Inhibition of cholesterol synthesis and esterification regulates high density lipoprotein interaction with isolated epithelial cells of human small intestine

D. D. Sviridov, M. Y. Pavlov, I. G. Safonova, V. S. Repin, and V. N. Smirnov

Institute of Experimental Cardiology, National Cardiology Research Center, 3rd Cherepkovskaya Street, 15, Moscow, 121552, USSR

Abstract The effect of two inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, lovastatin and monacolin L, and an inhibitor of acyl coenzyme A:cholesterol acyltransferase (ACAT), Sandoz compound 58-035, on the interaction of ^{125}I-labeled high density lipoprotein-3 (HDL_3) with isolated human enterocytes was studied. Both HMG-CoA reductase inhibitors inhibited cholesterol synthesis and ^{125}I-labeled HDL_3 binding and degradation by enterocytes; a strong correlation between changes in cholesterol synthesis and interaction of ^{125}I-labeled HDL_3 with cells was observed. Lovastatin caused reduction of the apparent number of ^{125}I-labeled HDL_3 binding sites without affecting the binding affinity. No changes of cell cholesterol content were observed after incubation of cells with lovastatin. Mevalonic acid reversed the effect of lovastatin on ^{125}I-labeled HDL_3 binding. Lovastatin blocked up-regulation of the HDL receptor in response to loading of cells with nonlipoprotein cholesterol and modified cholesterol-induced changes of ^{125}I-labeled HDL_3 degradation. Lovastatin also reduced HDL-mediated efflux of endogenously synthesized cholesterol from enterocytes. The ACAT inhibitor caused a modest increase of ^{125}I-labeled HDL_3 binding to enterocytes and significantly decreased its degradation; both effects correlated with inhibition of cholesteryl ester synthesis. The results allow us to assume that the intracellular free cholesterol pool may play a key role in regulation of the HDL receptor. —**Sviridov, D. D., M. Y. Pavlov, I. G. Safonova, V. S. Repin, and V. N. Smirnov.** Inhibition of cholesterol synthesis and esterification regulates high density lipoprotein interaction with isolated epithelial cells of human small intestine. *J. Lipid Res.* 1990. **31:** 1821–1830.

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High density lipoprotein (HDL) binding sites have been found on many cell types (for references see 1, 2). These binding sites satisfy the criteria of a classical biological receptor: the binding of HDL to cells is specific, saturable, high-affinity, reversible, can be regulated, and is accompanied by biological effects. Moreover, an HDL binding protein has recently been identified and partially characterized (3–5). The properties of HDL binding sites are similar for most cells and tissues; however, post-binding events vary from one case to another. The binding may be followed by rapid dissociation [fibroblasts (6–8), ovaries (9), smooth muscle cells (7), macrophages (8)], selective uptake of cholesteryl esters [adrenal cells (10), ovaries (10), hepatocytes (10–12), fibroblasts (12)], internalization and degradation of whole particles [hepatocytes (13) and enterocytes (6, 14, 15)], and internalization with subsequent retroendocytosis [macrophages (16, 17) and liver sinusoidal cells (18)]. These observations allow the assumption that different patterns of HDL–cell interactions may correspond to different functions of the HDL receptor in different cells and/or different metabolic situations.

One of the ways to investigate the biological function(s) of the receptor is to find certain biological stimuli to which the receptor responds, i.e., to study regulation of the receptor. The regulation of the HDL receptor has been under intensive study during recent years. All stimuli that have been found to affect the HDL receptor were directly or indirectly related to cell cholesterol metabolism. Among these stimuli are direct loading of cells with exogeneous cholesterol (19–21), changes of cell demand of cholesterol induced by hormones (22, 23), or factors regulating cell growth (24, 25). However, recent pharmacological findings allow more direct investigation of the relationship between the HDL receptor and cell cholesterol metabolism. Development of compactin-related compounds, inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (26) and Sandoz compound 58-035, an inhibitor of acyl coenzyme A:cholesterol acyltransferase (ACAT) (27, 28), i.e., inhibitors of

Abbreviations: ACAT, acyl CoA:cholesterol acyltransferase; BSA, bovine serum albumin; DMSO, dimethylsulfoxide; HDL, high density lipoprotein; HMG-CoA, 3-hydroxy-3-methylglutaryl CoA; LDS, lipoprotein-deficient serum; MEM, minimum essential medium; PBS, phosphate-buffered saline.

two key enzymes of cholesterol metabolism, allow an investigation of the regulation of the HDL receptor in response to changes in cholesterol synthesis and esterification.

We used isolated human small intestine epithelial cells (enterocytes) to study the effect of lovastatin and monacolin L, two inhibitors of HMG-CoA reductase (26), and Sandoz compound 58-035, an inhibitor of ACAT (27, 28), on HDL interaction with these cells. The HDL receptor on human enterocytes is well characterized (14, 21), and it was shown in a previous study that HDL can interact with these cells according to at least two patterns: binding-dissociation and binding-internalization-degradation (21). Cell cholesterol content, to some extent, regulates these two patterns of interaction (21). In the present study we demonstrate that compactin-related compounds inhibit both binding and degradation of ^{125}I-labeled HDL_3 by human enterocytes, and compound 58-035 inhibits degradation but stimulates binding of ^{125}I-labeled HDL_3 to these cells.

MATERIALS AND METHODS

Cells

A segment of middle jejunum was taken at autopsy from children of ages from several days to 10 years within 1 h after death. The predominant causes of death were congenital heart and brain defects. Small intestine epithelial cells (enterocytes) were isolated and maintained as previously described (14, 21).

Lipoproteins

High density lipoprotein (HDL_3) (d 1.125–1.216 g/ml) was isolated from blood plasma of healthy donors by sequential preparative ultracentrifugation (29). The homogeneity of HDL_3 was established by analytical ultracentrifugation. The protein composition was characterized by SDS-polyacrylamide gel electrophoresis (30); no apoE was found in the HDL_3 fraction. The lipoprotein concentration was evaluated by its protein content. Iodination was performed according to Bilheimer, Eisenberg, and Levy (31). The specific radioactivity of preparations was 80–200 cpm/ng protein.

