, both are healthy with normal plasma lipids, and there is no family history of premature coronary disease.

Lipid and lipoprotein values during this subject's course are shown in **Fig. 1A**. Dietary counseling was begun during month 11. The vertical line between months 43 and 44 indicates the initiation of lovastatin therapy. The long vertical arrows indicate the dates of liver biopsies; the short vertical arrows indicate the dates of lipoprotein turnover studies. Lipid levels clearly decreased prior to initiation of lovastatin, perhaps due to dietary therapy. Lovastatin was started at 20 mg/day and over 4 weeks increased to 80 mg/day, a dose he has continued to take for 3 years. Drug therapy was associated with a further decrease but did not normalize levels of total cholesterol (327 mg/dl at month 43, 233 mg/dl at month 74), and IDL + LDL cholesterol (247 mg/dl at month 43, 175 mg/dl at month 74). There was no consistent change in triglyceride levels after starting lovastatin and only a small effect on levels of HDL cholesterol (26 mg/dl at month 43, 31 mg/dl at month 74).

The medication was well tolerated. Serum levels of hepatic transaminases (AST and ALT) and gamma glutamyl transpeptidase (GGT) before and after initiation of lovastatin therapy are shown in Fig. 1B. As for Fig. 1A,

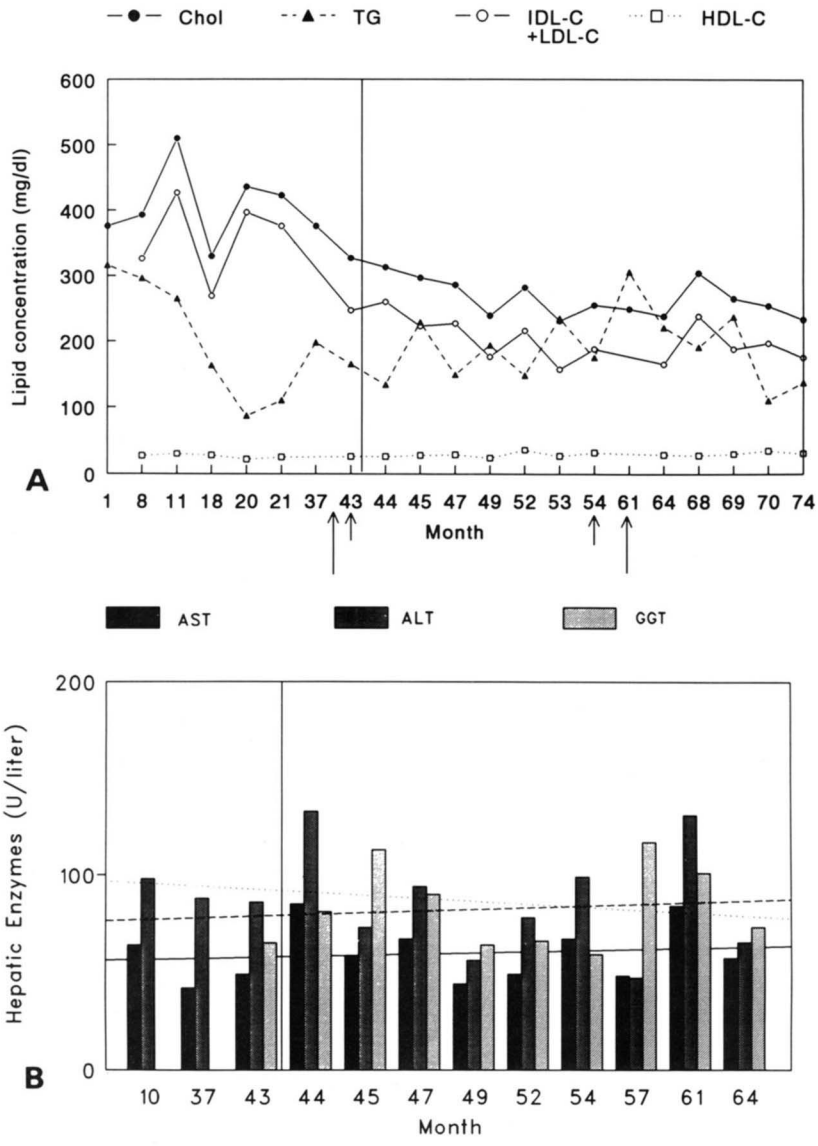

Fig. 1. Lipid and lipoprotein concentrations (panel A) and hepatic enzymes (panel B) in a subject with cholesteryl ester storage disease (CESD). For panel A, total cholesterol, triglyceride, IDL + LDL cholesterol, and HDL cholesterol concentrations in a subject with CESD were determined between December 1984 (month 1) and January 1991 (month 74). The vertical line between months 43 and 44 indicates initiation of lovastatin therapy. The short vertical arrows at months 43 and 54 indicate dates of kinetic studies. The long vertical arrows at months 39 and 61 indicate dates of liver biopsies. All determinations were performed on serum except for the determinations at months 43 and 54 which were performed on plasma. For panel B, serum levels of AST (normal < 40–50 units/liter), ALT (normal < 50–55 units/liter), and GGT (normal < 65–85 units/liter) were determined before and after initiation of lovastatin therapy (indicated by the vertical line between months 43 and 44). Trends for enzyme levels over time are represented by the solid horizontal line (AST), the dashed horizontal line (ALT), and dotted horizontal line (GGT).

