Cholesterol Homeostasis in Mononuclear Leukocytes from Patients with Familial Hypercholesterolemia Treated with Lovastatin

Fay C. Hagemenas and D. Roger Illingworth

Lovastatin is a potent competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase), the rate-limiting enzyme in cholesterol biosynthesis. The hypocholesterolemic effects of lovastatin in patients with primary hypercholesterolemia have been well established, but the extent to which this drug affects cellular cholesterol homeostasis in humans is less well delineated. Previous in vivo studies in animals and in vitro studies in cells grown in tissue culture have shown that the administration of either lovastatin or a related drug, mevastatin (compactin), results in compensatory increases in the mass of HMG CoA reductase and in cholesterol synthesis from 2-14C-acetate. Recent in vitro studies in cells grown in tissue culture have shown that the administration of lovastatin results in compensatory increases in the mass of HMG CoA reductase and a concurrent reduction in the rate of synthesis of LDL.

In the present study, we utilized freshly isolated mononuclear leukocytes from patients with heterozygous familial hypercholesterolemia (FH) treated with increasing doses of lovastatin. The high affinity receptor-mediated degradation of 125I-LDL in freshly isolated mononuclear cells from patients on 80 mg of lovastatin daily was determined at baseline and on lovastatin at a dose of 80 mg/day, LDL cholesterol levels fell progressively on lovastatin (30% reduction on 80 mg daily, p<0.005). These changes were paralleled by a 121% increase in the activity of HMG CoA reductase (p<0.05) and a 39% increase in cholesterol synthesis from 2-14C-acetate (p<0.005). Total and high affinity degradation of 125I-LDL increased from 27±3.3 and 12.1±1.6 ng/4x10^6 cells/4 hours on the diet only to 69.7±7.2 and 32.9±3.6 ng/4x10^6 cells/4 hours, respectively, (mean±SEM) in mononuclear cells isolated from patients on 80 mg of lovastatin daily (p<0.005). We conclude that the hypocholesterolemic effects of chronic lovastatin therapy are accompanied by an increase in high affinity degradation of LDL and an increased capacity for cholesterol biosynthesis in freshly isolated mononuclear leukocytes. (Arteriosclerosis 9:355-361, May/June 1989)

The 23 adult patients who participated in this study were diagnosed as having heterozygous FH on the basis of persistent primary hypercholesterolemia greater than 200 mg/dl for a period of at least 6 months. They were treated with sequentially increasing doses of lovastatin (10 to 80 mg/day in a twice-daily regimen). The in vitro activity of HMG CoA reductase and cholesterol synthesis from 2-14C-acetate was determined in mononuclear cells obtained under steady-state conditions after patients had spent 6 weeks on doses of 20, 40, or 80 mg/day. The total and high affinity degradation of 125I-LDL was determined at baseline and on lovastatin at a dose of 80 mg/day.

Methods

The 23 adult patients who participated in this study were diagnosed as having heterozygous FH on the basis of persistent primary hypercholesterolemia greater than 200 mg/dl for a period of at least 6 months. They were treated with sequentially increasing doses of lovastatin (10 to 80 mg/day in a twice-daily regimen). The in vitro activity of HMG CoA reductase and cholesterol synthesis from 2-14C-acetate was determined in mononuclear cells obtained under steady-state conditions after patients had spent 6 weeks on doses of 20, 40, or 80 mg/day. The total and high affinity degradation of 125I-LDL was determined at baseline and on lovastatin at a dose of 80 mg/day.
300 mg/dl, tendon xanthomas, an inheritance pattern consistent with autosomal dominant, and primary hypercholesterolemia in other family members with an absence of multiple phenotypes. All patients had normal thyroid, renal, and hepatic function, and none had diabetes. They had all received dietary instruction on a low fat (<30%) diet with a cholesterol intake of less than 300 mg/day. Nineteen patients participated in a sequential dose-response study with lovastatin, and four patients received a single oral dose of 100 mg 14C-labeled lovastatin to assess drug pharmacokinetics. The latter four patients were not taking any lipid-lowering drugs, had not previously been treated with lovastatin, and did not participate in the dose-response study. The control subjects were normolipidemic individuals who were age- and sex-matched with the treated group but who were not following any specific dietary prescription. All patients were seen on an outpatient basis in the Clinical Research Center of The Oregon Health Sciences University. Informed consent was obtained from each patient, and the protocol was approved by the Human Research Committee of this institution.

