Role of the Low Density Lipoprotein Receptor in Regulating the Content of Free and Esterified Cholesterol in Human Fibroblasts

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ABSTRACT The transfer of normal human fibroblasts from medium containing whole serum to medium devoid of lipoproteins produced a 90% decrease in the cellular content of cholesteryl esters and a 30% decrease in the free cholesterol content. When these lipoprotein-deprived cells were subsequently incubated with human low density lipoprotein (LDL), there was a 7-fold increase in the cellular content of esterified cholesterol and a 1.6-fold increase in the cellular content of free cholesterol. The concentration at which LDL produced its half-maximal effect in elevating cellular sterol content (30 μg/ml of LDL-cholesterol) was similar to the half-maximal concentration previously reported for high affinity binding of LDL to its cell surface receptor. High density lipoprotein (HDL) and whole serum from a patient with abetalipoproteinemia (neither of which contains a component that binds to the LDL receptor) did not produce a significant increase in the content of either cholesterol or cholesteryl esters in normal cells. Furthermore, in fibroblasts from patients with the homozygous form of familial hypercholesterolemia, which lack functional LDL receptors, LDL had no effect in raising the cellular content of either free or esterified cholesterol even when present in the medium at concentrations as high as 450 μg sterol/ml. It is concluded that LDL-receptor interactions constitute an important biochemical mechanism for the regulation of the cholesterol content of normal human fibroblasts. Moreover, when considered in light of current concepts of LDL metabolism in intact mammals, the present data suggest that a major function of plasma LDL may be to transport cholesterol from its site of synthesis in liver and intestine to its site of uptake in peripheral tissues.

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INTRODUCTION

In cultured human fibroblasts, cellular cholesterol synthesis is controlled by a negative feedback system in which the activity of the rate-controlling enzyme in cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase),[1] is inversely related to the content of extracellular low density lipoprotein (LDL) (1, 2). LDL, the major cholesterol-carrying lipoprotein in human plasma, suppresses the activity of HMG-CoA reductase by binding to specific, high affinity receptor sites located on the cell surface (3–5). Although the precise biochemical mechanism by which binding leads to enzyme suppression is not yet known, it has been shown that the binding of LDL leads to at least two other related enzymatic events: (*a*) the protein component of the lipoprotein is proteolytically degraded to its constituent amino acids (4), and (*b*) cholesteryl ester formation within the cell is markedly stimulated (6). High density lipoprotein (HDL), the other major cholesterol-carrying lipoprotein in plasma, does not bind to the LDL receptor and neither suppresses HMG-CoA reductase activity (1, 2) nor stimulates cellular cholesterol esterification (6).

Analysis of the multiple actions of the LDL receptor has been simplified by studies of cultured cells obtained from subjects with the homozygous form of familial hypercholesterolemia. These mutant cells manifest a

[1] *Abbreviations used in this paper:* FCS, fetal calf serum; GLC, gas-liquid chromatography; HDL, high density lipoprotein; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl-CoA reductase; LDL, low density lipoprotein; LPDS, lipoprotein-deficient serum; PBS, phosphate-buffered saline.

primary deficiency in the number of functional LDL receptors and hence are resistant to all of the known biochemical events mediated by this lipoprotein. In the homozygotes' cells, LDL does not bind with high affinity (3–5); the lipoprotein is not degraded at a normal rate (4); cellular cholesterol esterification is not stimulated (6); HMG-CoA reductase activity is not suppressed (2, 7); and cholesterol is overproduced (7, 8).

Recent studies have shown that incubation of normal cells with nonlipoprotein cholesterol can reproduce two of the events observed when LDL binds to its receptor —namely, suppression of HMG-CoA reductase activity (2, 9) and stimulation of cellular cholesteryl ester formation (6). Moreover, nonlipoprotein cholesterol elicits a normal response in the homozygotes' cells even though they do not respond to LDL (2, 6, 9). These observations have raised the possibility that LDL-mediated suppression of HMG-CoA reductase activity and stimulation of cholesteryl ester formation result from an action of the LDL receptor in permitting the net transfer of cholesterol from extracellular LDL to a site within the cell. If the cellular accumulation of cholesterol from LDL does indeed require the LDL receptor, this formulation would explain why nonlipoprotein cholesterol and other related sterols that can enter cells in the absence of the LDL receptor (10) are able to exert similar effects in normal and homozygotes' cells.

The present studies were designed to test the validity of the above hypothesis. Direct measurements have been made of the cellular content of free and esterified cholesterol in normal and mutant fibroblasts incubated in the presence and absence of plasma lipoproteins. The results indicate: (*a*) that LDL but not HDL produces a net increase in the cellular content of free and esterified cholesterol in normal cells, and (*b*) that this accumulation of cholesterol is markedly impaired in cells that lack the LDL receptor.

METHODS

Materials. [1,2-^{3}H]Cholesterol (56 Ci/mmol), cholesteryl oleate ([1-^{14}C]oleate) (50 mCi/mmol), [1-^{14}C]oleic acid (51.8 mCi/mmol), and D,L-3-hydroxy-3-methyl[3-^{14}C]glutary-CoA (7.67 mCi/mmol) were purchased from New England Nuclear (Boston, Mass.). Na ^{125}I (carrier free in 0.05 N NaOH) was obtained from Schwarz/Mann Div. (Becton, Dickinson & Co., Orangeburg, N. Y.). Sodium oleate, cholesterol, and stigmasterol were obtained from Applied Science Labs., Inc. (State College, Pa.). 7-Ketocholesterol was purchased from Steraloids, Inc. (Pawling, N. Y.). All sterols were greater than 99% pure as measured by gas-liquid chromatography (GLC). Silicic acid (100 mesh) was obtained from Mallinckrodt Chemical Works (St. Louis, Mo.). Celite was obtained from Johns-Manville Products Corp. (Denver, Colo.). 3% OV-17 on 100/120 mesh Gas-Chrom Q was obtained from Applied Science Labs, Inc. All other materials were obtained as previously described (9).

