THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 251, No. 11, Issue of June 10, pp. 3277-3286, 1976
Printed in U.S.A.

Restoration of a Regulatory Response to Low Density Lipoprotein in Acid Lipase-deficient Human Fibroblasts*

(Received for publication, January 15, 1976)

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Previous studies have shown that cultured fibroblasts derived from patients with genetic defects in lysosomal acid lipase (*i.e.* the Wolman Syndrome and Cholesteryl Ester Storage Disease) are defective in their ability to hydrolyze the cholesteryl esters contained in plasma low density lipoprotein (LDL). As a result, these mutant cells show a reduced responsiveness to the regulatory actions of LDL, as evidenced by a decreased LDL-mediated suppression of the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase and by a decreased LDL-mediated activation of cellular cholesteryl ester formation. In the current studies, the Wolman Syndrome and Cholesteryl Ester Storage Disease cells were grown in the same Petri dish with mutant fibroblasts derived from a patient with the homozygous form of Familial Hypercholesterolemia. Whereas pure monolayers of either the Familial Hypercholesterolemia cells (lacking cell surface LDL receptors) or the acid lipase-deficient cells (lacking cholesteryl ester hydrolase activity) responded poorly to LDL, the mixed monolayers developed lipoprotein responsiveness as measured by an enhancement of both LDL-mediated suppression of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity and LDL-mediated stimulation of cholesteryl ester formation. This effect was shown to result from the release of the lysosomal acid lipase from the Familial Hypercholesterolemia homozygote cells into the culture medium and its subsequent uptake by the acid lipase-deficient cells. The acquisition of this acid lipase activity enhanced the ability of the Wolman Syndrome and Cholesteryl Ester Storage Disease cells to respond to the lipoprotein by suppression of 3-hydroxy-3-methylglutaryl coenzyme A reductase and activation of cellular cholesteryl ester formation. These data emphasize the importance of the lysosomal acid lipase in the cellular metabolism of LDL cholesteryl esters and, in addition, demonstrate that delivery of this enzyme to genetically deficient cells can enhance the regulatory response to the lipoprotein.

The classic studies of Neufeld and co-workers have demonstrated that genetic deficiencies of lysosomal enzymes in cultured cells can be corrected by addition to the culture medium of the missing enzyme (reviewed in Ref. 1). Such correction is possible because cultured cells take up exogenous lysosomal enzymes by endocytosis and incorporate them into secondary lysosomes, their normal site of action (2-7). In the most extensively studied system, genetic deficiencies in lysosomal glycosidases or sulfatases can be alleviated by the addition to the culture medium of the missing hydrolase, and this, in turn, reduces the intralysosomal accumulation of unhydrolyzed mucopolysaccharides in the mutant cells (1-7).

Recently, we have studied two lysosomal enzyme deficiency states in which the enzyme deficiency leads not only to an accumulation of unhydrolyzed substrate within lysosomes but also to a failure of generation of a regulatory signal (8, 9). In these two lysosomal storage diseases, Cholesteryl Ester Storage Disease and the Wolman Syndrome, the genetic defect involves a lysosomal acid lipase (10-13).[1] This enzyme normally functions in fibroblasts to hydrolyze cholesteryl esters that enter the cell bound to low density lipoprotein (8, 9, 16), and thus it plays a critical role in a pathway by which normal human fibroblasts derive free cholesterol from LDL, the major cholesterol-carrying lipoprotein in human plasma (reviewed in Refs. 8 and 16). In the initial step in the LDL[2] pathway, the lipoprotein binds to a high affinity cell surface receptor. The

* This research was supported by grants from the American Heart Association, the National Foundation-March of Dimes, and the National Institutes of Health (GM 19258 and HL 16024).

‡ Established Investigator of the American Heart Association.

§ Recipient of a Research Career Development Award (GM 70,277) from the United States Public Health Service.

[1] The primary defect in both the Wolman Syndrome and Cholesteryl Ester Storage Disease appears to involve a deficiency in a single lysosomal acid lipase that possesses both cholesteryl ester hydrolase activity and triglyceride hydrolase activity (10-13). The two syndromes differ, however, in that Wolman Syndrome fibroblasts contain no detectable activity of this lipase, whereas in Cholesteryl Ester Storage Disease fibroblasts about 1 to 5% of normal enzyme activity is expressed (14, 15).

[2] The abbreviations used are: LDL, low density lipoprotein; [^{3}H]CL-LDL, [^{3}H]cholesteryl linoleate bound to low density lipoprotein, Tricine, *N*-tris(hydroxymethyl)methylglycine.

receptor-bound LDL is internalized through endocytosis and delivered to lysosomes, where its protein and cholesteryl ester components are hydrolyzed. The free cholesterol liberated from the degradation of LDL then enters the extralysosomal compartment where it elicits regulatory responses in two microsomal enzyme activities: (*a*) it suppresses 3-hydroxy-3-methylglutaryl coenzyme A reductase, reducing endogenous cholesterol synthesis, and (*b*) it activates an acyl-CoA:cholesterol acyltransferase, enhancing its own re-esterification and storage. When fibroblasts from patients with Cholesteryl Ester Storage Disease (9) and the Wolman Syndrome[3, 4] are incubated with LDL, the binding, uptake, and proteolytic degradation of the lipoprotein proceed normally, but the defective lysosomal acid lipase causes an accumulation of unhydrolyzed LDL-derived cholesteryl esters within lysosomes. The reduced rate of formation of free cholesterol in turn leads to an impaired suppression of 3-hydroxy-3-methylglutaryl-CoA reductase activity and a diminished rate of activation of cholesteryl ester formation within the cell (9).[3, 4]

