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Mutations Affecting the Binding, Internalization, and Lysosomal Hydrolysis of Low Density Lipoprotein in Cultured Human Fibroblasts, Lymphocytes, and Aortic Smooth Muscle Cells

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Studies comparing the metabolism of low density lipoprotein (LDL) in normal cells and in cells cultured from patients with homozygous familial hypercholesterolemia have disclosed the existence of a receptor for plasma LDL. This receptor has been identified on the surface of human fibroblasts, lymphocytes, and aortic smooth muscle cells. An extension of these studies to cell strains derived from patients with other single gene defects in cholesterol metabolism has provided additional insight into the normal mechanisms by which cells regulate their cholesterol content and how alterations in these genetic control mechanisms may predispose to atherosclerosis in man.

Key words: lipoproteins, receptors, smooth muscle cells, cholesterol, atherosclerosis, familial hypercholesterolemia

When cultured human fibroblasts are deprived of cholesterol, they synthesize a specific cell surface receptor that binds low density lipoprotein (LDL), the major cholesterol-carrying lipoprotein of human plasma (1–3). Binding of LDL to the receptor is the first step in a pathway – the LDL pathway – by which cells take up the lipoprotein and utilize its cholesterol. By regulating the number of cell surface LDL receptors, cells are able to control the rate of entry of cholesterol, thereby assuring themselves an adequate supply of the sterol while at the same time preventing its overaccumulation. Since plasma LDL is derived ultimately from lipoproteins synthesized in the liver or intestine, the LDL pathway constitutes a mechanism in vivo by which cholesterol can be delivered to peripheral tissues from the liver (the main site of cholesterol synthesis) or the intestine (the site of cholesterol absorption from the diet).

SEQUENTIAL STEPS IN THE LDL PATHWAY AS DELINEATED IN FIBROBLASTS

Once LDL has bound to the LDL receptor in normal fibroblasts the lipoprotein is internalized by adsorptive endocytosis and delivered to lysosomes (4–6) where the protein and cholesteryl ester components of the lipoprotein are hydrolyzed (7, 8). The resulting free cholesterol is then available to be used by the cell for membrane synthesis. When

Abbreviations: FH – familial hypercholesterolemia; HDL – high density lipoprotein; HMG CoA reductase – 3-hydroxy-3-methylglutaryl coenzyme A reductase; LDL – low density lipoprotein

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sufficient cholesterol has accumulated to satisfy this requirement, 3 regulatory events occur: 1) cholesterol synthesis is suppressed through a reduction in the activity of the rate-controlling enzyme, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase) (9); 2) excess lipoprotein-derived free cholesterol is reesterified for storage as cholesteryl esters through an activation of an acylCoA:cholesterol acyltransferase (10, 11); and 3) synthesis of the LDL receptor itself is diminished, thereby preventing further entry of LDL-cholesterol into the cell (12). The sequential steps in the LDL pathway are illustrated diagrammatically in Fig. 1.

MUTATIONS AFFECTING THE LDL PATHWAY IN FIBROBLASTS

At each step in the delineation of the LDL pathway in cultured fibroblasts, interpretation of the data has been clarified by the analysis of mutant fibroblasts derived from patients with genetic defects involving specific steps in the pathway. To date, 6 such mutations have been identified, 5 affecting the LDL pathway at the cellular level and one affecting the secretion of plasma LDL itself.