Cells. Skin biopsies were obtained with informed consent, and fibroblast cultures were established in our laboratory as previously described (1, 11). In all experiments except those in Fig. 2, the normal cells were derived either from the foreskin of a healthy newborn or from the nongenital skin of a healthy 10-yr-old boy, and the mutant hypercholesterolemic cell line was obtained from the nongenital skin of J. P., a 12-yr-old female subject with the homozygous form of familial hypercholesterolemia (7). All normal and mutant cell lines used in the present studies have been shown in previous studies to possess the typical phenotypic characteristics of the indicated genotype, as determined by measurements of LDL-mediated suppression of HMG-CoA reductase activity (2), [^{125}I]LDL binding and degradation (5), and LDL-dependent cholesteryl ester formation (6). All cells were grown in monolayer and were used between the 5th and 15th passage. Cell lines were maintained in a humidified incubator (5% CO_2) at 37°C in 75-cm^2 stock flasks containing 10 ml of growth medium consisting of Eagle's minimum essential medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with penicillin (100 U/ml); streptomycin (100 μg/ml); 20 mM Tricine, pH 7.4; 24 mM $NaHCO_3$; 1% (vol/vol) nonessential amino acids; and 10% (vol/vol) fetal calf serum (FCS). All experiments were carried out using a similar format: confluent monolayers of cells from the stock flasks were dissociated with 0.05% trypsin-0.05% EDTA solution (day 0), and an indicated inoculum of cells was seeded into a series of petri dishes containing growth medium with 10% FCS. Fresh growth medium with 10% FCS was added at intervals of 2–3 days as indicated in the figure legends. When the cells were in the late logarithmic phase of growth, each cellular monolayer was washed with phosphate-buffered saline (PBS), and various lipoprotein additions and incubations were made as indicated in the figure legends.

Measurement of cellular sterol content. To remove all nonspecificially bound extracellular lipoproteins, each cell monolayer was washed five times at 4°C as previously described (3, 4). Each of the first five washes was carried out with either 3- (60-mm dishes) or 5-ml (100-mm dishes) aliquots of buffer containing 50 mM Tris-Cl, pH 7.4; 0.15 M NaCl; and 2 mg/ml of bovine albumin stock solution, followed by one final wash with either 3 or 5 ml of buffer containing 50 mM Tris-Cl, pH 7.4 and 0.15 M NaCl. The washed cells were scraped with a rubber policeman, centrifuged (900 *g*, 3 min, 4°C), and resuspended in 1 ml of 0.15 M NaCl. After a 50-μl aliquot was removed for protein determination (12), the cell suspension was recentrifuged (900 *g*, 3 min, 24°C). To the cell pellet was added 2 ml of chloroform: methanol (2:1) containing as an internal standard for recovery tracer amounts (< 80 ng of sterol) of [1,2-^{3}H]cholesterol (2×10^5 cpm) and cholesteryl oleate ([1-^{14}C]oleate) (2×10^4 cpm). The mixture was then agitated for 30 s and allowed to stand at 24°C (or in some experiments at 50°C) for 30 min. To separate the phases, 0.5 ml of H_2O was added (13), and each tube was agitated for 30 s and then centrifuged (900 *g*, 5 min, 24°C). The bottom phase was washed once with 1.5 ml of pure upper-phase solvent consisting of chloroform: methanol: H_2O (15:240:235) (13), followed by recentrifugation and removal of the upper aqueous phase.

The bottom phase was evaporated to dryness under nitrogen, and the lipids were resuspended in 150 μl of benzene and applied to a column (100× 5 mm) packed with silicic acid: Celite (wt: wt, 2:1) equilibrated in benzene (14).

The cholesteryl esters were first eluted with a total of 3.3 ml of benzene. The free cholesterol was then eluted with a total of 2 ml of ethyl acetate. For the purposes of GLC, a second internal standard consisting of 7.5 μg of stigmasterol was added to each of the two fractions obtained from the silicic acid: Celite column.

The cholesterol fraction was evaporated to dryness under nitrogen, the residue was resuspended in 100 μl of benzene, and a 5-μl aliquot was removed for liquid scintillation counting of the [^{3}H]cholesterol. At this stage, the recovery of free cholesterol averaged 74%. Double-label counting indicated that there was no contamination with [^{14}C]cholesteryl oleate. The cholesterol fraction was then evaporated to dryness, resuspended in 15 μl of choloroform, and 2-μl aliquots were subjected to GLC for measurement of cholesterol content.

The cholesteryl ester fraction from the silicic acid: Celite column was evaporated to dryness and resuspended in 100 μl of benzene, and a 5-μl aliquot was removed for liquid scintillation counting of the [^{14}C]cholesteryl oleate. At this stage, the recovery of cholesteryl oleate averaged 67%. Double-label counting indicated that there was no contamination with [^{3}H]cholesterol. 400 μl of 0.625 N ethanolic potassium hydroxide was added to the remaining 95 μl of benzene solution containing the cholesteryl esters. The alkaline hydrolysis mixture was heated at 80°C for 30 min, the hydrolysis solution was then evaporated under nitrogen, and the residue was resuspended in 500 μl of H_2O. The free cholesterol was extracted by addition of 2 ml of cholorform: methanol (2:1), followed by centrifugation to separate the two phases, and removal of the upper aqueous phase. The lower phase was washed three times with 1.5 ml of pure upper-phase solvent (13) and then evaporated under nitrogen, and the residue was resuspended in 15 μl of chloroform. 2-μl aliquots were subjected to GLC for measurement of the hydrolyzed cholesterol content.