Lipoprotein-deficient serum (LDS) was obtained by repeated ultracentrifugation of fresh blood serum from healthy donors with a density > 1.25 g/ml for 48 h at 105,000 *g*. The final protein content of LDS was 50 mg/ml.

Binding assay

The incubation mixture contained 0.5×10^6 cells, 50 μl LDS, the indicated concentrations of lovastatin (Merck Sharp and Dohme), monacolin L (a kind gift from Dr. A. Endo), mevalonic acid (lactone; Sigma, St. Louis) or compound 58-035 (a kind gift from the Upjohn Co.), and minimum essential medium (MEM, Flow, Ayrshire, U.K.) in a total volume of 0.5 mg. Stock solutions of lovastatin and monacolin L were prepared in dimethylsulfoxide (DMSO) (4 mg/ml) and kept frozen. Compound 58-035 was prepared at 6 mg/ml in DMSO and kept frozen. The compounds were diluted with MEM immediately prior to use. The mixture was incubated in the wells of 24-well plates (Costar, Cambridge, MA) for 1 h at 37°C in a CO_2-incubator (5% CO_2, 95% air) with shaking in an orbital shaker at 60 rpm. After incubation, ^{125}I-labeled HDL_3 was added to the wells to a final concentration of 10 μg/ml, and wells were incubated for a further 2 h under the same conditions. To determine nonspecific binding, 100 μg of unlabeled HDL_3 was added in parallel incubations. It was demonstrated in preliminary experiments that an excess of unlabeled HDL_3 had no pronounced effect on evaluation of the high-affinity, specific component of the binding. After a second incubation the mixture was transferred into plastic tubes and cells were sedimented by centrifugation at 500 *g* for 5 min at 4°C. Media was used for determination of the amount of degraded ^{125}I-labeled HDL_3 and cells were washed three times with 10 ml of MEM containing 1 mg/ml bovine serum albumin (BSA, Sigma) and once with 10 ml of Dulbecco's phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} (Flow). Cells were then resuspended in 1 ml 0.05% trypsin/0.002% EDTA (Flow) and incubated for 5 min at 37°C. The reaction was stopped by adding 100 μl of fetal calf serum (Flow); cells were sedimented by centrifugation at 500 *g* for 10 min at 4°C. Radioactivity in the supernatant was determined in a gamma counter.

To study the effect of lovastatin on cholesterol-loaded cells, enterocytes (0.5×10^6) were preincubated for 1 h at 37°C with shaking at 60 rpm with the indicated concentration of cholesterol, added as 1% ethanolic solution, in the presence or absence of 0.1 ng/ml lovastatin. After incubation, cells were transferred into plastic tubes and washed twice with MEM, then returned to the wells, and ^{125}I-labeled HDL_3 binding to these cells was assessed as described above.

Degradation was determined as non-iodine trichloroacetic acid-soluble radioactivity in the incubation medium as previously described (14, 21).

Cholesterol synthesis and esterification

To determine the rate of cholesterol synthesis and esterification, cells (2×10^6) were incubated essentially as described above, but [2-^{14}C]acetate (Amersham, Buckinghamshire, U.K., sp act 50 mCi/mmol) (final concentration 10 μCi/ml) or [^{14}C]oleate-BSA complex was added to the incubation mixture instead of ^{125}I-labeled HDL_3. To prepare the [^{14}C]oleate-BSA complex, 100 μCi [1-^{14}C]oleic acid in toluene (Amersham, sp act 60 mCi/ mmol) was mixed with 1.4 mg KOH and the toluene was eva-

porated. PBS (1.5 ml) without Ca^{2+} and Mg^{2+} containing 4.24 mg BSA (essentially fatty acid-free, Sigma) was added and the mixture was shaken vigorously. The complex was stored at −20°C; 50 μl of this solution was added to each well (final concentration approximately 5 μCi/ml).

To determine the rate of cholesterol synthesis, after incubation with [^{14}C]acetate, cells were washed twice with PBS and dissolved in 1 M NaOH. Samples were saponified by incubation for 2 h at 100°C in 5 M KOH in 50% ethanol, and cholesterol was precipitated with digitonin (Sigma) according to Sperry and Webb (32).

To determine the rate of cholesterol esterification, after incubation with [^{14}C]oleate–BSA, cells were washed twice with PBS and lipids were extracted according to Folch, Lees, and Sloane Stanley, (33). After evaporation of the solvent, the lipids were dissolved in 50 μl chloroform–methanol 2:1 (v/v). Cholesteryl esters were isolated by thin-layer chromatography on Kieselgel plates (Merck, Darmstadt, F.R.G.) developed in petroleum ether–ethyl ester–acetic acid 85:15:1 (v/v/v).

Efflux of endogenously synthesized cholesterol

Cells (3×10^6) were pulse-incubated with [^{14}C]acetate (final concentration 10 μCi/ml) for 1 h at 37°C with shaking (60 rpm) in a CO_2-incubator. After incubation, unlabeled sodium acetate (Sigma) (final concentration 2 mM) and, when indicated, lovastatin (final concentration 0.1 ng/ml) were added, and the mixture was incubated further for 1 h as described above. After the second incubation, indicated amounts of HDL_3 were added to the incubation mixture; incubation was continued another hour under the same conditions. After incubation, cells were sedimented by centrifugation at 500 *g* for 10 min at 4°C. Incorporation of [^{14}C]acetate into digitonin-precipitable sterols in cells and media was determined as described above.