The mutation that has proved to have the greatest explanatory potential is the one found in patients with the homozygous form of receptor-negative familial hypercholesterolemia (FH), an autosomal dominant disorder (mutation No. 2 in Fig. 1). Fibroblasts from these FH homozygotes lack functional LDL receptors as determined by assays that are sufficiently sensitive to detect about 2% of the normal number (1, 13). As a result, these cells fail to bind and take up the lipoprotein with high affinity and, therefore, fail to hydrolyze either its protein or cholesteryl ester components. Because they are unable to utilize LDL-cholesterol, these homozygote cells must satisfy their cholesterol require-

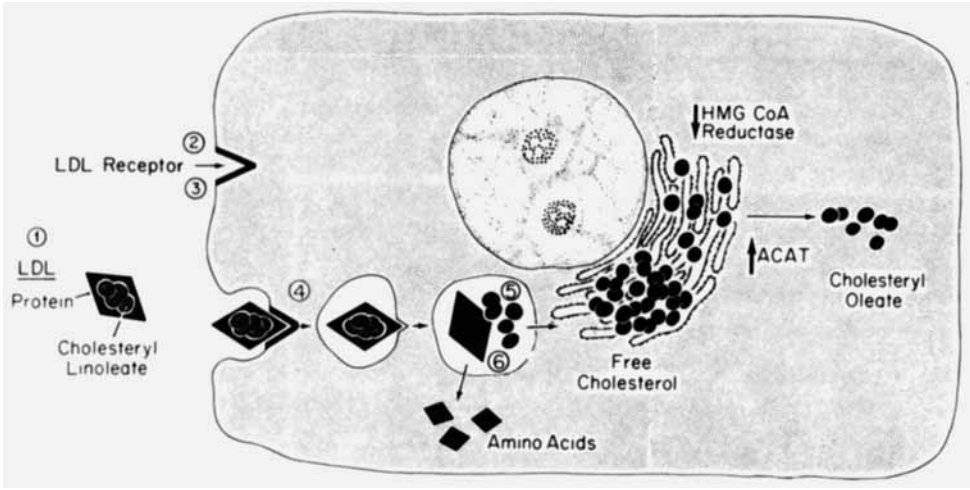

Fig. 1. Sequential steps in the LDL pathway in cultured human fibroblasts. The numbers indicate the sites at which mutations have been identified: 1) abetalipoproteinemia; 2) familial hypercholesterolemia, receptor-negative; 3) familial hypercholesterolemia, receptor-defective; 4) familial hypercholesterolemia, internalization defect; 5) Wolman disease; and 6) cholesteryl ester storage disease. HMG CoA reductase denotes 3-hydroxy-3-methylglutaryl coenzyme A reductase, and ACAT denotes fatty acyl-coenzyme A:cholesterol acyltransferase. (Modified from M.S. Brown and J.L. Goldstein, Science 191: 150–154, 1976. Copyright 1976 by the American Association for the Advancement of Science. Reprinted with permission.)

ment by synthesizing large amounts of cholesterol de novo even when high levels of LDL are present in the culture medium. Moreover, in these mutant cells LDL does not stimulate the formation of cholesteryl esters.

In addition to the cells in which LDL receptor activity is not detectable (receptor-negative), a second class of mutant fibroblasts has been observed in which the maximal number of functional LDL receptors is reduced to about 5–20% of normal (mutation No. 3 in Fig. 1). Patients with this mutation have been designated as having the receptor-defective type of homozygous FH (13). Both the receptor-defective and the receptor-negative mutations have been observed in fibroblasts obtained from subjects with the clinical phenotype of homozygous FH. Such patients manifest extremely high levels of plasma LDL (their LDL-cholesterol levels are about six- to tenfold above normal), accumulation of free and esterified cholesterol in interstitial spaces and within phagocytic cells of the skin and tendons, and severe atherosclerosis with myocardial infarction occurring as early as 18 months of age.

The heterozygous parents of both the receptor-negative and receptor-defective FH homozygotes are more mildly affected than the homozygotes. These heterozygotes manifest plasma LDL-cholesterol levels that are two- to fourfold above the normal level and they usually develop clinical signs of atherosclerosis between the ages of 30 and 60. Fibroblasts from heterozygotes with the receptor-negative mutation have been shown to synthesize about one-half the normal number of LDL receptors and thus their cells take up and degrade LDL at one-half the normal rate (14). The consequences that this 50% deficiency in LDL receptors creates for the regulation of cholesterol metabolism in this genetically dominant syndrome are discussed elsewhere (15).