In the present studies, we have attempted to determine whether correction of the deficient cholesteryl ester hydrolase activity in Wolman Syndrome and Cholesteryl Ester Storage Disease fibroblasts would restore the ability of these cells to respond to LDL. As a donor for the cholesteryl ester hydrolase activity, we have used a mutant fibroblast strain that is also deficient in its ability to respond to LDL but in which the genetic block involves a step in the LDL pathway that is different from the lysosomal acid lipase. These mutant cells, which were derived from a patient with the receptor-negative form of homozygous Familial Hypercholesterolemia, lack the cell surface LDL receptor (17) and therefore fail to take up the lipoprotein with high affinity (9, 16). As a result, the Familial Hypercholesterolemia cells, like the Cholesteryl Ester Storage Disease cells and the Wolman Syndrome cells, fail to show normal suppression of 3-hydroxy-3-methylglutaryl-CoA reductase and activation of the cholesteryl-esterifying system when incubated with LDL (8, 17). Our results indicate that the Familial Hypercholesterolemia cells can supply a cholesteryl ester hydrolase activity to the two mutant strains with acid lipase deficiency, and this, in turn, is able to enhance the ability of the latter cells to manifest a regulatory response to LDL.

EXPERIMENTAL PROCEDURE

Materials—[1-^{14}C]Oleic acid (51.8 mCi/mmol), [1-^{14}C]oleoyl-CoA (59 mCi/mmol), and [1-^{14}C]cholesterol (50 mCi/mmol) were purchased from New England Nuclear Corp. [1,2-^{3}H]Cholesterol (43 Ci/mmol) was purchased from Amersham/Searle Corp. ^{125}I-Sodium (carrier-free in 0.05 N NaOH) was obtained from Schwarz/Mann. Triton X-100 and chloroquine diphosphate were obtained from Sigma Chemical Co. Other chemicals, tissue culture supplies, and thin layer chromatographic and gas-liquid chromatographic materials were obtained from sources as previously reported (18, 19).

Cells—The normal human fibroblast strain used in this study was the same strain (D.S.) that has been used in our previous studies (9, 17–19). The regulation of lipoprotein and cholesterol metabolism in these cells is typical of that in 20 other normal fibroblast strains that have been studied in our laboratory and that were derived from skin biopsies of healthy adults and children. The Familial Hypercholesterolemia homozygote cell strain (M.C.) (17) displays biochemical characteristics typical of that of 12 other receptor-negative homozygote strains studied in our laboratory. The Wolman Syndrome strain (D.DiM.) was obtained from Dr. John O'Brien, University of California, San Diego. The Cholesteryl Ester Storage Disease strain (J.R.), originally provided to us by Dr. Arthur Beaudet (13), has been previously studied in our laboratory (9). All cells were grown in monolayer and were used between the 5th and 20th passage. Cell lines were maintained in a humidified incubator (5% CO_2) at 37° in 75-cm^2 stock flasks containing 10 ml of growth medium consisting of Eagle's minimum essential medium supplemented with penicillin (100 units/ml); streptomycin (100 μg/ml); 20 mM Tricine-chloride, pH 7.4; 24 mM $NaHCO_3$, 1% (v/v) nonessential amino acids; and 10% (v/v) fetal calf serum (19).

Unless otherwise stated, all experiments were carried out using a standard format: confluent monolayers of cells from stock flasks were dissociated with 0.05% trypsin/0.02% EDTA solution. On Day 0, a total of 1×10^5 fibroblasts, consisting either of cells from one strain or mixtures of cells from two different strains that were combined in varying ratios as indicated in the legends, were seeded into Petri dishes (60 × 15 mm) containing 3 ml of growth medium with 10% (v/v) fetal calf serum. On Day 3, the medium was replaced with 3 ml of fresh medium containing 10% fetal calf serum. On Day 5, when the cells were in late logarithmic growth, each monolayer was washed with 2 ml of Dulbecco's phosphate-buffered saline, after which 2 ml of fresh medium containing 5% human lipoprotein-deficient serum was added (final protein concentration, 2.5 mg/ml). All experiments were initiated either on Day 6 or Day 7 after the cells had been incubated with lipoprotein-deficient serum for 24 or 48 hours.

Preparation of Conditioned Medium—Confluent monolayers of cells from stock flasks were dissociated with 0.05% trypsin/0.02% EDTA solution. On Day 0, 1×10^6 cells per dish from a single cell strain were seeded into Petri dishes (60 × 15 mm) containing 3 ml of growth medium with 10% fetal calf serum. On Day 3, the medium was replaced with 3 ml of fresh medium containing 10% fetal calf serum. On Day 6, each monolayer was washed with 2 ml of Dulbecco's phosphate-buffered saline, after which 2 ml of fresh medium containing 5% human lipoprotein-deficient serum was added. After 24 hours the medium containing 5% human lipoprotein-deficient serum was removed, kept at 4°, and used within 24 hours as conditioned medium.