Cholesterol and protein assays

To determine the effect of lovastatin on cell total cholesterol content, enterocytes (4×10^6) were incubated in the presence or absence of lovastatin (final concentration 0.1 ng/ml) for 3 h at 37°C with shaking at 60 rpm in a CO_2-incubator. After incubation, cells were sedimented by centrifugation for 5 min at 500 *g* at 4°C and solubilized by incubation for 2 h at 60°C in 0.5% sodium deoxycholate (Sigma) in PBS. Total and free cholesterol content of aliquots was measured using a Boehringer Mannheim kit (CHOD-PAP enzymatic colorimetric method). Appropriate amounts of sodium deoxycholate were added to the standards. However, it was demonstrated, that this did not interfere with the enzymatic reaction.

Cell protein content and lipoprotein concentration were measured according to Bradford (34).

Statistics

Each experiment was done in duplicate or triplicate and reproduced 2–3 times on preparations of enterocytes obtained from the other donor. A comparison of the results obtained on different preparations gave a coefficient of variation about 20%. Statistical significance was calculated using Student's *t*-test. Binding parameters were calculated by Eadie-Hoffstee transformation using the program described by Zivin and Waud (35).

RESULTS

Effect of HMG-CoA reductase inhibitors on cholesterol synthesis and interaction of HDL with cells

To assess the effect of two compactin-related compounds, lovastatin and monacolin L, on cholesterol synthesis and interaction of ^{125}I-labeled HDL_3 with human enterocytes, cells were preincubated for 1 h with different concentrations of each compound and then incubated for further 2 h with [^{14}C]acetate or ^{125}I-labeled HDL_3 in the presence of the inhibitors. It was shown in preliminary experiments that extention of preincubation time up to 3 h had no additional effect on either the rate of cholesterol synthesis or ^{125}I-labeled HDL_3 interaction with enterocytes.

As expected, lovastatin caused a strong inhibition of cholesterol synthesis in enterocytes (**Fig. 1**). The effect was pronounced at lovastatin concentration as low as 0.001 ng/ml and reached a plateau at 0.1 ng/ml with inhi-

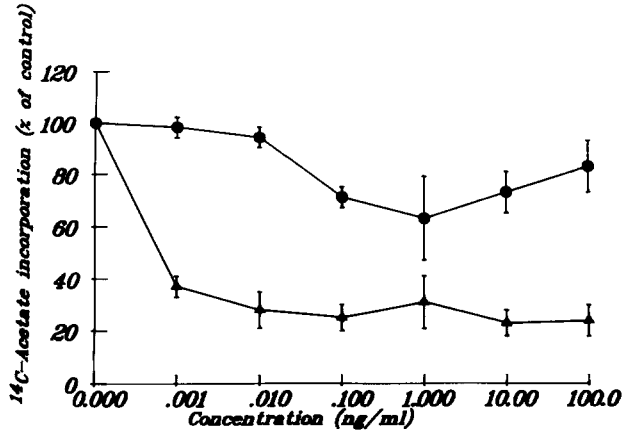

Fig. 1. Effect of lovastatin and monacolin L on cholesterol synthesis in human enterocytes. Cells (2×10^6) were preincubated with indicated concentrations of lovastatin (▲) or monacolin L (●) for 1 h at 37°C with shaking at 60 rpm. After preincubation, [^{14}C]acetate was added to a final concentration of 10 μCi/ml and samples were further incubated for 2 h at 37°C with shaking at 60 rpm. The amount of [^{14}C]acetate incorporated into digitonin-precipitable sterols was determined as described in Materials and Methods. Each point represents a mean ± SEM of triplicate determinations of a representative experiment. One hundred percent values correspond to 12434 ± 2525 dpm/mg cell protein for lovastatin and 19616 ± 1375 dpm/mg cell protein for monacolin L.

bition of cholesterol synthesis by 75%. Monacolin L appeared to be a less effective inhibitor of cholesterol synthesis (Fig. 1). Inhibition reached statistical significance at a concentration of about 0.1 ng/ml and a maximum inhibition of 37% was observed.

Preincubation of enterocytes with lovastatin induced dose-dependent inhibition of ^{125}I-labeled HDL_3 binding and degradation by these cells (**Fig. 2A**). In the presence of lovastatin, binding of ^{125}I- labeled HDL_3 to cells was inhibited by about 40% and degradation by about 60%. Binding saturation of ^{125}I- labeled HDL_3 was achieved at a lovastatin concentration of 0.1 ng/ml, and degradation saturation at 0.01 ng/ml. Monacolin L also caused inhibition of ^{125}I-labeled HDL_3 binding (Fig. 2B); however, its effect was less pronounced than that of lovastatin. Monacolin L caused a maximum 15% inhibition of ^{125}I-labeled HDL_3 binding to enterocytes ($P<0.05$). Unexpectedly, the effect of monacolin L on ^{125}I-labeled HDL_3 degradation was even more pronounced than that of lovastatin: it reduced degradation by about 75% (Fig. 2B). There was a strong correlation of the effects of these two compounds on cholesterol synthesis and ^{125}I-labeled HDL_3 binding to cells (**Fig. 3**) ($r = 0.95$, $P<0.001$). A similar correlation was observed between cholesterol synthesis and ^{125}I-labeled HDL_3 degradation by enterocytes ($r = 0.72$, $P<0.03$) (not shown). Saturation curves indicated that lovastatin treatment reduced ^{125}I-labeled HDL_3 binding to high-affinity binding sites on enterocytes (**Fig. 4**). Analysis of binding parameters demonstrated that lovastatin reduced the apparent number of binding sites (813 ng/mg cell protein vs 1115 ng/mg cell protein, $P<0.05$) without affecting binding affinity.