A third type of mutation in LDL uptake has been described in a patient who manifests a clinical syndrome indistinguishable from that of homozygotes with the receptor-negative and receptor-defective mutations (mutation No. 4 in Fig. 1). Fibroblasts from this patient are unique in that they are able to bind normal amounts of LDL at the receptor site but are unable to internalize the receptor-bound lipoprotein (16). As a result, in these cells, just as in the cells from receptor-negative FH homozygotes, high affinity degradation of LDL does not occur, and the lipoprotein does not suppress cholesterol synthesis nor does it stimulate cholesteryl ester formation. The existence of this internalization mutation indicates that the adsorptive endocytosis of LDL by cells requires at least 2 functionally distinct active sites, each of which can be altered by mutation, namely, a site that is required to bind LDL and a site that participates in the internalization of the lipoprotein after it is bound to the receptor site. Our preliminary genetic studies suggest that these 2 sites may reside on the same peptide chain.

To date, 22 strains of fibroblasts derived from patients in 11 countries who manifest the clinical phenotype of classic homozygous FH have been studied in detail in our laboratory. Of these 22 cell strains, 12 were found to be receptor-negative, 9 were receptor-defective, and 1 exhibited the internalization defect (Table I).

In addition to the 3 known mutations that produce the syndrome of familial hypercholesterolemia, 2 other mutations have been shown to affect the LDL pathway in fibroblasts (1–3). These mutations (mutations No. 5 and No. 6 in Fig. 1) occur in patients with the Wolman disease and cholesteryl ester storage disease, two autosomal recessive disorders in which cholesteryl esters accumulate abnormally in lysosomes of cells throughout the body. Studies by Patrick and Lake disclosed that the primary defect in the Wolman disease involves the absence of a lysosomal acid lipase that normally hydrolyzes both cholesteryl esters and triglycerides (17). In the related, but clinically less severe, syndrome

TABLE I. Biochemical Analysis of Fibroblast Strains Derived From 22 Subjects With the Clinical Syndrome of Homozygous FH

Biochemical Phenotype	Number of Subjects
Receptor-negative	12
Receptor-defective	9
Internalization defect	1

of cholesteryl ester storage disease the activity of this same lysosomal acid lipase is reduced to about 1–5% of normal (18). When incubated with LDL, fibroblasts from patients with both of these disorders bind and take up the lipoprotein normally and degrade its protein component at a normal rate (19, 20). However, the defect in lysosomal acid lipase activity prevents normal hydrolysis of the cholesteryl ester component of LDL. As a result, the cholesteryl esters of LDL accumulate within lysosomes and the lipoprotein fails acutely to suppress HMG CoA reductase or to activate the acyl-CoA:cholesterol acyltransferase (19, 20).

Clinically, in the Wolman disease the absence of the lysosomal acid lipase produces a massive accumulation of cholesteryl esters and triglycerides in nearly all body tissues and death ensues within the first year of life (21). In cholesteryl ester storage disease, the residual acid lipase activity is sufficient to allow survival to young adulthood. It is of importance that in the few of these young patients whose tissues have been studied at autopsy cholesteryl ester accumulation was particularly marked in the arterial wall and advanced atherosclerosis was noted (18, 21).

Another mutation that has proved useful in working out the LDL pathway is the one that produces the autosomal recessive disease abetalipoproteinemia (mutation No. 1 in Fig. 1). This genetic defect causes a block in either the synthesis or secretion of apoprotein B so that the plasma of these patients is devoid of LDL (22). Although their plasma contains cholesterol bound to other lipoproteins (mainly, high density lipoprotein [HDL]), HDL-cholesterol is not taken up efficiently by normal fibroblasts in tissue culture (7) and hence it fails to suppress HMG CoA reductase activity or stimulate cholesteryl ester formation in normal cells (9, 10). These observations support the conclusion that only those human cholesterol-carrying lipoproteins that contain apoprotein B – namely, LDL and VLDL – are able to bind to the LDL receptor and deliver cholesterol to fibroblasts.