Lipoproteins—Human LDL (density 1.019 to 1.063 g/ml) and human lipoprotein-deficient serum (density > 1.215 g/ml) were obtained from the plasma of healthy subjects and prepared by differential ultracentrifugation (19). Fetal calf lipoprotein-deficient serum was prepared as previously described (18). The concentration of LDL is expressed in terms of its protein content. The mass ratio of total cholesterol to total protein in LDL was 1.6:1, and about 75% of the total cholesterol was in an esterified form. ^{125}I-labeled LDL (specific activity, 467 cpm/ng of protein) was prepared as previously described (20). [^{3}H]Cholesteryl linoleate was prepared by the method of Goodman (21). [^{3}H]Cholesteryl linoleate bound to LDL ([^{3}H]CL-LDL) (specific activity, 307×10^3 cpm/nmol of total cholesteryl linoleate in LDL) was prepared as previously described (22). For experiments, the ^{125}I-LDL was diluted with native LDL to give the final specific activity indicated in the legend.

Incorporation of [1-^{14}C]Oleate into Cholesteryl Esters by Intact Fibroblast Monolayers—Monolayers were incubated with LDL and [1-^{14}C]oleate bound to albumin in growth medium supplemented with 5% human lipoprotein-deficient serum (23). After the indicated interval, the cells were washed and harvested, extracted with chloroform/methanol, and the cholesteryl [^{14}C]oleate isolated by thin layer chromatography as previously described (23). Esterification activity is expressed as the picomoles of cholesteryl [^{14}C]oleate formed per mg of total cell protein.

Total Binding and Degradation of ^{125}I-LDL by Intact Fibroblast Monolayers—Monolayers were incubated at 37° with ^{125}I-LDL in growth medium supplemented with 5% human lipoprotein-deficient serum as previously described (24). After the indicated interval, the medium was removed, treated with trichloroacetic acid, extracted with chloroform and hydrogen peroxide to remove free iodine (25), and an aliquot of the aqueous phase was counted to determine the amount of ^{125}I-labeled acid-soluble material formed by the cells and released to the medium (24). Previous studies have shown that the bulk of this acid-soluble material is [^{125}I]monoiodotyrosine (24). Degradation activity represents the cell-dependent rate of proteolysis and is expressed as the nanograms of ^{125}I-LDL degraded to acid-soluble material per mg of total cell protein. To determine the total amount of ^{125}I-LDL bound to the cells (*i.e.* the ^{125}I-LDL contained both on the cell surface and within the cell), after removal of the incubation medium the mono-

[3] J. L. Goldstein, J. H. Dees, L. M. Buja, and M. S. Brown (1976) manuscript in preparation.

[4] J. H. Dees, M. S. Brown, J. L. Goldstein, and L. M. Buja (1976) manuscript in preparation.

layers were washed six times and dissolved in 0.1 N NaOH as previously described (24). An aliquot of the dissolved cell solution was counted to determine the amount of bound LDL and another aliquot was used to measure cell protein content. Binding activity is expressed as the nanograms of ^{125}I-LDL bound to the cell per mg of total cell protein. Under the conditions of the experiment in Fig. 1, more than 90% of the observed total binding of ^{125}I-LDL represents receptor-dependent binding (24, 26).

Assay of Cholesteryl Ester Hydrolase Activity in Cell-free Extracts—Cells from either a single strain or from two different mutant strains were grown in Petri dishes as described above, and the cell monolayer from each dish was harvested by scraping into 1 ml of chilled buffer containing 50 mM Tris-chloride, pH 7.4/0.15 M NaCl. After centrifugation (900 × *g*, 3 min, 4°), the cell pellet was resuspended in 1 ml of the above buffer and washed once more in the same manner. Cell-free extracts were prepared by dissolving each pellet of fibroblasts in 0.1 ml of 1% Triton X-100 in water. Cholesteryl ester hydrolase activity was determined by a modification of the assay originally described by Beaudet *et al.* (13). Each assay contained the following in a final volume of either 50 or 200 μl: 0.1 M sodium acetate, pH 4.3; 1% Triton X-100; 0.5 mg/ml of bovine serum albumin; 5 mM 2-mercaptoethanol; 1 mM EDTA; the indicated amount of [^{3}H]cholesteryl linoleate; and the indicated amount of the detergent-solubilized extract. After incubation at 37° for the indicated time, each reaction was terminated with 2 ml of chloroform/methanol (2/1) containing an internal standard consisting of [^{14}C]cholesterol (30 μg, 500 cpm) and unlabeled cholesteryl linoleate (30 μg). The free [^{3}H]cholesterol was isolated and quantified as in the monolayer assay described below. Hydrolytic activity is expressed as the picomoles of [^{3}H]cholesterol formed from the hydrolysis of [^{3}H]cholesteryl linoleate per mg of extract protein. A blank value, which was determined by parallel incubations containing either no extract or a boiled extract, was subtracted from each experimental value. The blank value for free [^{3}H]cholesterol was 0.1 to 0.5% of the total amount of [^{3}H]cholesteryl linoleate that was present in each assay.