the vertical line between months 43 and 44 indicates the initiation of drug therapy. Trends for enzyme levels over time are represented by the solid horizontal line for AST, the dashed horizontal line for ALT, and dotted horizontal line for GGT. No hepatic enzyme levels were as high as three times the upper limit of normal. Serum levels of total protein, albumin, and globulins have remained normal with chronic drug treatment. In addition, therapy did not interfere with adolescent growth. Prior to treatment at month 43, height was just above the 5th percentile and weight just below the 5th percentile. After 2 years of lovastatin therapy, the subject was above the 10th percentile for height and above the 8th percentile for weight.

Lovastatin treatment was associated with a decrease in liver and spleen size. Within 6 months of therapy, liver span decreased from 15 cm to 11 cm and the initially palpable spleen was no longer palpable. Surprisingly, liver–spleen size continued to decrease with long-term therapy despite a lack of further changes in serum lipid or lipoprotein levels. **Fig. 2** shows liver–spleen scans obtained by the same technique at months 61 (12/89) and 77 (4/91) demonstrating clear decreases in liver and spleen size with continued lovastatin treatment. Another liver–spleen scan performed by a different technique just before therapy confirmed the presence of massive hepatomegaly with two-thirds of the liver visualized below the right costal margin.

TABLE 1. Effect of lovastatin on hepatic cholesterol and cholesteryl ester content in cholesteryl ester storage disease

	Hepatic Cholesterol		
	Unesterified	Esterified	Hepatic Protein
	μg/mg protein		*mg/g tissue wet weight*
Pre-treatment	35.5 ± 0.6	455.6 ± 18.0	0.131 ± 0.003
Treatment	31.4 ± 1.0[a]	335.7 ± 6.0[b]	0.128 ± 0.004

Liver biopsies were performed on a subject with cholesteryl ester storage disease 3 months before (Pre-treatment) and after 18 months (Treatment) of therapy with lovastatin. Determinations of the cholesterol and cholesteryl ester content of several different regions of each biopsy sample were performed by gas chromatography using 5 α-cholestane as internal standard.
[a] $P = 0.028$ versus pre-treatment biopsy.
[b] $P = 0.0028$ versus pre-treatment biopsy.