EXPRESSION OF THE LDL PATHWAY IN LYMPHOCYTES

All of the steps of the LDL pathway in human fibroblasts also have been identified in long-term human lymphoid cells maintained in suspension culture (23, 24) and in human lymphocytes freshly isolated from the bloodstream (25, 26). In particular, studies by Kayden et al. (23) and Ho et al. (24–26) have shown that the processes of cholesterol synthesis and esterification in lymphoid cells and in circulating lymphocytes are regulated through the LDL receptor. These investigators have also shown that lymphoid cells and lymphocytes from patients with the receptor-negative form of homozygous FH are markedly deficient in cell surface LDL receptor activity. Therefore, these cells exhibit the

same constellation of secondary defects (i.e., defective LDL uptake and degradation, overproduction of cholesterol, and failure to induce cholesteryl ester formation) as do the receptor-negative FH homozygote fibroblasts.

EVIDENCE FOR AN LDL RECEPTOR IN AORTIC SMOOTH MUSCLE CELLS AND ITS ABSENCE IN CELLS FROM A PATIENT WITH HOMOZYGOUS FH

Figure 2 shows the electron microscopic appearance of human aortic smooth muscle cells maintained in monolayer culture. As described for monkey smooth muscle cells (27), these human cells show abundant myofilaments and peripheral dense bodies and are therefore morphologically distinct from cultured human fibroblasts. Moreover, the human aortic smooth muscle cells, like the monkey smooth muscle cells, grow in a distinctive pattern characterized as "hills and valleys."

Previous studies have shown that cultured human aortic smooth muscle cells take up and degrade ^{125}I-LDL by a process that is saturable with respect to LDL concentration (28, 29). The data in Fig. 3 confirm that this uptake process is dependent on a high affinity cell surface receptor that is specific for LDL. In this experiment normal aortic smooth muscle cells were subjected to prior growth in the absence of lipoproteins. The cells were then incubated at 37°C with 20 μg protein/ml of ^{125}I-LDL either alone or in the presence of increasing concentrations of unlabeled LDL (density, 1.019–1.063 g/ml) or HDL (density, 1.085–1.215 g/ml). After 2 hr, the cell monlayers were washed extensively and the amount of ^{125}I-LDL bound to the receptor was determined by releasing it with heparin, a sulfated glycosaminoglycan that removes ^{125}I-LDL from its surface binding site (4). Whereas unlabeled LDL at a concentration of about 20 μg protein/ml produced a 50% competitive reduction of the binding of ^{125}I-LDL, unlabeled HDL did not compete significantly at concentrations as high as 900 μg protein/ml (Fig. 3A). Unlabeled LDL, but not unlabeled HDL, competitively inhibited the proteolytic degradation of ^{125}I-LDL (Fig. 3B). These data indicate that, as in human fibroblasts and lymphocytes, the proteolytic degradation of ^{125}I-LDL by aortic smooth muscle cells is dependent on high affinity cell surface binding.

Evidence that the LDL receptor on cultured human aortic smooth muscle cells is genetically the same as the LDL receptor on fibroblasts and lymphocytes is provided by study of aortic smooth muscle cells obtained from a patient with homozygous FH. Table II shows that the binding, uptake, and degradation of ^{125}I-LDL is markedly deficient in these mutant cells (< 2% of normal values).

Consistent with their deficiency in LDL binding, internalization, and degradation was the finding that the cells from the FH homozygote were markedly deficient in their ability to respond to LDL by suppressing the activity of HMG CoA reductase (Fig. 4) or by enhancing the rate of cholesteryl ester formation (Table III). That the latter defect in the mutant cells was not due to a primary abnormality in the cholesterol esterification system itself was evident from the finding that the FH homozygote cells were able to develop a normal rate of cholesteryl [^{14}C]oleate formation when stimulated with 25-hydroxycholesterol (Table III).