Uptake and Hydrolysis of [^{3}H]Cholesteryl Linoleate-LDL by Intact Fibroblast Monolayers—Monolayers were incubated with [^{3}H]CL-LDL in growth medium containing 5% human lipoprotein-deficient serum as previously described (22). After incubation at 37° for the indicated time, each monolayer was washed as previously described (22), and the cells were extracted with chloroform/methanol after the addition of an internal standard containing [^{14}C]cholesterol (30 μg, 500 cpm) and unlabeled cholesteryl linoleate (30 μg). The free and esterified [^{3}H]cholesterol were separated by thin layer chromatography on silica gel sheets sequentially developed in the same direction with benzene/ethyl acetate (2/1) and petroleum ether/benzene (2/1) as previously described (22). Hydrolytic activity is expressed as the picomoles of [^{3}H]cholesterol formed from the hydrolysis of [^{3}H]CL-LDL per mg of total cell protein. In order to determine a blank value for the small amount of free [^{3}H]cholesterol that was found in the cells in the absence of lysosomal hydrolysis, parallel monolayers were incubated under identical conditions except that the medium contained 75 μM chloroquine to inhibit lysosomal cholesteryl ester hydrolase activity (22). The total [^{3}H]cholesteryl linoleate uptake represents the sum of the cellular content of the unhydrolyzed [^{3}H]cholesteryl linoleate and hydrolyzed free [^{3}H]cholesterol. These calculations were corrected for the recovery of the [^{14}C]cholesterol from each dish, which averaged 85%.

Measurement of Cellular Content of Free and Esterified Cholesterol—The content of free and esterified cholesterol in fibroblasts was determined by a previously described method in which the steroids were extracted from washed cell pellets with chloroform/methanol, the free and esterified cholesterol fractions were separated on silicic acid/Celite columns, and the cholesterol content in each fraction was measured by gas-liquid chromatography (27). Correction for procedural losses (which averaged 25%) was made by utilizing [^{3}H]cholesterol, cholesteryl [^{14}C]oleate, and stigmasterol as internal standards (27).

Assay of 3-Hydroxy-3-methylglutaryl-CoA Reductase Activity in Cell-free Extracts—The rate of conversion of 3-hydroxy-3-methyl[3-^{14}C]glutaryl-CoA to [^{14}C]mevalonate was measured in extracts of detergent-solubilized cells as described (19) except that after lactonization the mixture was taken to dryness and the mevalonolactone was extracted once with 0.1 ml of chloroform/methanol (2/1) and spotted directly on the thin layer sheets (28). Enzyme activity is expressed as the picomoles of [^{14}C]mevalonate formed per min per mg of soluble protein.

Assay of Acyl-CoA:Cholesterol Acyltransferase in Cell-free Extracts—The rate of transfer of [^{14}C]oleate from [^{14}C]oleoyl-CoA to cholesterol was assayed as previously described (29). Cells were homogenized using a Dounce homogenizer and aliquots containing 51 to 115 μg of protein were incubated in 0.2 ml of solution containing: 50 mM potassium phosphate, pH 7.4; 2 mM dithiothreitol; 6 mg/ml of bovine serum albumin; and 90 μM [1-^{14}C]oleoyl-CoA (91 cpm/pmol). After 1 hour at 37°, the reactions were terminated with the addition of 4 ml of chloroform/methanol (2/1) and the cholesteryl [^{14}C]oleate was isolated by thin layer chromatography as previously described using [^{3}H]cholesteryl oleate as an internal standard to correct for procedural losses, which averaged 15% (23).

Other—The protein concentrations of extracts and whole cells were determined by a modification of the method of Lowry *et al.* (30), using bovine serum albumin as a standard. Unless otherwise stated, each value in all experiments represents the average of duplicate assays or incubations.

RESULTS

When either Wolman Syndrome cells or Cholesteryl Ester Storage Disease cells (both lacking cholesteryl ester hydrolase activity) were grown for 1 week in the same Petri dish with Familial Hypercholesterolemia homozygote cells (lacking LDL receptors), the LDL receptor activity in the resulting mixed monolayers was approximately proportional to the number of either Wolman Syndrome or Cholesteryl Ester Storage Disease cells that were initially seeded at Day 0 (Fig. 1). This result was similar whether the total cellular uptake of ^{125}I-LDL or the rate of its proteolytic degradation was used as an index of LDL receptor activity. Conversely, the total amount of cholesteryl ester hydrolase activity found in each Petri dish after disruption of the cells was inversely proportional to the number of Wolman Syndrome or Cholesteryl Ester Storage Disease cells that were initially seeded at Day 0 (Fig. 2). Considered together, the data in Figs. 1 and 2 indicate that under the conditions of these experiments the growth rates of all three cell strains were similar and that the proportion of cells present in each dish after 1 week of growth was similar to the proportions of cells initially seeded on Day 0. Moreover, the presence of the deficient cell strain did not alter the total amount of LDL receptor activity or cholesteryl ester hydrolase activity produced by the competent strain.

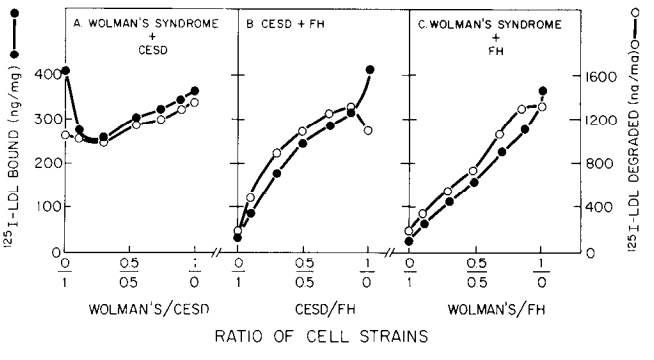

FIG. 1. Total binding and proteolytic degradation of ^{125}I-LDL in fibroblast monolayers composed of cells from two different mutant strains grown together in varying proportions. Monolayers containing mixtures of cell strains were prepared by seeding the cells together in the indicated ratios as described under "Experimental Procedure." On Day 7, each monolayer received 12 μg of protein/ml of ^{125}I-LDL (127 cpm/ng). After incubation at 37° for 6 hours, the total amount of ^{125}I-LDL bound to the cells (●) and the amount of ^{125}I-LDL that had been degraded proteolytically (O) were determined as described under "Experimental Procedure." *CESD*, Cholesteryl Ester Storage Disease; *FH*, Familial Hypercholesterolemia.