GLC was performed using the internal standard-area ratio technique employing stigmasterol as the internal standard. A Hewlett-Packard model 5750 research chromatograph with flame ionization detector and Hewlett-Packard model 3370 B integrator (Hewlett-Packard Co., Avondale, Pa.) were used in these analyses. The sterols were separated at 260°C on a 6-foot (2 mm ID) glass column packed with 3% OV-17 on 100/120 mesh Gas-Chrom Q. The flow rate of the nitrogen carrier gas was 45 ml/min. The flame ionization detector operated at hydrogen and air flow rates of 62 and 222 ml/min, respectively. Average elution times for cholesterol and stigmasterol were 243 and 338 s, respectively. Since the detector and integrator gave the same response (area of peak) to equal masses of cholesterol and stigmasterol, the amount of cholesterol in each sample was calculated by multiplying the peak area ratio (cholesterol: stigmasterol) by the amount of stigmasterol added to each sample (7.5 μg). Each microgram value was then divided by the fractional recovery of cholesterol and cholesteryl esters as determined by the scintillation counting procedure as described above. The final value, which represented the micrograms of cholesterol present as either free cholesterol or cholesteryl esters in the original sample, was then expressed as micrograms of sterol per milligram of total cell protein.

Other assays. The activity of HMG-CoA reductase in cell-free extracts (2), the specific high affinity binding and degradation of [^{125}I]LDL by cell monolayers (4), and the rate of [1-^{14}C]oleate incorporation into cholesteryl esters by cell monolayers (6) were measured exactly as described in the cited references. For the experiments in Figs. 8 and 9, the sodium oleate-albumin solutions were prepared by a modification (6) of the method described by Van Harken, Dixon, and Heimberg (15).

Lipoproteins. Human LDL (*d* 1.019–1.063 g/ml), HDL (*d* 1.063–1.215 g/ml), and lipoprotein-deficient serum (LPDS, $d > 1.215$ g/ml) were prepared from single 500-ml units of blood collected in 0.1% EDTA from healthy subjects who had been fasted for 15 h (2). Lipoproteins were fractionated by sequential flotation in a Beckman preparative ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 214,000 *g* (average) and 4–10°C for 16–24 h according to standard techniques (16) using solid KBr for density adjustment (17). The lipoprotein-deficient fraction of FCS, which was used only in the growth studies in Fig. 7, was prepared by a single centrifugation of FCS at 214,000 *g* for 48 h at 4–10°C after density adjustment to 1.215 using solid KBr (9). The isolated human and calf fractions were dialyzed at least 36 h at 4°C against three changes of at least 50 vol of buffer containing 10 mM Tris-Cl, pH 7.4; 0.15 M NaCl; and 0.3 mM sodium EDTA. After dialysis, the human LPDS was defibrinated with thrombin as previously described (2), sterilized by Millipore filtration (Millipore Corp., Bedford, Mass.), adjusted to a protein concentration of 50 mg/ml using the dialysis buffer, and stored at 4°C. After dialysis, the volume of the lipoprotein-deficient fraction of FCS was adjusted so as to equal that of the starting FCS (9). HDL and LDL each migrated as a homogeneous peak on lipoprotein electrophoresis (18). For the experiment in Fig. 5, blood samples from a patient with abetalipoproteinemia and from a healthy subject were collected in tubes without anticoagulants and allowed to clot at 24°C. The resulting whole serum was heated at 56°C for 20 min before addition to the growth medium to inactivate complement. The cholesterol content of sera and lipoprotein fractions was measured either by a modification of the method of Zak (19) or by GLC (see above). The protein content of the lipoprotein fractions was determined by the method of Lowry, Rosebrough, Farr, and Randall (12) using bovine serum albumin as a standard.

RESULTS

The content of free and esterified cholesterol in normal human fibroblasts varied according to the lipoprotein content of the culture medium (Fig. 1). In normal fibroblasts grown continuously in 10% FCS, nearly 40% of the total cholesterol was in an esterified form (Fig. 1A). When the FCS was removed from the medium and the cells incubated for 48 h in medium containing either no serum or the lipoprotein-deficient fraction of human serum, there was an 80–90% decline in the content of cholesteryl esters but only a 30% drop in the content of free cholesterol. The addition of human LDL to the medium at the time of removal of FCS largely prevented the decline in cholesteryl esters. When compared with normal fibroblasts, cells from a homozygote with familial hypercholesterolemia grown in 10% FCS contained a lower level of cholesteryl esters, and there was little change when the fetal calf lipoproteins were removed (Fig. 1B). Moreover, in contrast to the normal cells, the mutant cells did not show a significantly increased cholesteryl ester content in the presence of LDL.

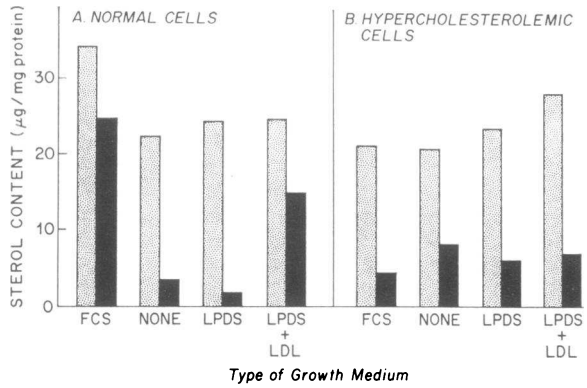

FIGURE 1 Content of free and esterified cholesterol in normal (A) and hypercholesterolemic (B) fibroblasts incubated in the presence and absence of serum lipoproteins. Cells were plated (day 0) at a concentration of 1.5×10^5 cells/100-mm petri dish in 7 ml of growth medium containing 10% FCS. On day 2, the medium was replaced with 7 ml of fresh growth medium containing 10% FCS. On day 4, each cell monolayer was washed with 5 ml of PBS, after which 5 ml of one of the following types of growth medium was added: 10% (vol/vol) FCS (8 mg protein/ml); no serum; 10% (vol/vol) human LPDS (5 mg protein/ml); or 10% (vol/vol) human LPDS (5 mg protein/ml) plus 85 μg/ml of LDL-cholesterol. After incubation at 37°C for 48 h (day 6), each cell monolayer was washed and harvested for measurement of sterol content as described in Methods. Each bar represents the mean value of duplicate dishes. The stippled bars refer to free cholesterol, and the solid bars refer to esterified cholesterol.