In fibroblasts the binding and uptake of LDL leads to an increase in the cellular content of esterified cholesterol (30). A similar phenomenon occurs in smooth muscle cells. Thus, in the experiment shown in Fig. 5, normal aortic smooth muscle cells were grown for 9 days in the absence of lipoproteins so as to achieve nearly complete depletion of their content of esterified cholesterol. When LDL was then added to

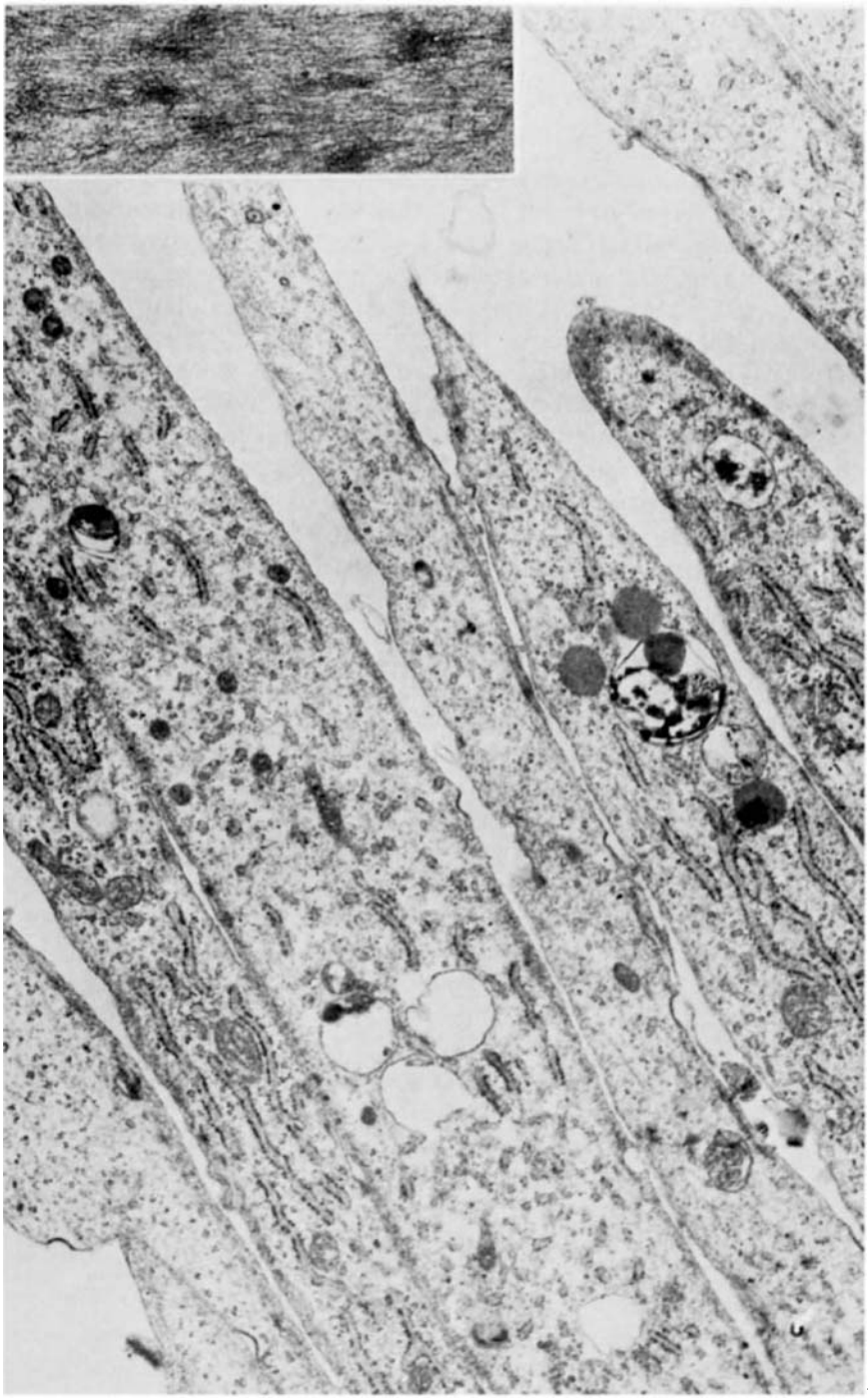

Fig. 2. Electron microscopic appearance of normal human aortic smooth muscle cells maintained in monolayer culture. The cells were cultured by the method of Ross (32) from explants of the thoracic aorta of a normal 6-month-old human fetus (28, 31). A cell monolayer was prepared as described in the legend to Table II. On day 19 of cell growth, the cells were fixed in situ for 30 min with 3% glutaraldehyde in 0.1 M sodium phosphate at pH 7.3. The cells were then scraped from the dish and centrifuged (5 min, 4°C, 12,000 rpm). The resulting cell pellet was postfixed with 2% OsO_4 in 0.1 M sodium phosphate at pH 7.3, dehydrated, embedded in araldite, and stained with uranyl acetate and lead citrate. Note the abundant myofilaments (inset) and peripheral dense bodies. Magnifications, × 14,850; inset, × 37,980.

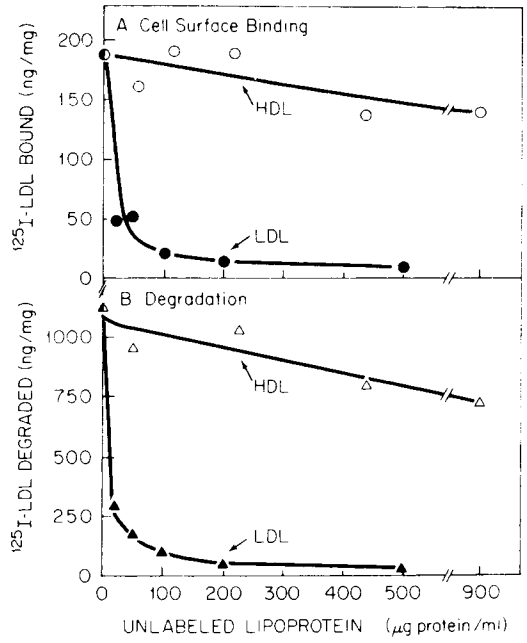