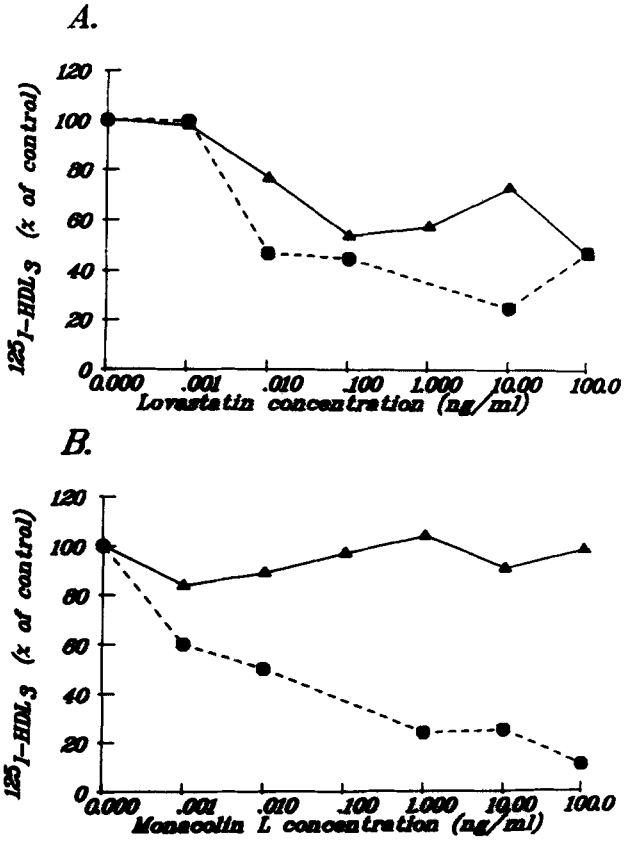

Fig. 2. Effect of lovastatin (A) and monacolin L (B) on interaction of ^{125}I-labeled HDL_3 with human enterocytes. Cells (0.5×10^6) were preincubated with indicated concentrations of lovastatin (A) or monacolin L (B) for 1 h at 37°C with shaking at 60 rpm. After preincubation, ^{125}I-labeled HDL_3 was added to a final concentration of 10 μg/ml and sample were further incubated for 2 h at 37°C with shaking at 60 rpm. The amount of bound (▲) and degraded (●) ^{125}I-labeled HDL_3 was determined as described in Materials and Methods. The curves represent specific binding and degradation (i.e., total minus nonspecific, determined in the presence of a 20-fold excess of unlabeled HDL_3). Each point represents a mean of duplicate determinations of a representative experiment. One hundred percent values correspond to: A. binding, 384 ng/mg cell protein; degradation, 367 ng/mg cell protein; B. binding, 582 ng/mg cell protein; degradation, 255 ng/mg cell protein.

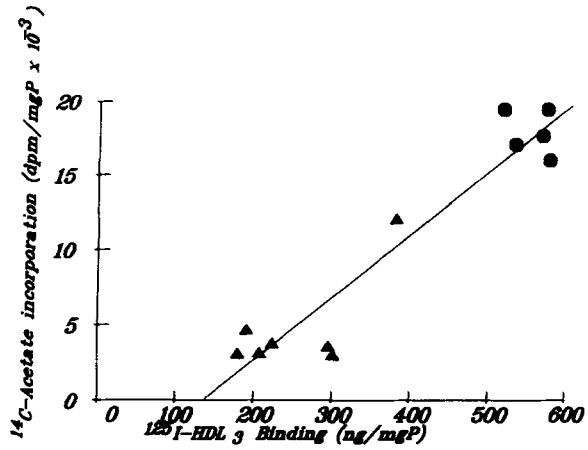

Fig. 3. Correlation between lovastatin- and monacolin L-induced changes in cholesterol synthesis and binding of ^{125}I-labeled HDL_3 to human enterocytes. The data are obtained from experiments described in Figs. 1 and 2; (▲), lovastatin (●), monacolin L.

Inhibition of cholesterol synthesis by lovastatin may deprive cells of cholesterol, and down-regulation of HDL_3 interaction with enterocytes by lovastatin may be attributed to a process opposite to that of up-regulation of HDL receptor by cholesterol loading (21). To assess this possibility, the effect of lovastatin on cell cholesterol content was measured. Incubation of enterocytes with lovastatin for 3 h did not result in a decrease of cell total cholesterol content (control: 43.6 ± 2.4 μg/mg cell protein; lovastatin: 51.8 ± 1.7 μg/mg cell protein; mean ± SEM, n = 4). Measurements of cell free cholesterol content showed that free cholesterol represents 90% of cell total cholesterol content. Changes of cell cholesteryl ester content (determined as a difference between cell total and free cholesterol contents) were, if any, below of the limits of the methodology.

The effect of lovastatin on ^{125}I-labeled HDL_3 interaction with enterocytes may be mediated by a product of de novo cholesterol synthesis; however, the possibility of a

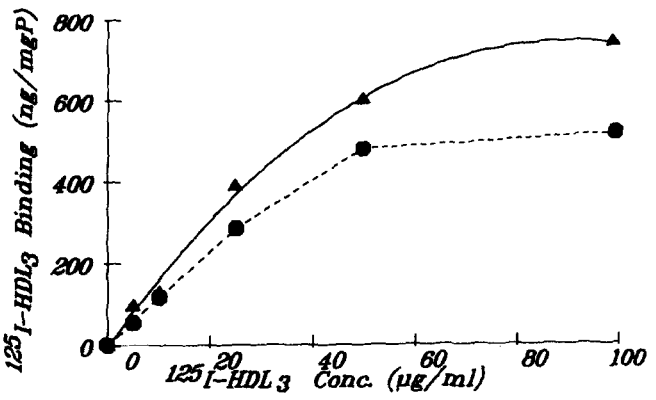

Fig. 4. The effect of lovastatin on saturation curves of ^{125}I-labeled HDL_3 binding to human enterocytes. Cells (0.5×10^6) were preincubated in the presence (●) or absence (▲) of 0.1 ng/ml lovastatin for 1 h at 37°C with shaking at 60 rpm. After preincubation, the indicated concentrations of ^{125}I-labeled HDL_3 were added and samples were further incubated for 2 h at 37°C with shaking at 60 rpm. The amount of bound ^{125}I-labeled HDL_3 was determined as described in Materials and Methods. Curves represent specific binding (i.e., total minus nonspecific, determined in the presence of a 10-fold excess of unlabeled HDL_3). Each point represents a mean of duplicate determinations of a representative experiment.

direct effect of lovastatin on HDL_3 binding cannot be excluded a priori. To distinguish between these two possibilities, the effect of simultaneous addition of lovastatin and mevalonic acid, which is a product of the reaction inhibited by lovastatin, was studied. These experiments may also be considered as a positive control of the study. It was demonstrated that the addition of mevalonic acid completely reversed the effect of lovastatin on binding of HDL_3 to enterocytes (**Table 1**). Mevalonic acid itself caused modest up-regulation of ^{125}I-labeled HDL_3 binding to cells (Table 1).