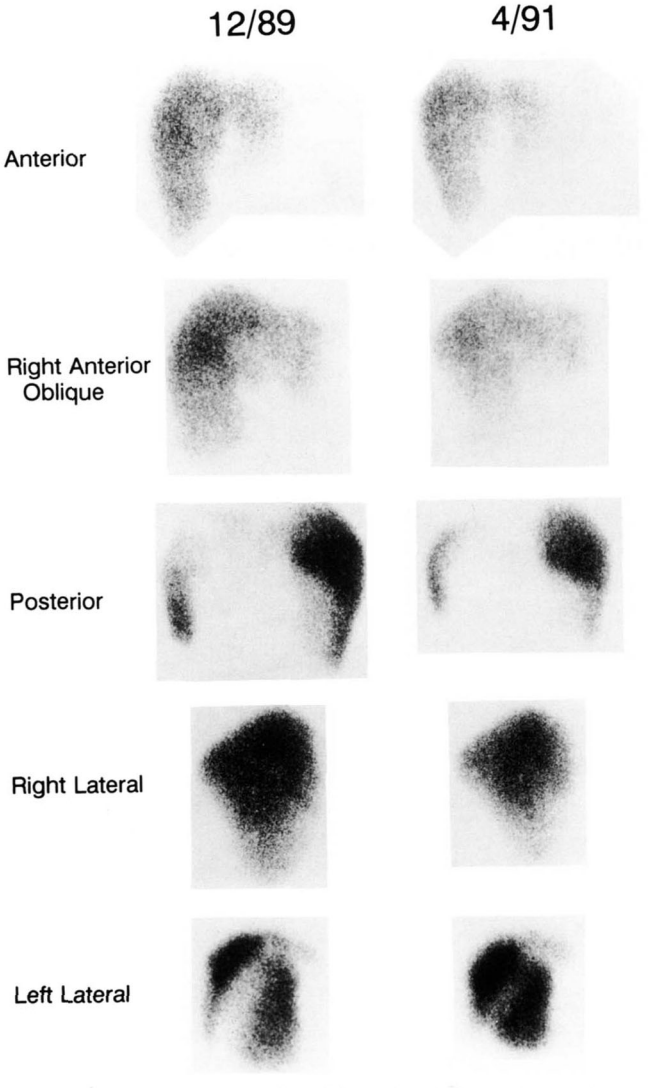

Fig. 2. Liver–spleen scans of a subject with cholesteryl ester storage disease on lovastatin therapy. Scans were performed by the same technique during the course of chronic therapy with lovastatin (80 mg/day). The 12/89 scan corresponds to month 61 and the 4/91 scan month 77 as numbered in Fig. 1.

Histologic features of liver biopsies performed before and during therapy were essentially identical. Specimens were characterized by portal-to-portal bridging fibrosis but no regenerative nodules and no significant inflammation.

Lovastatin therapy was associated with a 12% decrease in hepatic free cholesterol and a 26% decrease in cholesteryl ester (**Table 1**). The absolute levels of hepatic free cholesterol in Table 1 are nearly identical to those reported in cultured HepG2 cells (22) and in biopsies from other patients with CESD as well as controls (ref. 1, see Table 64-4). The mass of cholesteryl ester in normal cells is usually substantially less than the mass of unesterified cholesterol. However, hepatic cholesteryl ester mass in our subject was more than 10 times that of free cholesterol and was still considerably elevated despite improvement with therapy.

LDL receptor protein was induced in liver by lovastatin. Using tissue extracts prepared from different regions of liver biopsies to control for regional variation in LDL receptor expression, immunoblot experiments using an antibody specific for the LDL receptor were performed on pre-treatment and treatment liver samples. **Fig. 3** shows an immunoblot of CESD liver before (lane 1) and during (lane 2) lovastatin therapy with the position of the LDL receptor indicated by the arrow. In our hands,

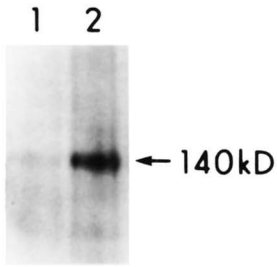

Fig. 3. Immunoblots of human LDL receptor protein in the liver of a subject with cholesteryl ester storage disease before and during lovastatin therapy. Tissue extracts of human liver were prepared and electrophoresed in SDS-polyacrylamide gels followed by transfer to nitrocellulose and detection with an antibody specific for the LDL receptor. No bands were seen on parallel blots probed with a nonspecific antibody. The LDL receptor position indicated by the arrow was determined by comparison with molecular weight standards and the position of the LDL receptor in extracts of human fibroblasts electrophoresed on the same gel. Lane 1 contained 25 μg protein from liver before lovastatin therapy. Lane 2 contained 25 μg protein from liver during lovastatin treatment.

the human LDL receptor consistently migrates to the 140 kDa position under nonreducing conditions. No bands were seen on parallel control blots using non-immune IgG instead of specific antibody. In four experiments (different blots utilizing extracts prepared from different regions of the liver biopsy specimens), the LDL receptor signal in lovastatin-treated liver was 412 ± 86% of pre-treatment liver (P = 0.0171). There was no difference in the hepatic concentration of a control protein, tubulin, determined by Ponceau-S staining of blots before and during lovastatin therapy. Specifically, protein staining showed no evidence that the proteins in the pre-treatment sample (lane 1) were degraded.