Fig. 3. Comparison of the ability of unlabeled LDL and HDL to compete with ^{125}I-LDL for cell surface binding (A) and degradation (B) in monolayers of normal human aortic smooth muscle cells. On day 0, 1×10^5 cells from stock flasks were seeded into each 60-mm petri dish containing 3 ml of growth medium with 10% fetal calf serum (16). On days 2, 4, 7, and 10, the cells received fresh growth medium containing 10% calf lipoprotein-deficient serum. On day 11, the medium was replaced with 2 ml of fresh medium containing 5% human lipoprotein-deficient serum, 20 μg protein/ml of ^{125}I-LDL (224 cpm/ng), and the indicated amount of either unlabeled LDL (density, 1.019–1.063 g/ml) (●, ▲) or unlabeled HDL (density, 1.085–1.215) (○, △). After incubation for 2 hr at 37°C, the medium was removed and its total content of ^{125}I-labeled, trichloroacetic acid-soluble material was measured (7). The cell monolayers were then washed by a standard procedure and the total amount of heparin-releasable ^{125}I-LDL was determined (4). Each value represents the average of duplicate incubations.

these cells, the cellular content of esterified cholesterol increased (Fig. 5B). The presence of HDL at concentrations up to 400 μg protein/ml did not affect the basal level of either free or esterified cholesterol nor did it influence the increment in esterified cholesterol achieved with LDL at either 10 or 50 μg protein/ml.