Study Protocol

In the patients with FH, hypolipidemic drugs were withdrawn a minimum of 4 weeks before the start of the baseline phase. No patient had been previously treated with probucol. After a 4-week baseline period during which time patients took a placebo capsule twice daily, each patient was treated sequentially with increasing doses of lovastatin of 10, 20, 40, and 80 mg daily (taken twice daily with breakfast and dinner). Each dose period was 6 weeks in duration, and patients were seen after 4 and 6 weeks on each dose. Blood samples were obtained in the mornings after an overnight fast of 12 to 15 hours for lipid and lipoprotein analysis19 and for clinical chemistry and hematologic determinations. Mononuclear cells were isolated by Ficoll gradient centrifugation as previously described20 from 40 cc blood (taken in heparin anticoagulant) obtained from each patient during the last week of each treatment period. Patients did not take lovastatin in the mornings that they attended clinic until after blood samples had been drawn. The mononuclear cells isolated by this method consisted of a mixture of 80% to 85% lymphocytes and 15% to 20% monocytes. The viability of the cell preparation was assessed by trypan blue exclusion, and purity was assessed by nonspecific esterase staining. Treatment with lovastatin did not alter the number or composition of the cell mixture.

Plasma concentrations of lovastatin (and possible metabolites) were determined at hourly intervals after the oral administration of a single 100-mg dose of 14C-labeled lovastatin (20 μCi) in four adult patients with heterozygous FH. The 14C radioactivity in plasma and in the isolated mononuclear cells was used as an indicator of the concentrations of lovastatin and metabolites at different times after the oral dose had been taken.

Analytical Methods

The concentrations of plasma lipids and lipoproteins were determined as previously described.19 Cholesterol synthesis from 2-14C-acetate, total and high affinity degradation of 125I-LDL, and total HMG CoA reductase were measured by previously described methods15,21,22 with the following modifications in the LDL receptor assay. Two changes in the LDL receptor assay have occurred since our previously reported studies15 and have yielded lower radioactivity in the blanks. The volumes used in the extraction of noniodide 125I have been modified: 1 ml out of the 2.0 ml incubation media is mixed with 1.0 cc 20% trichloroacetic acid (TCA) and 2.0 ml water; 1.0 ml of the TCA supernatant is removed and mixed with hydrogen peroxide and potassium iodide. We have also modified the blanks in the LDL receptor assay to include more protein, and we use 125I-LDL plus 500 mg unlabeled LDL, which has been added to the medium with the labeled LDL but no cells. When compared to our previous results,15 these changes in assay conditions have led to more reproducible and lower blanks and an overall increase in the measured cellular catabolism of 125I-LDL. All studies were performed in triplicate using mononuclear cells obtained from 40 cc of heparinized blood.

Statistical Analyses

Baseline comparison between metabolic parameters and lipid concentrations in the FH patients and controls were made using Students t test. The changes from baseline to 80 mg/day were assessed with both paired t tests and Wilcoxon's Signed Rank test. The results of using the Wilcoxon and paired t tests were in complete agreement. We report here the results of the paired t test. Partial correlation coefficients were computed from a multiple regression analysis. Comparisons between the hypolipidemic response to each dose of lovastatin was by analysis of variance.20