Regulation of HDL interaction with cholesterol-loaded cells

Cells loaded with cholesterol in the presence or absence of lovastatin were used to test further the effect of lovastatin on interaction of ^{125}I-labeled HDL_3 with enterocytes. In these experiments cells were incubated for 1 h with indicated concentrations of nonlipoprotein cholesterol as described previously (21) in the presence or absence of lovastatin (0.1 ng/ml). Then excess cholesterol was washed out and binding and degradation of ^{125}I-labeled HDL_3 were assessed as described in the Materials and Methods; lovastatin was included in the incubation mixture.

Consistent with our previous findings (21), loading of enterocytes with cholesterol resulted in up to 4-fold enhancement of ^{125}I-labeled HDL_3 binding in a dose-dependent manner (**Fig. 5A**). Lovastatin blocked this effect; in the presence of lovastatin binding of ^{125}I-labeled HDL_3 was increased only by 40% (Fig. 5A). As a result, ^{125}I-labeled HDL_3 binding to cholesterol-loaded enterocytes was 5.5-fold lower in the presence of lovastatin than in its absence.

In agreement with our previous observation (21), cholesterol loading caused dual-phase changes of ^{125}I-labeled HDL_3 degradation by enterocytes. Degradation was enhanced at low concentrations of cholesterol, reached maximum at 20 μg/ml, and returned to the control level when cholesterol concentration was further increased (Fig. 5B). In the presence of lovastatin, degradation of ^{125}I-labeled HDL_3 was enhanced as cholesterol concentration was increased. It reached maximum at cholesterol concentration 60 μg/ml, but did not decrease when cholesterol concentration was increased to 150 μg/ml. Unfortunately, further increase of cholesterol concentration was precluded due to adverse effects of increased amounts of ethanol, which was added as a solvent for the cholesterol. Therefore, two interpretations of these data are possible: lovastatin may either cancel the second phase of the effect of cholesterol or shift both phases to the higher concentrations of cholesterol.

Regulation of endogenously synthesized cholesterol efflux

Slotte, Oram, and Bierman (36) and Aviram, Bierman, and Oram (37) demonstrated that, while HDL receptors probably do not mediate cholesterol efflux from cell membrane, they may induce translocation of cholesterol from intracellular compartments to the cell membrane and its efflux. This process appears to be regulated by interferon (25). We tested the effect of lovastatin on HDL-mediated efflux of endogenously synthesized cholesterol from enterocytes. In these experiments cells were pulse-incubated with [^{14}C]acetate to label endogenously synthesized cholesterol, then incubated with or without lovastatin in the

TABLE 1. Effect of lovastatin, mevalonic acid, and their combination on ^{125}I-labeled HDL_3 binding to human enterocytes

	^{125}I-Labeled HDL_3 Binding	
Additions	ng/mg Cell Protein	% of Control
None	355 ± 11	100
Lovastatin	251 ± 22*	71
Mevalonic acid	407 ± 14*	115
Lovastatin and mevalonic acid	406 ± 25*	114

Cells (0.5×10^6) were preincubated at 37°C for 1 h in the media with no additions, or with lovastatin (0.1 ng/ml), or with mevalonic acid (lactone) (9 mM), or both. After preincubation, ^{125}I-labeled HDL_3 was added to a final concentration 10 μg/ml, and samples were incubated further for 2 h at 37°C with shaking at 60 rpm. The amount of bound ^{125}I-labeled HDL_3 was determined as described in Materials and Methods. Values represent specific binding (i.e., total minus nonspecific, determined in the presence of a 20-fold excess of unlabeled HDL_3). Each value represents mean ± SEM of quadruplicate determinations; *$P < 0.01$ (compared to no addition).