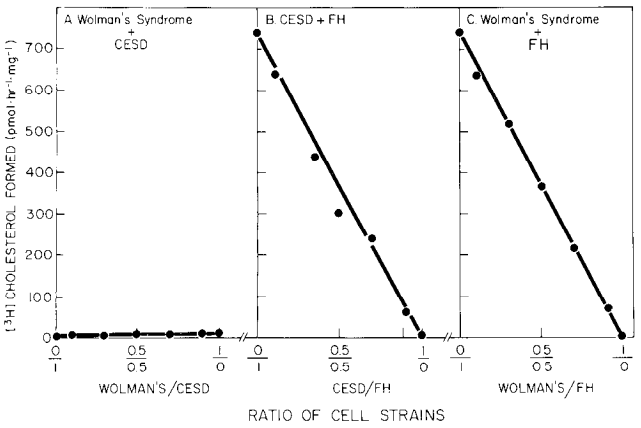

FIG. 2. Cholesteryl ester hydrolase activity in cell-free extracts of fibroblasts from different mutant strains grown together in varying proportions. Monolayers containing mixtures of two cell strains were prepared by seeding the cells together in the indicated ratios as described under "Experimental Procedure" except that on Days 3 and 5 the medium was replaced with 3 ml of fresh medium containing 10% fetal calf lipoprotein-deficient serum. On Day 6, the cell monolayers were harvested and cell-free extracts were prepared as described under "Experimental Procedure." Cholesterol ester hydrolase activity was determined by incubating aliquots of the indicated extract (25 to 35 μg of protein) with 6.7 nmol of [^{3}H]cholesteryl linoleate (91 cpm/pmol) in a final volume of 50 μl for 1 hour at 37° under standard assay conditions at pH 4.3. The amount of free [^{3}H]cholesterol formed was determined as described under "Experimental Procedure." *CESD*, Cholesteryl Ester Storage Disease; *FH*, Familial Hypercholesterolemia.

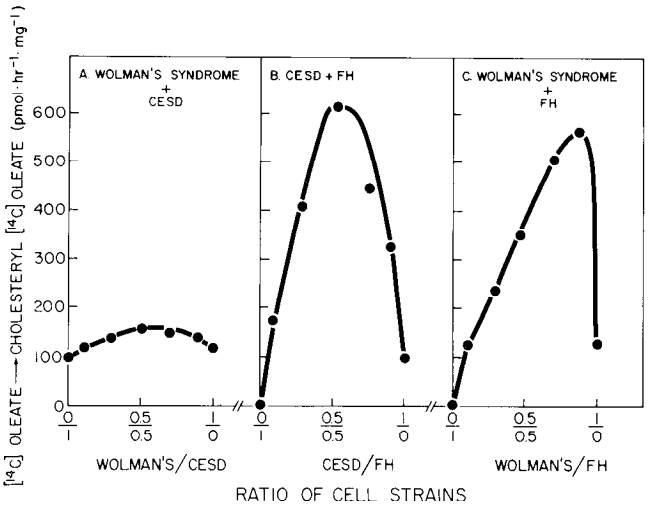

FIG. 3. LDL-stimulated cholesteryl ester formation in fibroblast monolayers composed of cells from two different mutant strains grown together in varying proportions. Monolayers containing mixtures of cell strains were prepared by seeding the cells together in the indicated ratios as described under "Experimental Procedure." On Day 7, each monolayer received 194 μg of protein/ml of LDL. After incubation at 37° for 5 hours, each monolayer was pulse-labeled for 2 hours with 0.1 mM [^{14}C]oleate-albumin (9550 cpm/nmol), after which the cells were harvested for determination of the cellular content of cholesteryl [^{14}C]oleate as described under "Experimental Procedure." In normal fibroblasts treated in the same way and studied simultaneously, the incorporation of [^{14}C]oleate into cholesteryl [^{14}C]oleate was 2539 pmol hr^{-1} mg protein^{-1}. *CESD*, Cholesteryl Ester Storage Disease; *FH*, Familial Hypercholesterolemia.

A strikingly different result was obtained when measurements were made of the ability of intact cells in these mixed cell monolayers to respond to LDL by an enhancement of cholesterol-esterifying activity. Normal fibroblasts incubated in the absence of LDL show no incorporation of [^{14}C]oleate into cholesteryl esters, but when LDL is added these cells markedly increase their cholesterol-esterifying capacity (23). This LDL-mediated stimulation of cholesteryl ester formation requires the binding and cellular uptake of the lipoprotein and the lysosomal hydrolysis of its cholesteryl esters (9, 16). As shown in Fig. 3, the Familial Hypercholesterolemia homozygote cells, which lack functional LDL receptors, do not incorporate [^{14}C]oleate into cholesteryl esters even in the presence of large amounts of LDL. Similarly, both Cholesteryl Ester Storage Disease and Wolman Syndrome cells, which are deficient in cholesteryl ester hydrolase activity, show only low rates of incorporation of [^{14}C]oleate into cholesteryl esters after a 7-hour incubation with LDL. However, when the Wolman Syndrome or Cholesteryl Ester Storage Disease cells were grown together in the same Petri dish with the Familial Hypercholesterolemia homozygote cells and the mixture then stimulated to form cholesteryl esters by incubation with LDL, the mixed monolayers showed a rate of [^{14}C]oleate incorporation into cholesteryl esters that was much greater than in either cell strain grown alone (Fig. 3, *B* and *C*). On the other hand, when the Cholesteryl Ester Storage Disease cells and Wolman Syndrome cells were grown together in the same Petri dish, there was little evidence that either cell strain was able to correct the defective response to LDL in the other strain (Fig. 3*A*).