Of particular relevance to atherosclerosis are the studies on the regulation of the LDL pathway in cultured human aortic smooth muscle cells (3, 31). These studies have demonstrated that aortic smooth muscle cells, like fibroblasts and lymphocytes, regulate the number of LDL receptors so that the cells take up and degrade only enough LDL to supply cellular needs for cholesterol. Because of this regulation, the addition of large amounts of native LDL to smooth muscle cells in culture does not induce an overaccumulation of cholesteryl esters of the type that is observed in the atherosclerotic lesion in vivo (31). These data thus emphasize the critical role of the regulation of the LDL receptor in protecting aortic smooth muscle cells against such an overaccumulation of cholesteryl esters.

TABLE II. Comparison of the Binding, Internalization, and Degradation of ^{125}I-LDL in Monolayers of Aortic Smooth Muscle Cells From a Normal Subject and a Patient With Homozygous FH

Genotype of aortic smooth muscle cells	Heparin-releasable ^{125}I-LDL	Heparin-resistant ^{125}I-LDL	Rate of degradation ^{125}I-LDL
	ng/mg	ng/mg	$ng \cdot 4hr^{-1} \cdot mg^{-1}$
Normal	125 (114)	504 (473)	1941 (1895)
FH homozygote	4.3 (2.3)	17 (7.5)	37 (21)

The homozygous FH smooth muscle cell strain was derived from an 8-year-old female (M.C.) whose clinical features have been described previously (33). Segments of M.C.'s thoracic aorta were obtained at the time that M.C. underwent aortic valve replacement in October, 1976. Smooth muscle cells were cultured as described in the legend to Fig. 2. On day 0, 1.5×10^5 cells from stock flasks were seeded into each 60-mm Petri dish containing 3 ml of growth medium with 10% fetal calf serum. On day 3, each cell monolayer received 3 ml of fresh medium with 10% fetal calf serum. On day 5, the medium was replaced with 3 ml of fresh medium containing 5% human lipoprotein-deficient serum. On day 7, each monolayer received 2 ml of medium containing 5% human lipoprotein-deficient serum and 10 μg protein/ml of ^{125}I-LDL (233 cpm/ng) in the absence and presence of 340 μg protein/ml of unlabeled LDL. After incubation for 4 hr at 37°C, the total amounts of heparin-releasable ^{125}I-LDL (4), heparin-resistant ^{125}I-LDL (4), and ^{125}I-LDL degradation (7) were determined. Each value represents the average of triplicate incubations. The numbers in parentheses represent the amount of binding, internalization, or degradation that was due to the specific, high affinity process. These values were determined from the difference between values obtained in the absence and presence of the excess unlabeled LDL (7, 15).

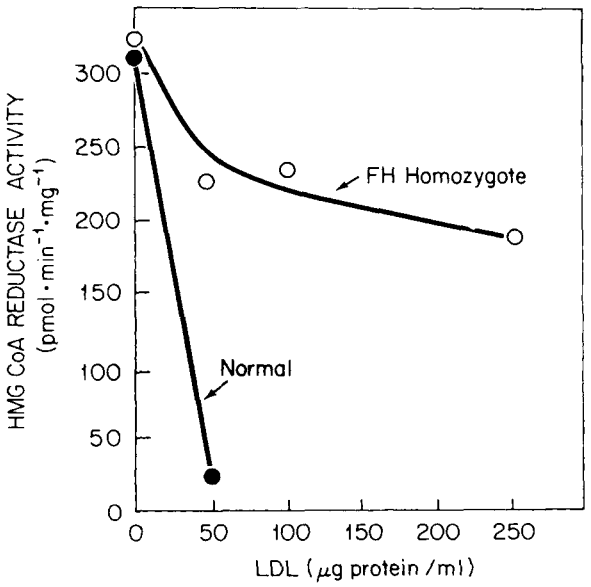

Fig. 4. Comparison of LDL-mediated suppression of HMG CoA reductase activity in monolayers of aortic smooth muscle cells from a normal subject and a patient with homozygous FH. Monolayers were prepared as described in the legend to Table II. On day 7, after incubation for 48 hr in the absence of lipoproteins, each monolayer received 2 ml of medium containing 5% human lipoprotein-deficient serum and the indicated concentration of LDL. After incubation for 6 hr at 37°C, the cells in each dish were harvested for measurement of HMG CoA reductase activity (9). Each value represents the average of duplicate incubations.