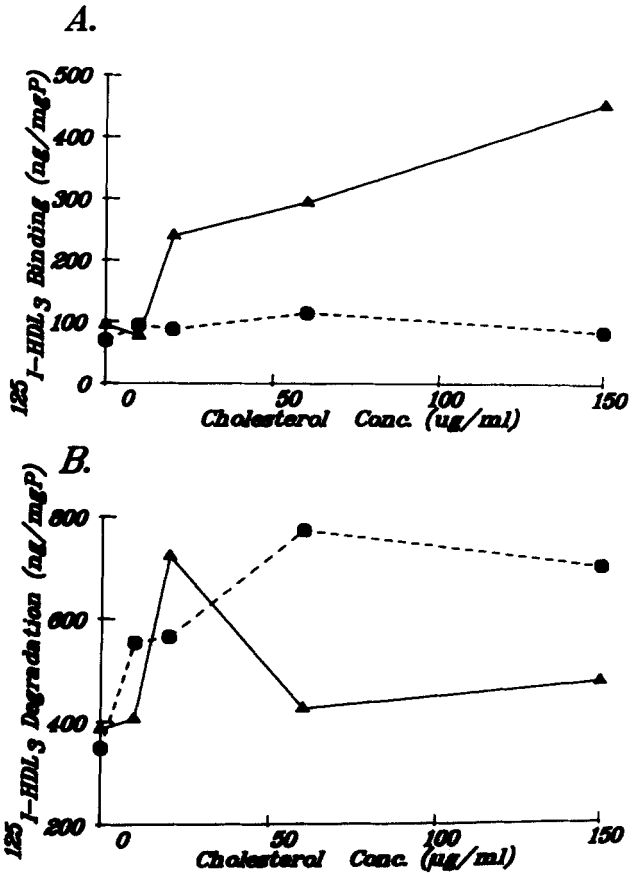

Fig. 5. Effect of lovastatin on binding (A) and degradation (B) of ^{125}I-labeled HDL_3 by cholesterol-loaded human enterocytes. Cells (0.5×10^6) were preincubated with the indicated concentrations of cholesterol in the presence (●) or absence (▲) of 0.1 ng/ml lovastatin for 1 h at 37°C with shaking at 60 rpm. After preincubation, cells were transferred into plastic tubes and washed twice with MEM, then returned to wells and incubated with 10 μg/ml ^{125}I-labeled HDL_3 in the presence or absence of 0.1 ng/ml lovastatin for a further 2 h at 37°C with shaking at 60 rpm. The amount of bound (A) and degraded (B) ^{125}I-labeled HDL_3 was determined as described in Materials and Methods. Curves represent specific binding (A) and degradation (B) (i.e., total minus nonspecific, determined in the presence of a 20-fold excess of unlabeled HDL_3). Each point represents a mean of duplicate determinations of a representative experiment.

presence of unlabeled acetate, and then with different concentrations of HDL_3. The amount of labeled cholesterol in the cells and medium was measured (see Materials and Methods). It was demonstrated that HDL_3 causes a dose-dependent decrease of the amount of newly synthesized cholesterol in cells (**Fig. 6A**). During 1 h incubation, HDL_3 promoted efflux of about 2.1×10^3 cpm/ng cell protein in the absence of lovastatin and about 1.6×10^3 cpm/ng cell protein in its presence ($P < 0.05$), i.e., lovastatin reduced HDL_3-mediated efflux of newly synthesized cholesterol by about 25%. A similar effect was observed when the amount of newly synthesized cholesterol that appeared in the incubation media was analyzed (Fig. 6B).

Effect of ACAT inhibitor on cholesterol esterification and HDL interaction with cells

In order to investigate further the link between cholesterol metabolism and HDL receptor, the effect of the Sandoz compound 58-035, an inhibitor of ACAT, on interaction of ^{125}I-labeled HDL_3 with human enterocytes was tested. As expected, compound 58-035 appeared to be an effective inhibitor of cholesterol esterification in enterocytes (**Fig. 7**). Cholesterol esterification was inhibited by up to 80% in a dose-dependent manner. The effect was pronounced at 5 μg/ml and reached a plateau at 20 μg/ml. Preincubation of enterocytes with compound 58-035 resulted in a moderate increase of ^{125}I-labeled HDL_3 bind-

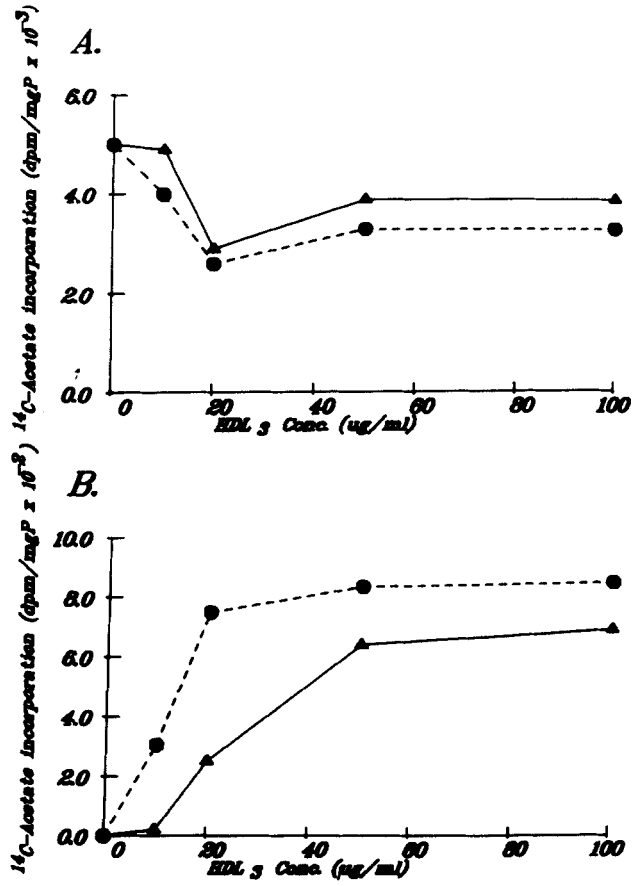

Fig. 6. Effect of lovastatin on HDL-mediated efflux of endogenously synthesized cholesterol from human enterocytes (A) and its appearance in the medium (B). Cells (3×10^6) were pulse-incubated with [^{14}C]acetate (final concentration 10 μCi/ml) for 1 h at 37°C with shaking at 60 rpm. After incubation, unlabeled sodium acetate (final concentration 2 mM) was added, and the mixture was incubated in the presence (▲) or absence (●) or 0.1 ng/ml lovastatin for 1 h under the same conditions. After the second incubation, indicated amounts of HDL_3 were added to the incubation mixture and its was incubated for another 1 h as before. Incorporation of [^{14}C]acetate into digitonin-precipitable sterols in cells (A) and media (B) was determined as described in Materials and Methods. Each point represents a mean of duplicate determinations of a representative experiment.