The enhanced responsiveness to the LDL-mediated stimulation of cellular cholesteryl ester formation was associated with an enhanced ability of the mixed cellular monolayers to hydrolyze the cholesteryl esters of LDL. Fig. 4*B* shows that when [^{3}H]cholesteryl linoleate-labeled LDL was incubated with pure monolayers of either the Wolman Syndrome cells or the Familial Hypercholesterolemia homozygote cells, little [^{3}H]cholesterol was formed, in the former case because of the deficient cholesteryl ester hydrolase activity (8)[3, 4] and in the latter case because of the deficient binding and uptake of the LDL (22). However, mixed monolayers containing both cell strains showed an enhanced ability to hydrolyze the exogenously added [^{3}H]cholesteryl linoleate-labeled LDL (Fig. 4*B*).

In addition to an enhanced cellular cholesterol-esterifying activity, cells in the mixed monolayers also developed an enhanced ability to respond to LDL by suppression of 3-hydroxy-3-methylglutaryl-CoA reductase activity. When grown as a pure monolayer, the Familial Hypercholesterolemia homozygote cells showed only 4% suppression of enzyme activity when incubated for 6 hours with LDL and the Cholesteryl Ester Storage Disease cells showed 20% suppression under the same conditions (Table I). On the other hand, when LDL was added to a mixed monolayer of the two cell strains, a 46% suppression of enzyme activity was observed. It should be noted that in the absence of LDL the mixed monolayer showed an 3-hydroxy-3-methylglutaryl-CoA reductase activity that was higher than that of either of the two pure monolayers. This has been a consistent finding in our studies; its cause is not known.

To determine the mechanism underlying the enhanced ability of mixed monolayers to hydrolyze the LDL-bound cholesteryl esters and to show a biologic response to LDL, experiments were performed in which conditioned medium

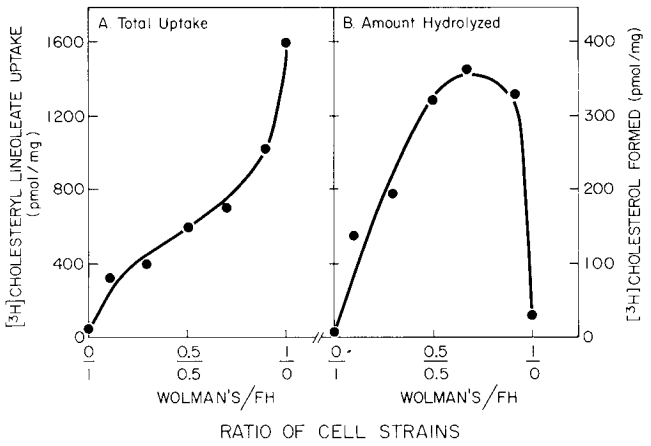

FIG. 4. Total cellular uptake and hydrolysis of [^{3}H]cholesteryl linoleate-labeled LDL in fibroblast monolayers composed of Wolman Syndrome and Familial Hypercholesterolemia (*FH*) homozygote cells grown together in varying proportions. Monolayers containing mixtures of the two cell strains were prepared as described under "Experimental Procedure" except that on Days 3 and 5 the medium was replaced with 3 ml of fresh medium containing 10% fetal calf lipoprotein-deficient serum. On Day 7, each monolayer received 2 ml of medium containing 5% human lipoprotein-deficient serum and 5 μg protein/ml of [^{3}H]CL-LDL (307 × 10^3 cpm/nmol of cholesteryl linoleate). After incubation at 37° for 6 hours, the cellular content of esterified and free [^{3}H]cholesterol was measured as described under "Experimental Procedure." *Graph A* shows the total (free plus esterified) [^{3}H]cholesterol content of the cells and *Graph B* shows the amount of free [^{3}H]cholesterol that was liberated from the hydrolysis of [^{3}H]cholesteryl linoleate-labeled LDL. In calculating the latter value, a blank value representing the amount of free [^{3}H]cholesterol that accumulated in the cells in the presence of chloroquine was subtracted from each point (see "Experimental Procedure"). This blank value ranged between 5 and 10% of the total [^{3}H]cholesterol uptake. In normal fibroblasts treated in the same way and studied simultaneously, the total uptake of [^{3}H]cholesterol was 1730 pmol/mg of protein and the amount of [^{3}H]cholesterol linoleate hydrolyzed was 1320 pmol/mg of protein.