Messenger RNA levels for the LDL receptor, apoB, and gamma actin were determined in liver biopsies before and during lovastatin therapy by solution hybridization using radiolabeled RNA probes. With this assay, in vitro-transcribed, radioactive antisense RNA complementary to a short region of a known mRNA is hybridized with total RNA followed by digestion with ribonucleases which degrade single-stranded RNA. The segment of mRNA hybridized to an RNA probe is protected from digestion and can be visualized on gels. Protected fragments of the predicted sizes are shown for LDL receptor message (359 nt), apoB message (215 nt), and actin message (267 nt) at the arrows in **Fig. 4**. Two major protected fragments were consistently seen for apoB, a pattern very similar to that reported by Azrolan and Breslow (17) in cultured hepatocytes despite the fact that these investigators used an RNA probe complementary to a different region of the apoB message. A comparison of the human apoB sequence used in our riboprobe with homologous sequences in GenBank yielded no non-apoB sequences that could account for the protected pattern observed, decreasing the possibility that our assay is not specific for apoB mRNA. The protected fragments in Fig. 4 were generated using total RNA from the lovastatin-treatment biopsy (month 61).

The levels of apoB, LDL receptor, and actin mRNA in CESD liver before and during lovastatin therapy are shown in **Table 2**. Essentially identical decreases (73–79%) were detected for each of the messages assayed. There was no difference in the quality of the RNA samples as determined by ethidium staining of denaturing agarose gels. Pre-treatment and treatment apoB and LDL receptor mRNA levels were not different when absolute values were standardized to levels of actin mRNA (Table 2). The absolute values for apoB mRNA in the treatment sample are similar to those reported recently in HepG2 cells (17), and the estimated levels of LDL receptor mRNA per cell in the treatment sample are similar to those estimated for hamster liver (25) using solution hybridization with single-stranded DNA probes. The absolute values for the LDL receptor message, however, are higher than those reported in the livers of nonhuman primates (26) and mice (27).

Fig. 5 depicts the turnover of radiolabeled LDL before (closed circles) and after (open circles) 1 year of lovastatin treatment. There was no significant difference in the fractional catabolic rate for ^{131}I-labeled LDL (0.424 ± 0.005 [mean ± SD] pools/day before and 0.432 ± 0.005 pools/day after treatment) confirming the results of Ginsberg and colleagues (3). However, there was a 34% decrease (247 mg/dl at month 43, 162 mg/dl at month 54) in IDL + LDL cholesterol levels measured by ultracentrifugation associated with lovastatin treatment. LDL production rate was decreased by 33% from 45.4 to 30.4 mg/kg

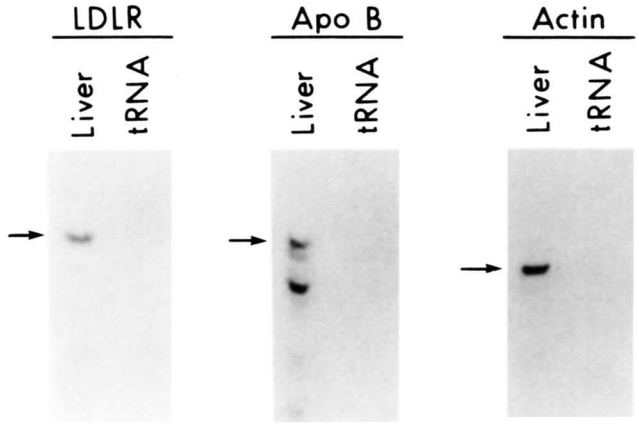

Fig. 4. Denaturing gel electrophoresis of RNase-protected RNA probes for the LDL receptor, apolipoprotein B, and actin in human liver. Three aliquots of total RNA (20 μg each) prepared from the liver of a subject with cholesteryl ester storage disease and three aliquots of tRNA were hybridized with ^{32}P-labeled RNA probes for the human LDL receptor, human apolipoprotein B, and human gamma-actin. After 12–16 h, mixtures were incubated with ribonucleases followed by electrophoresis on sequencing gels. The arrows denote protected fragments of 359 nucleotides for LDL receptor message, 215 nucleotides for apoB message, and 267 nucleotides for actin message.