Results

Studies of cholesterol synthesis, HMG CoA reductase, and LDL receptor activity in freshly isolated mononuclear leukocytes were conducted on cells isolated from 19 patients with heterozygous FH who had last taken a dose of lovastatin the previous evening (12 to 15 hours earlier) and who had been receiving constant doses of lovastatin for a period of 6 weeks. To ascertain whether appreciable amounts of lovastatin and metabolites were still present in plasma and in the isolated mononuclear cells at this time, pharmacokinetic studies were conducted in four patients with heterozygous FH, each of whom had received a single dose of 100 mg of 14C-labeled lovastatin (20 μCi) at 8:00 a.m. and from whom blood samples were taken periodically for 96 hours thereafter. As illustrated in Figure 1, 14C-lovastatin (and metabolite) concentrations increased rapidly in plasma after oral administration of lovastatin, and peak levels occurred after 3 hours. Plasma concentrations decreased rapidly thereafter, and concentrations of 14C-lovastatin and metabolites were undetectable in plasma samples obtained after more than 12 hours after the initial dose. Additionally, mononuclear cells isolated at these later time points did not show measurable 14C label (data not shown). Thus, for the mononuclear cell studies, the cells were obtained at a time when plasma concentrations...
Figure 1. The time course (hrs) of appearance of 14C lovastatin (and metabolites) (µg/ml) in plasma. Four patients with heterozygous familial hypercholesterolemia, who had never previously received lovastatin, took a single 100-mg dose of 14C lovastatin (20 µCi) at 8:00 a.m. Blood samples were taken over a period of 96 hours. The lovastatin was taken after an overnight fast. Results represent the means±SEM from separate studies in four patients.

The effect of sequentially increasing doses oflovastatin (taken twice daily) on the plasma concentrations of lipids and lipoproteins is shown in Figure 2. Plasma concentrations of total cholesterol decreased from 373±13 (mean±SEM) to 306±10 mg/dl after 4 to 6 weeks on 10 mg of lovastatin twice daily, 291±12 mg/dl on 20 mg twice daily, 272±11 mg/dl after 4 to 6 weeks on 40 mg daily, and 253±10 mg/dl after 4 to 6 weeks on 80 mg daily. The latter value corresponds to a 33% reduction in total cholesterol (p<0.005). Changes in the plasma concentrations of total cholesterol were paralleled by reductions in LDL cholesterol, which fell from an initial value of 296±11 mg/dl at baseline to values of 240±10, 222±11, 202±10, and 185±8 mg/dl, respectively, on doses ofLovastatin of 10, 20, 40, and 80 mg/day. These correspond to reductions in the plasma concentrations of LDL cholesterol of 19%, 25%, 32%, and 38%, respectively, on the progressively increasing doses of lovastatin (p<0.005). Plasma concentrations of HDL cholesterol remained stable (52±4 mg/dl at baseline, 51±4 mg/dl on 80 mg daily of lovastatin). Plasma concentrations of triglycerides, however, fell modestly on all doses oflovastatin and decreased from an initial value of 128±21 mg/dl at baseline to 87±10 mg/dl on 10 mg of lovastatin daily, 95±15 mg/dl on 20 mg daily, 88±12 mg/dl on 40 mg oflovastatin daily, and 87±19 mg/dl on the 80-mg daily regimen. Overall, these changes in the concentrations of plasma lipids and lipoproteins in 19 patients with heterozygous FH are similar to those previously reported and confirm the potent hypocholesterolemic effect of this drug in these patients.

The influence of increasing doses of lovastatin on the biosynthesis of cholesterol from 2-14C-acetate by freshly isolated mononuclear leukocytes was examined in 19 patients with heterozygous familial hypercholesterolemia who were studied at baseline and then after 6 weeks on 20, 40, or 80 mg oflovastatin/day. These studies provide comparative data on the influence of chronic lovastatin therapy on the rates of conversion of radiolabeled acetate into cholesterol by intact mononuclear leukocytes obtained 12 to 15 hours after the last dose of lovastatin had been taken by the patient. Although the concentrations oflovastatin and its active metabolites in the mononuclear leukocytes and homologous serum were not directly determined, these studies in freshly isolated mononuclear leukocytes provide, we believe, the best comparative data on the effects of chronic lovastatin therapy on cholesterol biosynthesis in intact mononuclear leukocytes studied in vitro. The assays were performed at a time when cellular concentration oflovastatin and metabolites would be at their predicted nadir on the twice-daily lovastatin dosage.
The effects of increasing doses of lovastatin (mg/day) on cholesterol synthesis from \(^{14}\)C-acetate by freshly isolated mononuclear leukocytes from patients with heterozygous familial hypercholesterolemia (●). Mononuclear leukocytes were isolated from 40 cc of heparinized blood obtained 12 to 15 hours after the last dose of lovastatin and incubated with \(^{14}\)C-acetate as described in the Methods section. The data represent the means±SEM from duplicate assays on mononuclear cells isolated from 19 patients studied on each dose of lovastatin. Normal control values (n=22) are shown as -•-. Normal control values (n=22) are shown as -•-