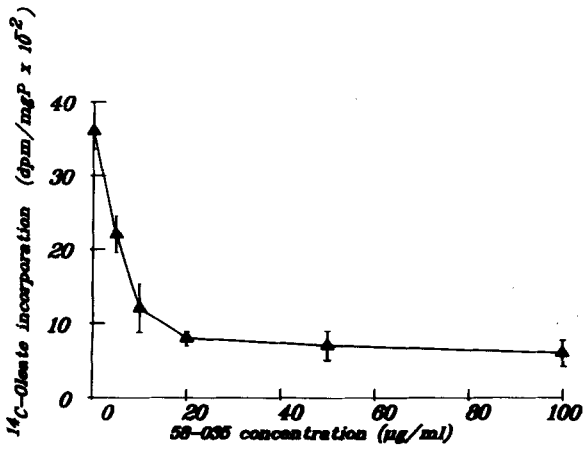

Fig. 7. Effect of compound 58-035 on cholesterol esterification in human enterocytes. Cells (2×10^6) were preincubated with the indicated concentrations of compound 58-035 for 1 h at 37°C with shaking at 60 rpm. After preincubation, [^{14}C]oleate–BSA complex was added to a final concentration of approximately 5 μCi/ml and samples were incubated for a further 2 h at 37°C with shaking at 60 rpm. The amount of [^{14}C]oleate incorporated into cholesteryl esters was determined as described in Materials and Methods. Each point represents the mean ± SEM of triplicate determinations of a representative experiment.

ing to enterocytes (**Fig. 8**). The binding was increased maximally by 25%; this modest enhancement was statistically significant ($P<0.01$) and reproducible. The effect of compound 58-035 on degradation of ^{125}I-labeled HDL_3 was more pronounced: it was decreased in dose-dependent manner by about 40% (Fig. 8). There was a strong negative correlation between cholesterol esterification and ^{125}I-labeled HDL_3 binding ($r = -0.81$, $P<0.02$)

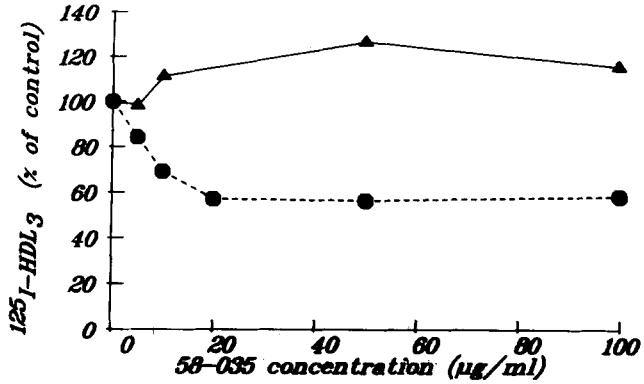

Fig. 8. Effect of compound 58-035 on interaction of ^{125}I-labeled HDL_3 with human enterocytes. Cells (0.5×10^6) were preincubated with the indicated concentrations of compound 58-035 for 1 h at 37°C with shaking at 60 rpm. After preincubation, ^{125}I-labeled HDL_3 was added to a final concentration of 10 μg/ml and samples were incubated for a further 2 h at 37°C with shaking at 60 rpm. The amount of bound (▲) and degraded (●) ^{125}I-labeled HDL_3 was determined as described in Materials and Methods. Curves represent specific binding and degradation (i.e., total minus nonspecific, determined in the presence of a 20-fold excess of unlabeled HDL_3). Each point represents a mean of two independent experiments. One hundred percent values correspond to: binding, 224 ng/mg cell protein; degradation, 977 ng/mg cell protein.

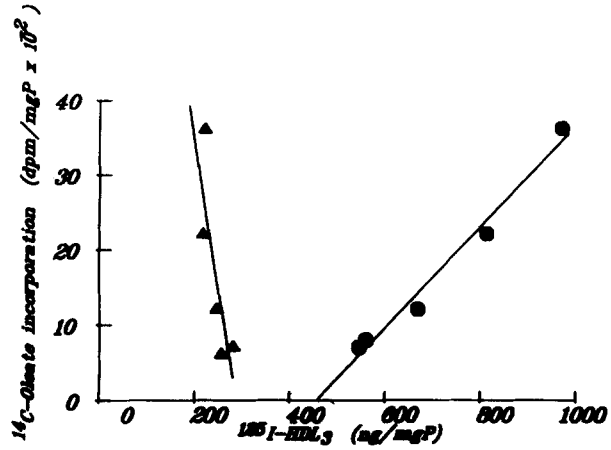

Fig. 9. Correlation between compound 58-035-induced changes in cholesterol esterification and interaction of ^{125}I-labeled HDL_3 with human enterocytes. The data in Fig. 9 were obtained from Figs. 7 and 8; (▲), binding; (●), degradation.

as a result of compound 58-035 and a strong positive correlation between cholesterol esterification and ^{125}I-labeled HDL_3 degradation ($r = 0.99$, $P<0.001$) (**Fig. 9**).

DISCUSSION

Since high density lipoproteins play a central role in the cholesterol transport system (38), it is widely believed that the receptors for this lipoprotein mediate cholesterol exchange between cells and HDL. Although it has recently been demonstrated that HDL receptors are not required for the movement of cholesterol from cell membranes to HDL (37, 39), alternative mechanisms for the involvement of these receptors in the efflux of cholesterol from cells were proposed. These include HDL-induced specific translocation of cholesterol from intracellular compartments to the cell surface (36, 37) and retroendocytosis (16–18). Other evidence for the involvement of HDL receptors in cholesterol metabolism is the known pattern of the regulation of the receptor activity. Thus, on the one hand, loading of cells that can use HDL as a cholesterol acceptor with both lipoprotein and nonlipoprotein cholesterol results in up-regulation of the HDL receptor (19–21); a similar effect was observed when cell demand for cholesterol was decreased by inhibition of cell proliferation (25). Increase of cell demand for cholesterol by stimulation of cell growth results in down-regulation of the receptor (24). On the other hand, hormone-induced increase of demand of cholesterol in cells that use HDL as a source of cholesterol results in up-regulation of the HDL receptor (22, 23). These regulatory patterns indicate that the HDL receptor is regulated in response to changes of cell cholesterol content or demand for it. Another approach was used in the current study to address the question of the relationship between the HDL

receptor and cholesterol metabolism. The effect of inhibitors of two key enzymes of cholesterol metabolism, HMG-CoA reductase and ACAT, on interaction of ^{125}I-labeled HDL_3 with human enterocytes was assessed.