To document further the difference in cholesteryl ester content in normal and hypercholesterolemic[2] cells and to obviate a variability that was associated with different batches of FCS,[3] the free and esterified cholesterol content in cell lines from five normal subjects and five homozygotes with familial hypercholesterolemia was compared in the presence and absence of a high concentration of LDL (Fig. 2). When grown in the lipoprotein-deficient fraction of human serum for 48 h, both the normal and mutant cells exhibited a low cholesteryl ester content. However, whereas the addition of LDL to the medium during the last 24 h raised the cholesteryl ester content in each of the normal cell lines, it had little effect on the hypercholesterolemic cells (Fig. 2A and B). As a result, in the presence of LDL the mean cholesteryl ester content of the normal cells was about sevenfold higher than in the hypercholesterolemic cells with no overlap between the two groups. In the same experiment, LDL consistently caused a 1.6-fold increase in the free cholesterol content of each of the normal cell lines, whereas it had no consistent elevating effect in the hypercholesterolemic cells (Fig. 2C and D).

The diminished LDL response in the homozygotes' cells suggested that in normal cells the accumulation of sterols in the presence of LDL required the action of the LDL receptor. This conclusion was supported by

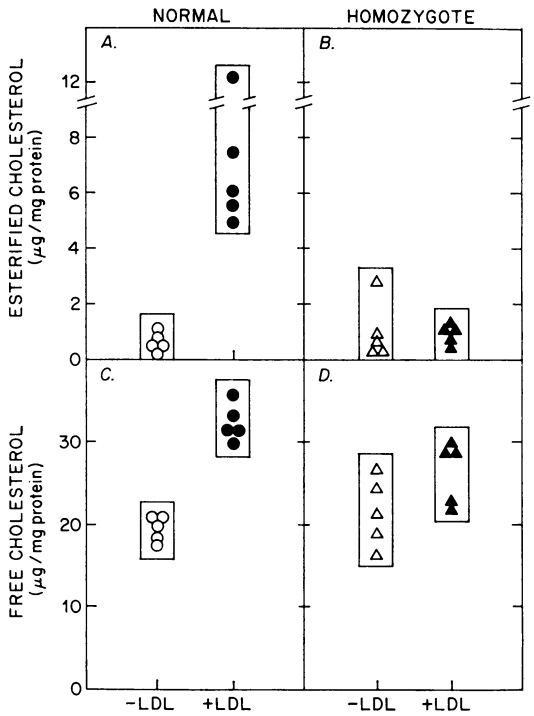

FIGURE 2 Content of free and esterified cholesterol in fibroblasts from five normal subjects (○, ●) and five homozygotes with familial hypercholesterolemia (△, ▲) incubated in the presence and absence of LDL. Each of the normal and mutant cell lines used in these studies has been previously characterized biochemically and was shown to have a phenotype consistent with the indicated genotype (5). The 5 mutant cell lines are the same as those reported previously (5, 6), and the 5 normal cell lines were chosen at random from among the 13 normal cell lines previously characterized (5). Cells were plated (day 0) at a concentration of 3×10^5 cells/100-mm petri dish in 7 ml of growth medium containing 10% FCS. On day 4, the medium was changed to fresh medium containing 10% FCS. On day 6, all of the cell monolayers were washed with 5 ml of PBS, after which 5 ml of fresh growth medium containing 5% human LPDS (2.5 mg protein/ml) was added. After 24 h (day 7), 375 μg of LDL-cholesterol was added in a volume of 10 μl to half of the dishes for each cell line. After a further incubation for 24 h at 37°C, each cell monolayer was washed and harvested for measurement of sterol content as described in Methods. Each point represents the mean value of duplicate dishes.

[2] For the sake of convenience, the term "hypercholesterolemic cells" is used to designate the mutant cell lines derived from subjects with the homozygous form of familial hypercholesterolemia.

[3] In the course of these studies, it was observed that the cholesteryl ester content of normal fibroblasts grown in 10% FCS varied from 10 to 25 μg sterol/mg protein, depending on the batch of FCS used.

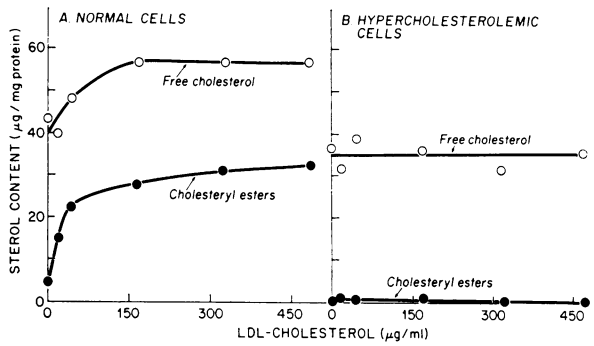

FIGURE 3 Effect of increasing concentrations of LDL on the content of free (○) and esterified (●) cholesterol in normal (A) and hypercholesterolemic (B) fibroblasts. Cells were plated (day 0) at a concentration of 1×10^5 cells/60-mm petri dish in 3 ml of growth medium containing 10% FCS. On day 4, the medium was replaced with fresh growth medium containing 10% FCS. On day 7, each cell monolayer was washed with 3 ml of PBS, after which 2 ml of fresh growth medium containing 5% human LPDS (2.5 mg protein/ml) was added. After 24 h (day 8), the medium was replaced with 2 ml of fresh growth medium containing 5% human LPDS and the indicated concentration of LDL-cholesterol added in a volume of 1–30 μl. After a further 24 h, each cell monolayer was washed and harvested. For each data point the cells from three dishes were pooled for measurement of sterol content as described in Methods.