As illustrated in Figure 3, rates of incorporation of \(^{14}\)C-acetate into cholesterol by freshly isolated mononuclear cells from the FH patients were lower than that seen in cells from control subjects studied under similar conditions. During treatment of the FH patients withLovastatin, cholesterol synthesis from \(^{2}\)\(^{14}\)C-acetate by their mononuclear cells showed little change on 20 mg/day ofLovastatin, but at 40 mg and 80 daily, a significant increase over baseline occurred (p<0.005). When compared to values at baseline, rates of \(^{2}\)\(^{14}\)C-acetate incorporation into cholesterol increased by 1.0% on 20 mg daily (NS), by 30.0% on 40 mg daily (p<0.005), and by 39.2% on 80 mg daily (p<0.005).

HMG CoA reductase activity was assayed on a solubilized preparation isolated from freshly isolated mononuclear leukocytes obtained from control subjects and FH patients at baseline and on sequentially increasing doses ofLovastatin (Figure 4). The baseline activity of HMG CoA reductase from mononuclear cells from patients with familial hypercholesterolemia was significantly higher (p<0.25) than that seen in similar preparations from normal control subjects in whom blood was obtained under fasting conditions between 8:00 A.M. and 10:00 A.M. (8.04±2.18 pmol/4x10^6 cells/30 minutes in FH patients vs. 3.37±0.8 pmol in 22 controls, mean±SEM).

Sequential data were obtained from mononuclear cell preparations isolated from 12 patients at baseline and on Lovastatin doses of 20, 40, or 80 mg/day. In contrast to the studies of \(^{2}\)\(^{14}\)C-acetate incorporation into cholesterol by intact mononuclear leukocytes, in which anyLovastatin within the cells would be expected to decrease cholesterol biosynthesis, the isolation of the membrane fraction which...
Table 1. Influence of Lovastatin on Degradation of 125I-LDL by Freshly Isolated Mononuclear Leukocytes

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<td>Total</td>
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Values are the means±SEM for triplicate assays.

Freshly isolated mononuclear cells (4×10⁶ cells) were incubated for 4 hours with 125I-labeled LDL (25 μg/ml) in the presence or absence of unlabeled LDL (500 μg/ml). Total degradation reflects 125I-LDL degradation in the presence of only 125I-LDL, nonspecific degradation is 125I-LDL degraded in the presence of unlabeled LDL, and specific degradation is the difference between them. Mononuclear cells were isolated from 19 patients with heterozygous familial hypercholesterolemia on diet only (baseline) and during chronic therapy with lovastatin, the last dose was taken 12 to 15 hours before venipuncture for cell isolation.

Partial correlation coefficients were computed from a multiple regression analysis, and comparisons of the response at baseline and on different doses of lovastatin determined by analysis of variance. At baseline, total and LDL cholesterol were weakly correlated with HMG CoA reductase activity, but the differences were not significant (r = -0.30 and -0.37, respectively). High affinity degradation of 125I-LDL by mononuclear leukocytes isolated from patients with heterozygous FH who were receiving 80 mg/day of lovastatin was significantly correlated with the concentrations of LDL cholesterol (r = -0.66, p < 0.01), but no correlation was evident between the degradation of 125I-LDL and LDL cholesterol concentrations at baseline. Similarly, there was no correlation between changes in LDL cholesterol concentrations and changes in the high affinity degradation of 125I-LDL by freshly isolated mononuclear leukocytes from patients with heterozygous FH during treatment withLovastatin.