TABLE III. Comparison of LDL-Mediated Stimulation of Cholesteryl Ester Formation in Monolayers of Aortic Smooth Muscle Cells From a Normal Subject and a Patient With Homozygous FH

Genotype of aortic smooth muscle cells	Addition to medium	[^{14}C] Oleate incorporated into cholesteryl [^{14}C] oleate
		pmol·hr^{-1}·mg^{-1}
Normal	None	17
	LDL, 50 μg protein/ml	867
FH homozygote	None	1.5
	LDL, 200 μg protein/ml	1.0
	25-Hydroxycholesterol, 5 μg/ml	1,620

Monolayers of smooth muscle cells were prepared as described in the legend to Table II. On day 7, after incubation for 48 hr in the absence of lipoproteins, each monolayer received 2 ml of medium containing 5% human lipoprotein-deficient serum and the indicated addition. After incubation for 5 hr at 37°C, the cells in each dish were pulse-labeled for 2 hr at 37°C with 0.1 mM [^{14}C] oleate-albumin (8,700 cpm/nmol), after which each monolayer was harvested for determination of the cellular content of cholesteryl [^{14}C] oleate (10). Each value represents the average of duplicate incubations.

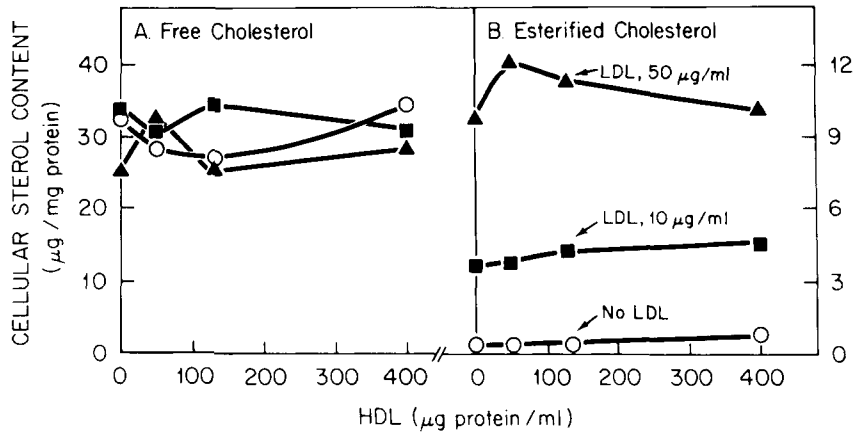

Fig. 5. Inability of HDL to prevent the LDL-mediated accumulation of esterified cholesterol in normal human aortic smooth muscle cells. On day 0, 1.5 × 10^5 cells were seeded into each 60-mm petri dish containing 3 ml of growth medium with 10% fetal calf serum (16). On days 2, 4, and 7, the cells received 3 ml of growth medium containing 10% calf lipoprotein-deficient serum. On day 9, the medium was replaced with 2 ml of fresh medium containing 5% human lipoprotein-deficient serum, the indicated concentration of LDL (density, 1.019–1.063 g/ml) and the indicated concentration of HDL (density, 1.085–1.215 g/ml). After incubation at 37°C for 48 hr, the cell monolayers were washed, harvested, and pooled (2 dishes per sample), and their content of free (A) and esterified (B) cholesterol was determined (30). Each value represents the average of duplicate samples.

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