TABLE 2. Effect of lovastatin on hepatic apolipoprotein B, LDL receptor, and gamma-actin mRNA levels in cholesteryl ester storage disease

	ApoB mRNA	LDL Receptor mRNA	Actin mRNA
Pre-treatment	508 ± 98	245 ± 39	18.7 ± 2.8
Treatment	106 ± 31	66 ± 15	4.9 ± 0.3
Change	↓79%	↓73%	↓74%
mRNA standardized to actin mRNA (arbitrary units)			
Pre-treatment	27.2	13.1	
Treatment	21.6[a]	13.5[a]	

Liver biopsies were obtained 3 months before (Pre-treatment) and after 18 months of therapy (Treatment). mRNA levels were determined by solution hybridization in two or three separate assays using radiolabeled RNA probes after documenting by gel electrophoresis that RNA of the predicted size for each probe was protected from RNase digestion (Fig. 4). Assay tubes contained between 3 and 6 μg of total RNA and, for each riboprobe, Pre-treatment and Treatment samples were assayed at the same time. Data in the first two rows are expressed as pg/μg of total RNA (mean ± SE).

[a]Not significantly different from Pre-treatment.

per day (pre-treatment weight 43.2 kg, treatment weight 48.4 kg).

Size exclusion chromatography (**Fig. 6**) of pre-treatment plasma (closed circles) and plasma after 2 years of lovastatin therapy (open circles) showed that the lovastatin-associated decrease in total cholesterol was accounted for by substantial decreases in VLDL and IDL cholesterol. However, despite the striking induction in hepatic LDL receptor protein with therapy (Fig. 3), LDL cholesterol was essentially unchanged. These data suggest that decreased LDL production caused by decreased levels of the LDL precursors VLDL and IDL is offset by delayed clearance of LDL particles (consistent with the finding of no increase in fractional catabolic rate [Fig. 5] despite increased LDL receptor protein [Fig. 3]) resulting in no net change in plasma LDL cholesterol levels.

To test the hypothesis that lovastatin physically alters LDL particles and disrupts their normal clearance, gradient gel electrophoresis of LDL was performed (**Fig. 7**). LDL from a normal control (lane 1) had a mean particle diameter of 28.1 nm. LDL from our CESD subject before lovastatin (month 42, see Fig. 1A) was more homogeneous in size than control LDL and had a mean particle diameter of 27.7 nm (lane 2). LDL from our subject after 20 months of lovastatin treatment (month 63) showed an increased size range and a mean particle diameter of 28.4 nm (lane 3). The same increased size range and particle diameter in comparison to control LDL and pre-treatment LDL was seen using LDL obtained after 11 months of lovastatin therapy (month 54).

As LDL appeared to be altered by lovastatin therapy, we tested the hypothesis that pre-treatment and treatment particles would interact differently with the LDL receptor in vitro, thus providing a explanation for the lack of an increase in the FCR of autologous LDL (Fig. 5) despite an increase in LDL receptor mass (Fig. 3). LDL binding

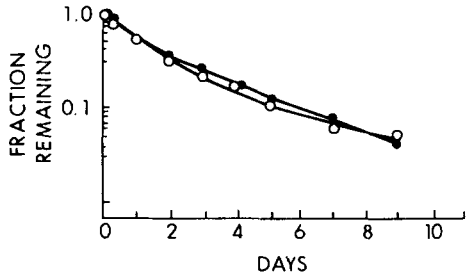

Fig. 5. Turnover of ^{131}I-labeled LDL in a subject with cholesteryl ester storage disease before and during lovastatin therapy. The vertical axis represents the fraction of initial trichloroacetic acid-precipitable radioactivity remaining in the plasma compartment over time. Closed circles represent baseline studies and open circles represent studies performed after 1 year of lovastatin therapy.

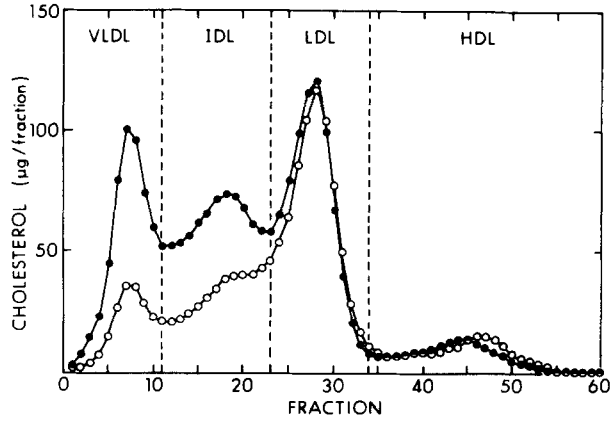

Fig. 6. Separation of plasma lipoproteins by size exclusion chromatography in cholesteryl ester storage disease (CESD). One ml of plasma from a subject with CESD before therapy (closed circles) and 2 years after initiation of therapy with lovastatin (open circles) was applied to two Superose 6B columns in series and eluent fractions were assayed for cholesterol content. The identification of fractions representing specific lipoproteins was confirmed by performing the same separation on control plasma (not shown).