from one cell strain was added to pure monolayers of another cell strain. Table II shows that culture media previously incubated with either normal or Familial Hypercholesterolemia homozygote fibroblasts was capable of enhancing the ability of the Cholesteryl Ester Storage Disease cells to respond to LDL by increasing their cholesterol-esterifying activity. On the other hand, none of the conditioned media was capable of bestowing LDL responsiveness on the Familial Hypercholesterolemia homozygote cells (Table II). In addition to enhancing the LDL-mediated cholesterol-esterifying activity, the conditioned medium produced by the Familial Hypercholesterolemia homozygote cells enhanced the ability of the Cholesteryl Ester Storage Disease cells to respond to LDL by suppression of 3-hydroxy-3-methylglutaryl-CoA reductase activity (Fig. 5). Considered together, the data in Table II and Fig. 5 suggest that the metabolic correction observed in the mixed monolayer experiments of Figs. 3 and 4 was the consequence of the production of a factor by the Familial Hypercholesterolemia homozygote cells that was released into the medium and subsequently taken up by the acid lipase-deficient cells.

The action of conditioned medium in restoring the LDL-mediated stimulation of cholesteryl ester formation in the Cholesteryl Ester Storage Disease cells was time-dependent (Fig. 6) and was proportional to the concentration of the medium added to the cells (Fig. 7). The data in Fig. 8 show

TABLE I

LDL-mediated suppression of 3-hydroxy-3-methylglutaryl-CoA reductase activity in fibroblast monolayers composed of cells from two different mutant strains grown together in equal proportions

Monolayers containing only FH cells, only CESD cells, or a 1:1 mixture of both cell strains were prepared as described under "Experimental Procedure." On Day 7, each monolayer received either no LDL or 39 μg of protein/ml of LDL as indicated. After incubation for 6 hours at 37°, cells were harvested for measurement of HMG-CoA reductase activity as described under "Experimental Procedure." In the same experiment, normal fibroblasts incubated in the absence and presence of LDL showed HMG-CoA reductase activities of 109 and 8 pmol min^{-1} mg $protein^{-1}$, respectively (93% suppression). Each value represents the result of separate incubations.

Cell strain in monolayers	HMG-CoA[a] reductase activity		Average per cent suppression by LDL
	No LDL	+ LDL	
	pmol min^{-1} mg protein^{-1}		
FH homozygote	150,160 (155)[b]	130,166 (148)	4
CESD	110,110 (110)	97,85 (91)	20
FH homozygote + CESD	200,200 (200)	100,110 (105)	46

[a] The abbreviations used are: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; FH, Familial Hypercholesterolemia; CESD, Cholesteryl Ester Storage Disease.

[b] The figures in parentheses represent the average of the duplicate incubations.

TABLE II

Effect of conditioned medium on LDL-mediated stimulation of cholesteryl ester formation in fibroblast monolayers from normal subject and patients with homozygous Familial Hypercholesterolemia and Cholesteryl Ester Storage Disease

Cell monolayers were prepared and conditioned medium was obtained as described under "Experimental Procedure." On Day 7, each monolayer of the indicated cell strain received 2 ml of conditioned medium obtained from the indicated cell strain. After incubation at 37° for 24 hours, each dish received 100 μg of protein/ml of LDL and the incubations were continued for 5 hours. Each monolayer was then pulse-labeled for 2 hours with 0.1 mM [^{14}C]oleate-albumin (10,908 cpm/nmol), after which the cells were harvested for determination of the cellular content of cholesteryl [^{14}C]oleate as described under "Experimental Procedure." Each value represents the average of duplicate incubations. FH, Familial Hypercholesterolemia; CESD, Cholesteryl Ester Storage Disease.

Recipient of conditioned medium	Cell strain used to condition medium	[^{14}C]Oleate → cholesteryl [^{14}C]oleate
		pmol hr^{-1} mg protein^{-1}
Normal cells	None (fresh medium)	1040
Normal cells	Normal	1110
Normal cells	FH homozygote	1760
Normal cells	CESD	1360
FH homozygote cells	None (fresh medium)	2.7
FH homozygote cells	Normal	0.0
FH homozygote cells	FH homozygote	0.0
FH homozygote cells	CESD	2.4
CESD cells	None (fresh medium)	65
CESD cells	Normal	230
CESD cells	FH homozygote	880
CESD cells	CESD	88

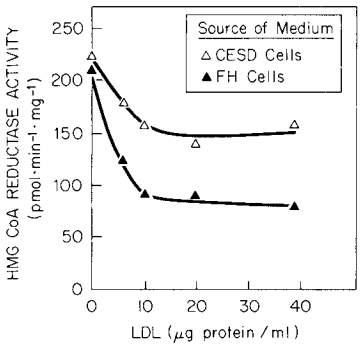

FIG. 5. Enhancement of LDL-mediated suppression of 3-hydroxy-3-methylglutaryl-CoA (*HMG-CoA*) reductase activity by incubation of Cholesteryl Ester Storage Disease (*CESD*) fibroblasts with conditioned medium from Familial Hypercholesterolemia (*FH*) homozygote cells. Monolayers of cells were grown as described under "Experimental Procedure." On Day 7, each dish of Cholesteryl Ester Storage Disease cells received 2 ml of conditioned medium derived from either Cholesteryl Ester Storage Disease cells (△) or Familial Hypercholesterolemia homozygote cells (▲). After incubation for 19 hours at 37°, each dish received the indicated concentration of LDL. After a further incubation for 6 hours, cells were harvested for measurement of 3-hydroxy-3-methylglutaryl-CoA reductase activity as described under "Experimental Procedure."