Discussion

The present study has examined parameters of cholesterol homeostasis in freshly isolated mononuclear leukocytes from patients with well-characterized heterozygous FH treated with lovastatin. Upon isolation from whole blood, mononuclear leukocytes from patients treated with lovastatin were found to have an increased HMG CoA reductase activity, higher rates of cholesterol biosynthesis from 2-14C-acetate, and increases in both total and high affinity degradation of 125I-LDL. With the exception of the increase in nonspecific 125I-LDL degradation, changes in the other parameters are all in a direction consistent with changes that would increase the delivery of cholesterol to the cells via either de novo synthesis or uptake from plasma using the LDL receptor pathway.

It is important to emphasize that the changes observed in cholesterol homeostasis in freshly isolated mononuclear leukocytes from patients with heterozygous FH treated withLovastatin occurred in cells isolated 12 to 15 hours after the last dose of drug had been taken. Although the concentration ofLovastatin and metabolites within monocytes and lymphocytes at these time points is unknown, in single dose studies with 14C-labeledLovastatin, we did not find detectable 14C-labeledLovastatin in mononuclear cells or in plasma at 12 hours after an oral dose. Similar pharmacokinetic studies were not conducted during chronic therapy withLovastatin, and it is possible that, under such conditions, significant concentrations ofLovastatin and active metabolites are present in plasma 12 to 15 hours after the last dose.

However, we believe that our data on cholesterol synthesis from 2-14C-acetate in freshly isolated mononuclear leukocytes is most compatible with a virtual absence ofLovastatin in plasma and in the mononuclear cells at the time of study; if significant amounts of the active drug had been present, we would have expected to see a reduction.
in the conversion of 2-\textsuperscript{14}C-acetate to cholesterol, whereas the results documented an increase. Therefore, we believe that the increases in cholesterol synthesis in freshly isolated mononuclear leukocytes seen in our studies are attributable to compensatory changes similar to those reported from previous in vivo or in vitro studies,\textsuperscript{11-14} which result from the inhibitory effect of lovastatin on cholesterol biosynthesis when this drug is present within the cells. Decreases in the plasma concentrations of LDL, or possibly changes in LDL composition, may also have promoted an increase in cholesterol efflux from the mononuclear cells, which indirectly stimulated a compensatory increase in cholesterol synthesis.

The decrement in LDL cholesterol per milligram of lovastatin administered to patients with heterozygous FH is greater at low doses (10 and 20 mg/day) than at higher doses and is consistent with the ability of this drug to act as a competitive inhibitor of HMG CoA reductase. Although we did not assess changes in LDL degradation by mononuclear leukocytes obtained from patients on different doses of lovastatin, cholesterol biosynthesis from 2-\textsuperscript{14}C-acetate did not change significantly in mononuclear cells isolated from patients on the 20 mg/day dose of lovastatin, whereas the measured activity of HMG CoA reductase assayed on a solubilized mononuclear cell preparation increased by 62%. These observations suggest that low doses (e.g., 20 mg/day) of lovastatin may promote relatively greater increases in LDL receptor expression than do compensatory increases in cellular cholesterol biosynthesis as measured by the incorporation of radiolabeled acetate into cholesterol. It is also possible that these doses of lovastatin may induce an increase in the mass of HMG CoA reductase, which is present largely in inactive form in the cells under physiological conditions. With higher doses of lovastatin, further increases in cellular HMG CoA reductase occur, which are paralleled by an increase in the cellular biosynthesis of cholesterol.

Our results in freshly isolated mononuclear leukocytes isolated from FH patients during treatment with lovastatin provide evidence that changes in cholesterol homeostasis do occur in human cells. Our findings are consistent with results of other investigators who have reported parallel changes in the livers of experimental animals.\textsuperscript{11-14} Under circumstances where lovastatin is present within cells, cholesterol synthesis is inhibited, and the expression of high affinity LDL receptors is increased\textsuperscript{24-26}; the latter changes contribute to the hypocholesterolemic effects of lovastatin.