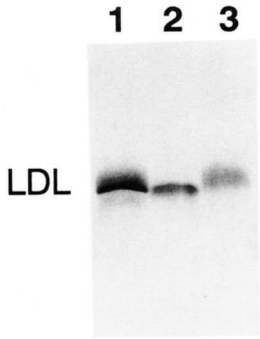

Fig. 7. Gradient gel electrophoresis of control and cholesteryl ester storage disease (CESD) LDL particles. LDL particles from a control subject (lane 1) and from a subject with CESD before treatment (lane 2) and during treatment with lovastatin (lane 3) were electrophoresed on 2–16% gradient gels. Mean particle size was determined by comparison to the migration of standards with known diameters.

studies assessing the competition for ^{125}I-labeled LDL binding to HepG2 and human fibroblast LDL receptors by pre-treatment and treatment (as well as control) LDL particles are shown in **Table 3**. By two-way analysis of variance, there was no difference in the competition of the different LDL particles for binding to the LDL receptor.

DISCUSSION

Cholesteryl ester storage disease is rare. However, the hyperlipidemia seen in this disorder occurs in a setting of normal LDL receptor function and overproduction of apoB-containing lipoproteins, features which probably characterize a substantial proportion of members of the general population with hyperlipidemia. The results of the current study may therefore have implications for the average person with polygenic hypercholesterolemia treated with HMG-CoA reductase inhibitors and for the effects of such therapy on human lipid metabolism in general. Nevertheless, our results were derived from a single patient and must be viewed as interesting observations that require further exploration.

Most importantly for our patient, lovastatin administered at the highest recommended dose (80 mg/day) was clinically beneficial, decreasing liver size in a disorder frequently characterized by progressive hepatomegaly as well as improving serum lipids, in particular lowering VLDL + IDL but not LDL concentrations, and decreasing hepatic cholesteryl ester content. Our results confirm the recent initial report of decreased hepatic cholesteryl ester content in CESD with lovastatin treatment (8). Our results also confirm previous findings of improved but not normalized serum lipids in this disorder (3, 8) although one group has recently reported that lovastatin at half the dose used in our subject did not improve serum lipoproteins in three females with CESD (28). Prior reports have not shown changes in liver size with treatment (3, 8, 28) perhaps because of the use of lower doses of lovastatin. The reduction in organ size observed clinically and by scans (Fig. 2) was likely due to reduced hepatic lipid storage, occurred in the absence of further changes in serum lipids, and was observed despite continued adolescent growth. Interference with the mevalonate pathway through HMG-CoA inhibition can disrupt cell growth (29). Fortunately, the use of 80 mg/day of lovastatin did not prevent continued adolescent growth in our subject, a clinical observation which supports recent in vitro findings that conventional doses of lovastatin sufficient to inhibit sterol synthesis do not affect processes related to regulation of cell growth (30).

There was a substantial increase in LDL receptor protein in lovastatin-treated liver (Fig. 3). We are unaware of prior reports of visualization of an increase in human LDL receptor protein with HMG-CoA reductase inhibition although a recent study did report increased LDL binding to human liver homogenates after treatment with pravastatin, another HMG-CoA reductase inhibitor (31). Our results are important because LDL binding assays do not strictly examine the interaction between LDL and the LDL receptor. LDL has been shown to bind specifically

TABLE 3. Competition for ^{125}I-labeled LDL binding to fibroblast and HepG2 LDL receptors by unlabeled control LDL, and by LDL from a subject with cholesteryl ester storage disease (CESD) before and during treatment with lovastatin

Cell Type	^{125}I-Labeled LDL + EDTA-Saline	^{125}I-Labeled LDL + Control LDL	^{125}I-Labeled LDL + CESD Pre-treatment LDL	^{125}I-Labeled LDL + CESD Lovastatin LDL
Fibroblasts	43.3 ± 1.0	20.8 ± 0.9	21.7 ± 1.6	17.0 ± 0.9
HepG2 cells	7.57 ± 0.10	5.70 ± 0.25	5.41 ± 0.23	5.72 ± 0.41