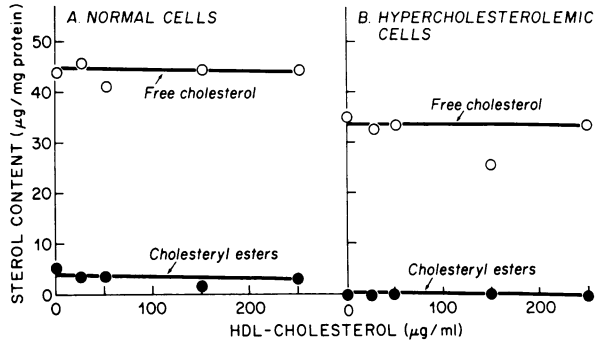

FIGURE 4 Effect of increasing concentrations of HDL on the content of free (○) and esterified (●) cholesterol in normal (A) and hypercholesterolemic (B) fibroblasts. Cells were grown exactly as described in the legend to Fig. 3 except that on day 8 the medium contained the indicated concentrations of HDL-cholesterol rather than LDL-cholesterol. Cells were incubated for 24 h, washed, harvested, and pooled for measurement of sterol content as described in the legend to Fig. 3.

a study of the relation between cellular cholesterol content and the concentration of the lipoprotein in the medium (Fig. 3). When incubated in the presence of increasing concentrations of LDL for 24 h, the content of free and esterified cholesterol in normal cells reached a maximum at an LDL level of about 100 μg/ml of LDL-cholesterol.[4] Half-maximal accumulation of sterol occurred at an LDL-cholesterol concentration in the range of 30 μg/ml (equivalent to 20 μg/ml of LDL-protein). This value is similar to the previously reported concentration for half-maximal binding of LDL to its receptor (i.e., 10–15 μg/ml of LDL-protein [4]). In the hypercholesterolemic cells, which are deficient in functional LDL receptors, no significant increase in the cellular content of free or esterified cholesterol was observed at LDL-cholesterol levels up to 450 μg/ml (Fig. 3B).

In contrast to the LDL effect, HDL, when added to the medium to give cholesterol concentrations up to 250 μg/ml, did not produce a significant change in the cellular content of free or esterified cholesterol in either the normal or hypercholesterolemic fibroblasts (Fig. 4). Similarly, whereas normal human serum raised the cholesteryl ester content of normal cells, serum from a patient with abetalipoproteinemia, which is devoid of LDL (20), had no such effect, even when added to the medium at cholesterol concentrations much higher than those at which normal serum was effective (Fig. 5). Considered together, the data in Figs. 3–5 suggest that

[4] In the LDL used in these studies, the ratio between total cholesterol content (micrograms) and the total protein content (micrograms) was 1.6. 72% of the total cholesterol in this LDL preparation was in an esterified form.

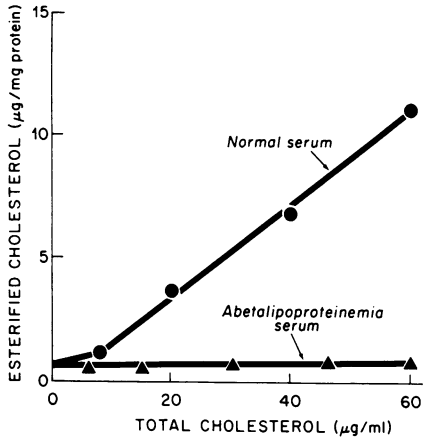

FIGURE 5 Failure of abetalipoproteinemic serum to increase the cholesteryl ester content of normal fibroblasts. Cells were grown exactly as described in the legend to Fig. 2 except that on day 7 the medium was replaced with 5 ml of fresh growth medium containing either normal serum (●) or abetalipoproteinemic serum (▲) to give the indicated final concentration of total cholesterol on the medium. After incubation at 37°C for 24 h, each cell monolayer was washed and harvested for measurement of esterified cholesterol content as described in Methods. To obtain comparable total cholesterol values for the two sera, the normal whole serum was diluted fivefold with human LPDS before addition to the medium. The concentration of free and esterified cholesterol in the undiluted normal serum was 52 and 148 mg/dl, respectively, and in the abetalipoproteinemic serum these respective values were 12.5 and 18 mg/dl. Each point represents the data from a single dish.

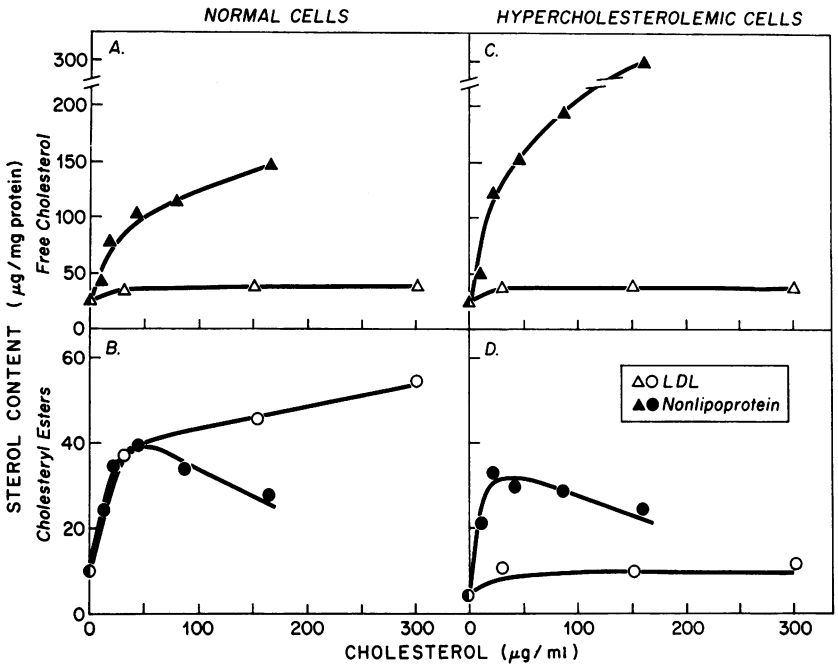

FIGURE 6 Comparison of the effect of increasing concentrations of LDL-cholesterol (△, ○) and nonlipoprotein cholesterol (▲, ●) on the content on free (△, ▲) and esterified (○, ●) cholesterol in normal (A, B) and hypercholesterolemic (C, D) fibroblasts. Cells were grown exactly as described in the legend to Fig. 3 except that on day 8 the medium contained 30 μl of ethanol and the indicated concentration of cholesterol added either as LDL or nonlipoprotein sterol dissolved in ethanol. Cells were incubated for 24 h, washed, harvested, and pooled for measurement of sterol content as described in the legend to Fig. 3.

the net transfer of cholesterol from human lipoproteins to cultured fibroblasts requires the presence of both apolipoprotein B and the LDL receptor. These requirements for cholesterol accumulation by cells are the same as those previously demonstrated for lipoprotein-mediated suppression of HMG-CoA reductase activity (2) and stimulation of cellular cholesteryl ester formation (6).