The effects obtained in the present study are at variance with the recent report of Freeman et al.\textsuperscript{27} These researchers noted a reduction in the activity of HMG CoA reductase in intestinal biopsy samples from patients with primary hypercholesterolemia during treatment with lovastatin, as compared to the activity in biopsies taken before drug therapy. However, because lovastatin is excreted in bile, it is possible that the lower activity of HMG CoA reductase observed in the biopsy samples from patients treated with lovastatin may have been influenced by the presence of active drug in the samples. The latter view is consistent with recent studies from our own laboratory in which rates of cholesterol biosynthesis from 2-\textsuperscript{14}C-acetate have been shown to decrease by 50% to 60% in mononuclear leukocytes obtained from patients with heterozygous FH 2 to 4 hours after oral administration of a single dose of lovastatin (Hagemenas FC, Illingworth DR, unpublished results).

The overall magnitude of lipid lowering observed in the patients with heterozygous FH on different doses of lovastatin in this study is similar to that previously reported from our laboratory and by others.\textsuperscript{3,4,7,8,10} The hypolipiemic effects of lovastatin have also been recently shown to result in 30% to 35% reductions in the 24-hour urinary excretion of mevalonic acid.\textsuperscript{28} The latter measurements, however, reflect the integrated plasma concentrations of mevalonic acid and have been shown to parallel changes in cholesterol biosynthesis.\textsuperscript{29} These results are not incompatible with the results from the present study, which demonstrate what we believe to be compensatory increases in cholesterol biosynthesis and rates of catabolism of \textsuperscript{125}I-LDL in freshly isolated mononuclear leukocytes from patients with heterozygous FH on chronic therapy with lovastatin, but in whom the cells were studied 12 to 15 hours after the last dose of lovastatin. The present results indicate that such compensatory changes, if they occur in the liver, are insufficient to cause a loss of hypcholesterolemic efficacy in most, but not all, patients during long-term therapy with lovastatin.\textsuperscript{30} However, when therapy withLovastatin is stopped or interrupted, an increased mass of HMG CoA reductase may contribute to the rapid rise in LDL cholesterol concentrations that occurs in patients with heterozygous FH upon discontinuation of treatment withLovastatin.\textsuperscript{9,10} Further studies to examine the time course of changes in cholesterol homeostasis in mononuclear leukocytes from patients with heterozygous FH during the initiation of treatment withLovastatin and following its temporary withdrawal are currently in progress.

In addition to examining the effects ofLovastatin therapy on parameters of cholesterol homeostasis in freshly isolated mononuclear leukocytes from patients with heterozygous FH, our results also provide comparative data on cholesterol synthesis from 2-\textsuperscript{14}C-acetate, HMG CoA reductase activity, and the degradation of \textsuperscript{125}I-LDL by mononuclear cell preparations isolated from normal control subjects (on an ad lib diet) and patients with heterozygous FH (who were on a low-fat, low-cholesterol diet). As anticipated, rates of high affinity degradation of \textsuperscript{125}I LDL by freshly isolated mononuclear leukocytes from patients with heterozygous FH were about half that seen in similar cell preparations obtained from the control subjects. This is consistent with the predicted 50% reduction in the number of cellular LDL receptors in these patients, all of whom met stringent criteria for the diagnosis of heterozygous FH. Surprisingly, rates of synthesis of cholesterol from 2-\textsuperscript{14}C-acetate were found to be higher in freshly isolated mononuclear leukocytes from the control subjects versus FH patients, whereas the total activity of HMG CoA reductase in solubilized microsomes was higher in cells isolated from the FH patients. It is unclear whether these differences are wholly or in part related to dietary differences between the control and the FH patients or to possible differences in either the mass of HMG CoA reductase or the proportion of the enzyme present in the
active versus inactive forms in mononuclear cells isolated from the FH patients as compared to the control subjects.

The validity of freshly isolated human mononuclear cells as a model system in which the effects of dietary perturbations or pharmaceutical manipulations that raise or lower plasma lipoprotein levels on in vivo parameters of cholesterol homeostasis can be assessed in an accessible cell line is well established. The present results serve to further validate this model system.

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