Cultured human fibroblasts and HepG2 cells were grown in lipoprotein-deficient serum followed by determination of specific ^{125}I-labeled LDL binding at 4°C by standard techniques. Binding assays were performed using 15 μg/ml ^{125}I-labeled LDL with no added unlabeled LDL (EDTA-saline condition), or with 7.5 μg apoB/ml unlabeled control LDL, 7.5 μg apoB/ml unlabeled pre-treatment LDL, or 7.5 μg apoB/ml lovastatin LDL. Similar results were obtained using higher amounts of unlabeled LDL. Data are expressed as ng of LDL specifically bound/mg cell protein (mean ± SE).

to proteins other than the LDL receptor (12). However, our results do not prove that the increased LDL receptor mass detected by Western blotting represents an increase in functional LDL receptors. These studies were performed in a diseased liver and it is possible that these receptors do not cycle normally between the cytoplasm and the cell surface.

Lovastatin administered in very high doses (equivalent to 750–3000 mg per day in our 48 kg subject) to hamsters and rabbits results in an increase in hepatic LDL receptor mRNA levels and LDL receptor protein (25) presumably by inhibition of cholesterol synthesis. Despite a reduction in free cholesterol (Table 1) and an impressive increase in LDL receptor protein in human liver, hepatic LDL receptor mRNA levels determined by a quantitative assay decreased with lovastatin (Table 2). Similar decreases were seen for apoB mRNA levels and for the message levels of a structural gene unrelated to lipid metabolism, gamma actin. If LDL receptor and apoB mRNA levels are expressed relative to actin levels, no specific change in message levels occurred with therapy. We do not believe there is a trivial explanation for the observed decreases. We were unable to detect any difference in the quality of the pre- and post-therapy RNA assayed, the assay used to quantitate message levels is not particularly subject to differences in RNA integrity since only a small fragment of each message is assayed, and RNA experiments were done before liver samples were processed for the experiments that detected large increases in LDL receptor protein in treated liver. Ideally, we would also have liked to assay HMG-CoA reductase mRNA levels which would be predicted to increase substantially with therapy. However, available RNA was limited considering the number of analyses performed on the liver biopsies (mRNA levels, free and esterified cholesterol, and LDL receptor protein).

We speculate that the observed decreases in message levels for apoB, the LDL receptor, and gamma actin are related to the striking decrease in liver size observed with lovastatin. The reduction in liver size may have been associated with a generalized, nonspecific decrease in transcription. Less likely, lovastatin alone, independent of the complex events associated with a reduction in hepatomegaly, may have regulated LDL receptor, apoB, and actin mRNA levels in parallel although the physiologic basis of such regulation is unclear since LDL receptor protein increased. Parallel regulation of sterol responsive and "housekeeping" genes is not unprecedented. Le Cras, Gherardi, and Bowyer (32) have recently shown that in lovastatin-treated HepG2 cells mRNA levels for β actin and glyceraldehyde-3-phosphate dehydrogenase change in parallel with message levels for the LDL receptor and HMG-CoA reductase, suggesting that lovastatin may have nonspecific effects on the general level of gene transcription.

The finding that lovastatin clearly decreased hepatic sterol concentrations, increased LDL receptor protein levels, and decreased serum levels of the apoB-containing lipoproteins VLDL and IDL without specific effects on apoB or LDL receptor message levels is consistent with posttranscriptional regulation of apoB and LDL receptor expression in human liver. As our mRNA data were obtained at only two time points in a single patient with an uncommon disease, it is premature to conclude that posttranscriptional mechanisms for regulation of apoB and the LDL receptor exist in human liver. However, a growing body of evidence indicates that apoB secretion is regulated posttranscriptionally (33–35), probably at the level of apoB degradation (36).