The requirement for LDL and its receptor for suppression of HMG-CoA reductase activity and stimulation of cellular cholesteryl ester formation can be obviated by the addition to the medium of free cholesterol in a nonlipoprotein form (2, 6, 9). Similarly, the data in Fig. 6 show that nonlipoprotein cholesterol can elevate the cellular content of free and esterified cholesterol in both normal and hypercholesterolemic cells. In normal cells at sterol concentrations up to 50 μg/ml, nonlipoprotein cholesterol was equally as effective as LDL-cholesterol in raising the cellular cholesteryl ester content (Fig. 6B). This occurred despite the fact that more than two-thirds of the LDL-cholesterol in the medium was already in an esterified form. Despite the similar increase in cholesteryl ester content, the free cholesterol content rose to a much greater extent in the presence of nonlipoprotein cholesterol as compared with LDL-cholesterol (Fig. 6A and B). Whereas LDL-cholesterol had no effect in the hypercholesterolemic cells, nonlipoprotein cholesterol produced an increase in cholesteryl esters similar to that in the normal cells (Fig. 6D), confirming that these mutant cells have no basic defect in the mechanism for esterifying cholesterol provided the sterol becomes available to the cell (6).

The physiological significance of the observation that LDL rather than HDL functions to deliver cholesterol from plasma to cells is indicated by the results of the growth experiment in Fig. 7. When normal fibroblasts are cultured in the presence of 7-ketocholesterol but in the absence of a source of exogenous cholesterol, the suppression of endogenous cholesterol synthesis by 7-ketocholesterol, a sterol that specifically reduces HMG-CoA reductase activity, is so marked that cell growth ceases (9). This inhibition of cell growth could be effectively prevented by the addition to the medium of either nonlipoprotein cholesterol or LDL (Fig. 7). On the other hand, HDL, when added at a cholesterol concentration equivalent to that of LDL, was unable to sustain a normal rate of growth, presumably because it, unlike LDL, was unable to cause a net transfer of its cholesterol into the cells. However, the fact that cell number did not decline in the presence of HDL, as it did with 7-ketocholesterol alone, suggests that this

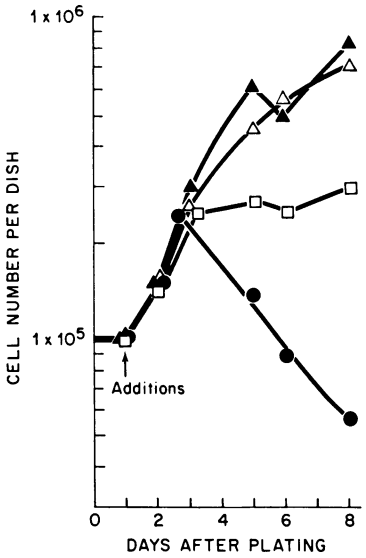

FIGURE 7 Effect of LDL, HDL, and nonlipoprotein cholesterol on the growth of normal fibroblasts cultured in the presence of 7-ketocholesterol. Cells were initially plated (day 0) at a concentration of 1×10^5 cells/dish in 3 ml of growth medium containing 10% lipoprotein-deficient FCS. 24 h after plating (day 1), the medium was changed to 3 ml of growth medium containing 10% lipoprotein-deficient FCS, 4.8 μg of 7-ketocholesterol added in 2 μl of ethanol, and one of the following additions: ▲, 45 μg of cholesterol added in 2 μl of ethanol; △, 20 μg of LDL-cholesterol and 2 μl of ethanol; □, 21 μg of HDL-cholesterol and 2 μl of ethanol; or ●, 2 μl of ethanol. Fresh medium containing lipoprotein-deficient FCS, 7-ketocholesterol, and the indicated addition was added on days 3 and 6 after plating. Cell number was determined at the indicated time by counting trypsinized cells using a hemocytometer. Each value represents the mean of duplicate counts of duplicate dishes.

lipoprotein may play some undefined role in preserving the integrity of cells depleted of cholesterol.

We have previously shown that in normal cells the addition of LDL to the medium produces a 40-fold increase in the ability of the cells to incorporate [^{14}C]-oleate into the fatty acid portion of cholesteryl esters (6). To determine whether the availability of long-chain fatty acids is an important limiting factor in the ability of cells to form cholesteryl esters, normal fibroblasts were incubated with increasing concentrations of oleate in the presence and absence of LDL (Fig. 8). At zero oleate concentration, the addition of LDL caused the expected sixfold increase in cholesteryl esters. The addition of increasing amounts of oleate caused only an additional 1.5-fold increase in cholesteryl ester content, a rise that was similar both in the presence and absence of LDL. Thus, the availability of exogenous free fatty acids does not appear to be a major limiting factor in the ability of normal cells to accumulate cholesteryl esters.

The relation between the time courses of the LDL-dependent accumulation of cholesterol and the other known functions of the LDL receptor in normal cells was examined in the experiments shown in Fig. 9. At each of the two different concentrations of LDL, high affinity LDL binding to its receptor reached a maximum between 2 and 4 h after addition of the lipoprotein (Fig. 9A). On the other hand, the degradation of the protein component of LDL was linear with time up to 8 h after an initial lag (Fig. 9B). The major suppression of HMG-CoA reductase activity occurred between 2 and 6 h (Fig. 9C). The ability of the cells to incorporate oleate into the fatty acid portion of cellular cholesteryl esters, as determined by repeated pulse labeling, increased progressively after a brief lag (Fig. 9D). The free and esterified cholesterol content of the cells showed little change at the lower LDL level, but both increased with time at the higher LDL level (Fig. 9E and F). The data in Fig. 9 show that at the lower level of LDL marked suppression of HMG-CoA reductase occurred without any significant rise in the total cellular content of free or esterified cholesterol. Whereas these data do not exclude the possibility that enzyme suppression results from an increase in either free or esterified cholesterol contained in a small pool, they do indicate that large increases in cellular sterol content are not necessary to bring about suppression of HMG-CoA reductase activity.