It was demonstrated that preincubation of human enterocytes with lovastatin results in a dose-dependent inhibition of HDL_3 binding and degradation. Lovastatin caused a decrease in the number of HDL binding sites, while the binding constant remained unchanged. It may be concluded that inhibition of cholesterol synthesis results in down-regulation of the HDL receptor, probably in response to a shortage in cholesterol supply. A similar effect of lovastatin on ^{125}I-labeled HDL_3 binding was observed for rat adrenal cortical cells (D. D. Sviridov and N. Fidge, unpublished results).

Three lines of evidence suggest that the effect of lovastatin on interaction of HDL_3 with enterocytes is probably mediated by products of de novo cholesterol synthesis. First, mevalonic acid completely reversed the effect of lovastatin. Second, a strong correlation was observed between cholesterol synthesis and interaction of HDL_3 with enterocytes. Third, monacolin L compound, which has a structure similar to that of lovastatin but is a less effective inhibitor of HMG-CoA reductase, had a similar, but less pronounced, effect on both cholesterol synthesis and binding of ^{125}I-labeled HDL_3 to enterocytes. No decrease of cell cholesterol content was observed after incubation of cells with lovastatin. It may be assumed that the effect of lovastatin is mediated by endogenously synthesized cholesterol or some of its precursors. One may speculate that it is the same mediator(s) that causes up-regulation of HMG-CoA reductase and HMG-CoA synthase (40) and may act at the gene level. In contrast to our findings, no direct link between cholesterol synthesis and HDL binding to rat enterocytes in vivo was demonstrated by Kagami, Fidge, and Nestel (41).

Loading of enterocytes with cholesterol resulted in up-regulation of ^{125}I-labeled HDL_3 binding, which is consistent with our previous finding (21). Lovastatin nearly blocked this effect and this resulted in an even greater effect of lovastatin on binding of HDL_3 to cholesterol-loaded cells. Dual phase changes of HDL_3 degradation in response to loading of cells with cholesterol were demonstrated previously (21) and confirmed in this study. Lovastatin caused a shift in the first phase (increased HDL_3 degradation) to higher cholesterol concentrations and either canceled the second phase (decreased HDL_3 degradation) or also shifted it to higher concentrations of cholesterol. Aviram et al. (37) demonstrated that loading of cells with nonlipoprotein cholesterol resulted in accumulation of cholesterol in the cell membrane followed by slow translocation into intracellular compartments. It is possible to assume that, if only intracellular cholesterol participates in the regulation of the HDL receptor, the effect of simultaneous action of nonlipoprotein cholesterol and lovastatin in a short-term experiment may be explained by interaction of two opposite processes: enrichment of intracellular cholesterol pool by translocation of cholesterol from the cell membrane and its depletion by inhibition of de novo cholesterol synthesis. Alternatively, only endogenously synthesized cholesterol (or its precursors) regulate the HDL receptor, but cholesterol synthesis itself is under the influence of loading of cells with nonlipoprotein cholesterol.

It was demonstrated that down-regulation of the HDL receptor by lovastatin results in a supression of HDL-mediated efflux of endogenously synthesized cholesterol from cells. This finding supports studies (25, 36, 37) claiming that the HDL receptor is involved in the translocation of intracellular cholesterol to the cell membrane and its efflux, and further emphasizes the physiological significance of receptor regulation. Further investigations are required to understand the mechanism of this process; however, the possibility of retroendocytosis cannot be excluded.

The effect of lovastatin on HDL binding and degradation may explain an elevation of plasma HDL after treatment of patients with this drug (42). A decreased amount of cell-bound HDL and supression of its catabolism may result in an elevation of plasma HDL. In addition, it was demonstrated by Monge et al. (43) that incubation of HepG2 cells with HDL down-regulates apoA-I synthesis in these cells. Decreased HDL binding to lovastatin-treated cells may have an opposite effect, which may also contribute to the elevated plasma HDL content induced by lovastatin.

Treatment of cells with lovastatin probably results in a depletion of the intracellular free cholesterol pool. To test an opposite situation, the effect of compound 58-035, an inhibitor of ACAT, was studied. Inhibition of esterification should result in enrichment of intracellular free cholesterol. Treatment of cells with compound 58-035 caused a modest but reproducible enhancement of HDL_3 binding; i.e., the effect was opposite to that of lovastatin. The effect of compound 58-035 on binding of ^{125}I-labeled HDL_3 was less pronounced than that of lovastatin, probably because of the short preincubation time. Tabas, Weiland, and Tall (28) showed that the effect of compound 58-035 on down-regulation of low density lipoprotein receptor on macrophages is increased after 4 h preincubation. Unfortunately, long incubations are precluded when isolated enterocytes are used (14). In contrast to binding, degradation of ^{125}I-labeled HDL_3 by enterocytes was unexpectedly inhibited when cells were treated with compound 58-035. One may speculate that in a situation of excess intracellular cholesterol it would be physiologically reasonable to combine a decrease HDL_3 degradation, which results only in cholesterol influx, with an increase

in its binding, which may result in both an efflux and influx of cholesterol. However, the mechanism of the differential regulation of binding and degradation, found in this and previous (21) studies, is poorly understood at the present time. A similar effect of ACAT inhibitor on HDL interaction with macrophages was observed by Schmitz et al. (17, 44), and it is possible to assume that similar mechanisms (i.e., these involving retroendocytosis) may work in enterocytes as well. However, no direct data to confirm this suggestion are presented in the current study.

The results allow us to assume that a specific intracellular free cholesterol pool (or pool of cholesterol precursor(s)) may play a key role in regulation of the HDL receptor. The existence of specific intracellular regulatory cholesterol pools was originally proposed by Tabas et al. (28) and confirmed by numerous studies. Recently, Bilhartz, Spady, and Dietschy (45) demonstrated that the newly synthesized cholesterol pool regulates biliary sterol secretion. More studies are needed to identify the cholesterol pool that may be responsible for regulation of the HDL receptor.

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