Sterol-mediated regulation of the LDL receptor is thought to be primarily transcriptional through the interaction of sterols with a sterol regulatory element (SRE-1) in the 5' flanking region of the LDL receptor gene (29). However, sterol-mediated down-regulation of the LDL receptor occurs in cells transfected with a retroviral vector containing the LDL receptor cDNA but not the 5' flanking region of the LDL receptor gene (37), consistent with the existence of sterol-responsive posttranscriptional mechanisms in mammalian cells. Recent evidence from studies of cultured human fibroblasts also supports the existence of sterol-responsive posttranscriptional mechanisms (consisting predominantly of altered translational efficiency) for regulation of LDL receptor expression (38). Sterol-responsive posttranscriptional mechanisms have clearly been shown to be genetically distinct from sterol-responsive transcriptional mechanisms for regulation of the HMG-CoA reductase gene (2). We are unaware of prior reports of the effects of HMG-CoA reductase inhibition on LDL receptor mRNA levels in human liver. The current study, by demonstrating that a decrease in hepatic sterol is associated with a striking increase in LDL receptor protein without a specific effect on LDL receptor RNA levels, thus provides support for the existence of sterol-responsive posttranscriptional mechanisms affecting LDL receptor expression in human liver. Our findings, however, do not imply that sterol-mediated transcriptional regulation did not occur at any time in our patient. Our results, for obvious ethical reasons, reflect a single treatment time point. It is entirely possible that liver tissue obtained soon after initiation of drug therapy (before changes in liver size were seen) would have shown an increase in LDL receptor message.

Ironically, although lovastatin induced the LDL receptor in human liver, clearance of LDL through the LDL receptor pathway did not appear to mediate the improvement in serum cholesterol observed in our subject with CESD. Since acid lipase deficiency causes accumulation of LDL-derived cholesteryl ester, an increase in LDL receptor-mediated metabolism of LDL would increase hepatic cholesteryl ester, the opposite of what we observed (Table 1). The fractional catabolic rate of LDL was not increased by lovastatin therapy (Fig. 5) and there was no decrease in LDL cholesterol measured by size exclusion

chromatography (Fig. 6) although VLDL and IDL cholesterol were lower with therapy. While the latter finding might be explained by apoE-mediated enhanced affinity of certain lipoproteins for the LDL receptor, such a mechanism is unlikely because hepatic cholesteryl ester content decreased. It is also possible that lovastatin has an independent effect on apoE receptor expression or that lovastatin reduces the cholesteryl ester content of lipoprotein particles that are removed normally by the apoE and LDL receptors, but again these possibilities are difficult to reconcile with the observed decrease in hepatic cholesteryl ester content; both would be associated with increased total hepatic uptake of lipoproteins and hepatic lipids would accumulate unless lovastatin independently improves the lysosomal hydrolytic defect in CESD.

Our results are more likely explained by a dominant inhibitory effect of lovastatin on the production of apoB-containing lipoproteins. Decreased lipoprotein production was the dominant effect of lovastatin observed in another patient with CESD (3), and appears to account for the hypolipidemic effect of lovastatin in familial combined hyperlipidemia (5) and in primary moderate hypercholesterolemia (6), disorders characterized by apoB overproduction and presumably normal LDL receptor function. In contrast, lovastatin clearly increases LDL fractional catabolic rate in patients with deficient LDL receptors (39).

Lovastatin induction of the LDL receptor in liver was not associated with a decrease in LDL cholesterol (Fig. 6) or an increase in LDL fractional catabolic rate (Fig. 5) but was associated with altered mobility of LDL particles as determined by gradient gel electrophoresis (Fig. 7). We interpret these results as indicating that lovastatin treatment decreases the production rate of LDL (by decreasing the concentration of the precursor particles VLDL and IDL) but LDL cholesterol levels do not change because decreased production is offset by decreased clearance (inferred from the lack of change in LDL cholesterol and LDL fractional catabolic rate despite increased levels of hepatic LDL receptor protein) caused by lovastatin-associated alteration of LDL particles. This alteration interferes with the ability of human LDL particles to bind to the LDL receptor in vivo but not in vitro since there was no difference in the ability of pre-treatment LDL and lovastatin LDL to interact with LDL receptors on cultured cells (Table 3). Strikingly similar results were reported by Berglund and colleagues (40) who observed impaired interaction of lovastatin-treated guinea pig LDL with the LDL receptor in vivo in animals but not in studies with cultured cells. These authors, however, were unable to detect any difference between control and lovastatin-treated guinea pig LDL by gradient gel electrophoresis. The current report provides the first evidence that the apparent impaired interaction of lovastatin-treated LDL with the LDL receptor in vivo is associated with altered LDL particles.

In summary, chronic lovastatin therapy was well tolerated by a patient with CESD and resulted in improved serum lipids and decreased liver-spleen size. Lovastatin therapy in our single patient with CESD appeared to paradoxically increase hepatic LDL receptor protein without increasing LDL metabolism through the LDL receptor pathway.

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