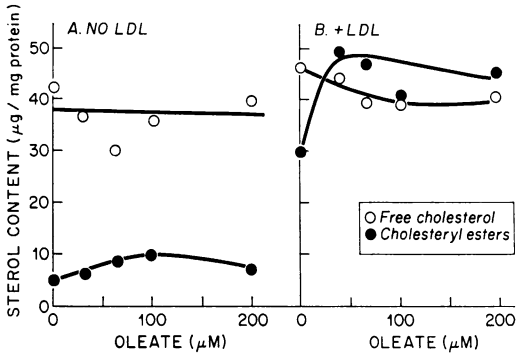

FIGURE 8 Effect of increasing concentrations of oleate on the content of free (○) and esterified (●) cholesterol in normal fibroblasts incubated in the absence (A) and presence (B) of LDL. Cells were grown exactly as described in the legend to Fig. 3 except that on day 8 the medium was replaced with 2 ml of fresh growth medium containing 5% human LPDS (2.5 mg protein/ml), 75 μg/ml of LDL-cholesterol as indicated, and the indicated concentration of oleate-albumin solution added in a volume of 5–30 μl. After a further 24 h, each cell monolayer was washed and harvested. For each data point, the cells from three dishes were pooled for measurement of sterol content as described in Methods.

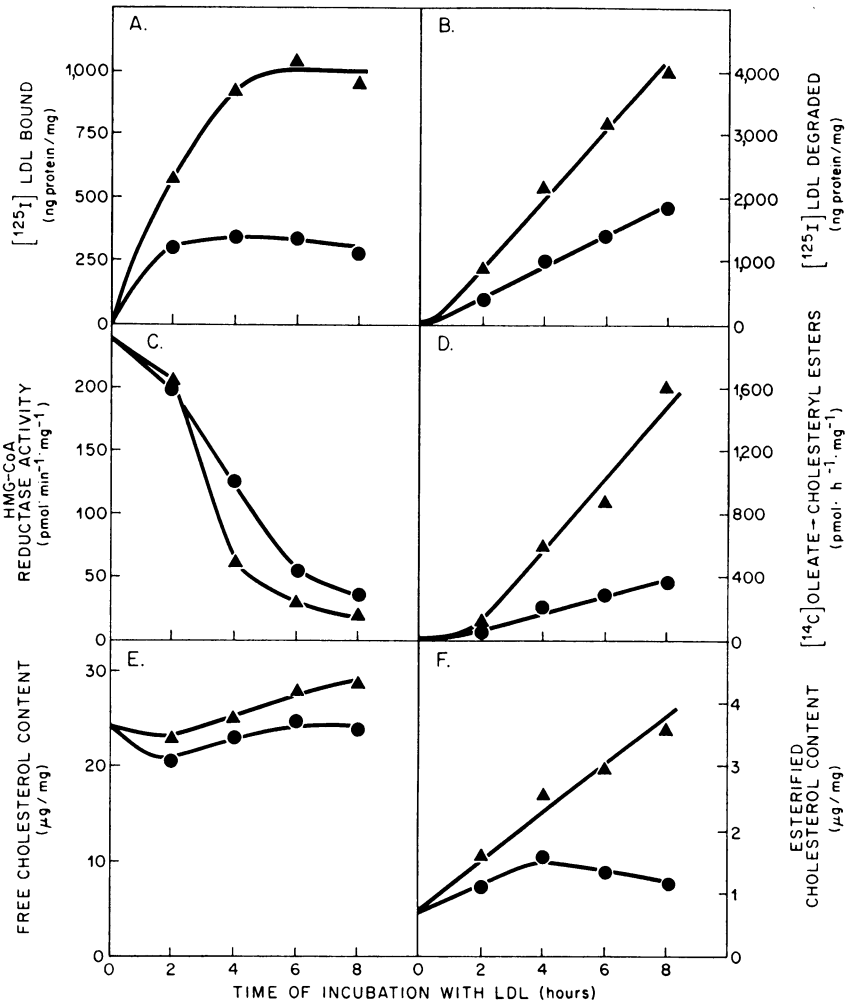

FIGURE 9 Manifestations of LDL-receptor interactions in normal fibroblasts incubated with LDL at 5 (●) and 25 (▲) μg protein/ml for varying time intervals. Cells from one normal subject were plated (day 0) into 94 petri dishes (60 mm) at a concentration of 1×10^5 cells/dish in 3 ml of growth medium containing 10% FCS. Cells were grown exactly as described in the legend to Fig. 3 except that on day 8 the medium was replaced with 2 ml of fresh growth medium containing 5% human LPDS and either [^{125}I]LDL (120 cpm/ng protein, exps. A and B) or unlabeled LDL (exps. C–F) at a concentration of either 5 (●) or 25 (▲) μg protein/ml. After incubation at 37°C for the indicated time, the following determinations were made. High affinity [^{125}I]LDL binding (A) and degradation (B): The medium was removed, its content of ^{125}I-labeled, trichloroacetic acid-soluble degradative products was measured (4), and the amount of ^{125}I radioactivity bound to the cells was determined (4). High affinity binding and degradation were calculated by subtracting the amount of radioactivity bound or degraded in the presence of 395 μg protein/ml of unlabeled LDL from that bound or degraded in its absence (4). At both concentrations of LDL, the high affinity binding and degradation (plotted above) accounted for more than 80% of the total radioactivity bound or degraded in the absence of the unlabeled LDL. HMG-CoA reductase activity (C): Cells were harvested, cell-free extracts were prepared, and HMG-CoA reductase activity was determined as described (2). [1-^{14}C]oleate incorporation into cholesteryl esters (D): 30 min before each indicated time point, cell monolayers were pulse-labeled with [1-^{14}C]-oleate (56 cpm/pmol) bound to albumin (6) at a final oleate concentration of 0.1 mM. After 30 min, the cells were harvested and the content of [^{14}C]cholesteryl esters was determined (6). Cellular content of free (E) and esterified (F) cholesterol: Cells from two dishes were washed, harvested, pooled, and the content of free and esterified cholesterol was determined as described in Methods. In all experiments (A–F), each data point represents the mean of duplicate determinations.

DISCUSSION

As a result of the previous studies of Bailey and coworkers (21) and Rothblat, Hartzell, Mialhe, and Kritchevsky (*22, 23*), it is known that mammalian cells cultured in the presence of animal serum synthesize little cholesterol, but are able to take up exogenous cholesterol contained in the serum lipoproteins of the medium. The present studies indicate that in human fibroblasts the exogenous cholesterol that enters these cells in the presence of serum is derived mainly from LDL and that this transfer requires the action of the LDL receptor. Evidence for the specific involvement of LDL and its receptor in this process rests on the following observations: (*a*) the hyperbolic relation between the cellular accumulation of free and esterified cholesterol and the concentration of LDL in the medium resembles the previously reported saturation curve for LDL binding to its receptor (4); (*b*) HDL, which does not bind to the LDL receptor (3), does not cause a net increase in the cellular content of either free or esterified cholesterol under conditions in which LDL causes an increase in both forms of the sterol; (*c*) cells from homozygotes with familial hypercholesterolemia, which lack the LDL receptor, do not shown an increase in cellular free or esterified cholesterol when incubated with LDL; (*d*) serum from a patient with abetalipoproteinemia, which is devoid of LDL (20), does not raise the content of free and esterified cholesterol in normal cells; and (*e*) cells in which endogenous sterol synthesis is suppressed by 7-ketocholesterol are able to derive enough exogenous sterol from LDL to grow normally, whereas their growth ceases in the presence of HDL.

In the present studies, we have measured directly the levels of cellular cholesterol in the presence of various plasma lipoproteins. At any given time, this sterol content reflects the end result of several opposing events: (*a*) a balance between the rates of cellular influx and efflux of sterols; (*b*) a balance between the rates of hydrolysis of cholesteryl esters and esterification of free cholesterol; and (*c*) the rate of endogenous synthesis of cholesterol. Although the method of direct measurement of cellular sterol levels has two advantages over experiments using radioisotopes (it permits an assessment of the end result of a complex physiologic process, and it avoids the problems of pool size and isotope exchange), this direct method has a major limitation in that it does not permit separate examination of the rates of cellular influx and efflux of cholesterol. Nevertheless, the close correlation between the net cellular accumulation of total cholesterol from LDL and the observed rate of degradation of the protein component of LDL suggests that this LDL receptor-mediated process produces a unidirectional flux of sterol into the cell. Moreover, the fact that sterol accumulates in the normal cells in the presence of LDL at a time when endogenous cholesterol synthesis is suppressed indicates that the increased cellular cholesterol is derived from LDL (6).

In addition to the specific LDL receptor-mediated process for sterol uptake described in this paper, we have observed a different type of uptake process in which radiolabeled free cholesterol of lipoproteins exchanges with cellular cholesterol apparently on a molecule-for-molecule basis.[5] In contrast to the LDL receptor-mediated process, this exchange process does not lead to a change in cellular sterol content, it can involve the cholesterol of HDL as well as LDL, and it can be observed in both hypercholesterolemic and normal fibroblasts[5] as well as in erythrocyte ghosts (24). This exchange process appears to resemble that previously reported by Bailey and Butler, who measured the uptake of [^{3}H]cholesterol from HDL by cultured cells (25). Because in our experiments this exchange process produces no net change in cellular cholesterol content and because it does not exhibit a clear-cut requirement for either a specific lipoprotein or a specific membrane receptor, its physiological function has been difficult to define.

Assuming that the LDL receptor-mediated process produces a net influx of cholesterol into normal cells, it would seem likely that some other process exists to permit the net efflux of sterols from cells. In this regard, Stein and Stein (26) and Bates and Rothblat (*27*) have presented data in other mammalian cell culture systems to suggest that HDL under some circumstances may selectively enhance the efflux of cellular sterol. Although such an HDL effect was not observed in the present study (Fig. 4), our experiments were carried out under conditions in which the cellular cholesterol content was already low due to prior growth of the cells in LPDS. Additional studies are in progress to determine whether a specific mechanism exists for eliciting net efflux of sterols in human fibroblasts.

A consideration of the present data, in light of current concepts of LDL metabolism in mammals, suggests that an important function of LDL may be to transport cholesterol from its site of synthesis in liver and intestine to its site of uptake in peripheral tissues. Liver and intestine, which are the only two tissues known to synthesize the protein component of LDL (28, 29), also have a much higher rate of cholesterol synthesis than do other tissues and are thought to be the ultimate source of at least 80% of the cholesterol in plasma (30). Unlike liver and intestine, peripheral tissues in the body (such as skin, adipose tissue, and muscle) resemble cultured fibroblasts in that they have much

[5] Unpublished observations.

lower rates of cholesterol synthesis (31, 32) and appear to derived a significant fraction of their steady-state content of cholesterol from plasma lipoproteins (33).

If the LDL receptor functions in the body as it does in cultured cells, all of its known actions—namely, binding and degradation of the lipoprotein, transfer of its cholesterol into the cell, suppression of HMG-CoA reductase activity, and stimulation of cellular cholesterol ester formation—would constitute a major biochemical mechanism by which nonhepatic and nonintestinal cells preferentially take up exogenous LDL-cholesterol and suppress endogenous cholesterol synthesis. Since liver and intestine synthesize and secrete large amounts of cholesterol despite levels of plasma LDL that are sufficient to suppress cholesterogenesis in peripheral tissues, it would seem likely that these two secretory tissues possess control mechanisms other than LDL-receptor interactions. Indeed, it is known that in liver, cholesterogenesis is suppressed primarily by dietary cholesterol carried in chylomicrons (30), and in intestine, cholesterogenesis is suppressed mainly by bile acids (30).

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