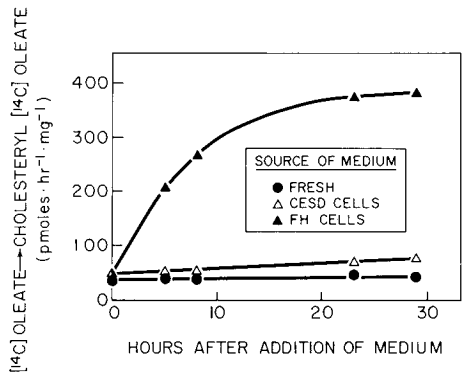

FIG. 6. Enhancement of LDL-stimulated cholesteryl ester formation by incubation of Cholesteryl Ester Storage Disease (*CESD*) fibroblasts with conditioned medium from Familial Hypercholesterolemia (*FH*) homozygote cells: effect of time of incubation with conditioned medium. Monolayers of cells were grown as described under "Experimental Procedure." On Day 7, each dish of Cholesteryl Ester Storage Disease cells received 2 ml of one of the following types of medium: fresh medium containing 5% human lipoprotein-deficient serum (●), conditioned medium derived from Cholesteryl Ester Storage Disease cells (△), or conditioned medium derived from Familial Hypercholesterolemia homozygote cells (▲). After incubation at 37° for the indicated interval, each dish received 195 μg of protein/ml of LDL and 5 hours later the cells were pulse-labeled for 2 hours with 0.1 mM [¹⁴C]oleate-albumin (11,201 cpm/nmol), after which the cells were harvested for determination of the cellular content of cholesteryl [¹⁴C]oleate as described under "Experimental Procedure."

that the enhanced cholesterol-esterifying activity elicited by the conditioned medium was dependent on the presence of LDL. Moreover, after incubation of the conditioned medium with the Cholesteryl Ester Storage Disease cells, the corrective factor appeared to be taken up by the cells; hence, subsequent removal of the conditioned medium did not prevent the enhanced response to LDL (Fig. 8).

Enhancement of the LDL-stimulated incorporation of [¹⁴C]oleate into cholesteryl [¹⁴C]oleate by conditioned medium (as measured in intact cells) was associated with an activation

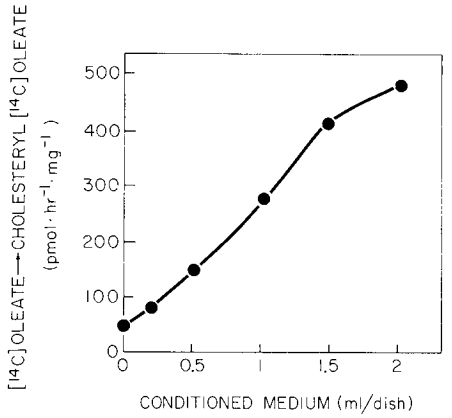

FIG. 7. Enhancement of defective LDL-stimulated cholesteryl ester formation by incubation of Cholesteryl Ester Storage Disease fibroblasts with conditioned medium from Familial Hypercholesterolemia homozygote cells: effect of concentration of conditioned medium. Monolayers of cells were prepared and conditioned medium was obtained from Familial Hypercholesterolemia homozygote cells as described under "Experimental Procedure." On Day 7 of growth each dish of Cholesteryl Ester Storage Disease cells received the indicated volume of conditioned medium plus the appropriate volume of fresh medium to bring the final volume to 2 ml. After incubation for 24 hours at 37°, each dish received 188 μg of protein/ml of LDL and 5 hours later the cells were pulse-labeled for 2 hours with 0.1 mM [¹⁴C]oleate-albumin (11,200 cpm/nmol), after which the cells were harvested for determination of the cellular content of cholesteryl [¹⁴C]oleate as described under "Experimental Procedure."

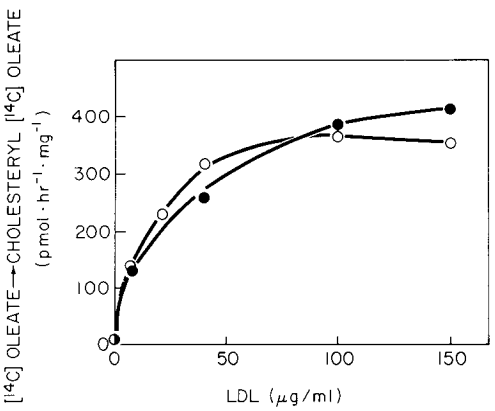

FIG. 8. Cholesteryl ester formation in Cholesteryl Ester Storage Disease fibroblasts after incubation with conditioned medium from Familial Hypercholesterolemia homozygote cells: effect of concentration of LDL. Monolayers of Cholesteryl Ester Storage Disease cells were prepared and conditioned medium from Familial Hypercholesterolemia homozygote cells was obtained as described under "Experimental Procedure." On Day 6, each dish of Cholesteryl Ester Storage Disease cells received 2 ml of conditioned medium. After incubation for 24 hours at 37°, the conditioned medium from one group of dishes was removed and replaced with 2 ml of fresh medium containing 5% human lipoprotein-deficient serum (○). In the other group of dishes, the conditioned medium was not removed (●). To both groups of dishes the indicated concentration of LDL was added. After incubation for 5 hours at 37°, each dish was pulse-labeled for 2 hours with 0.1 mM [¹⁴C]oleate-albumin (11,201 cpm/nmol), after which the cells were harvested for determination of the cellular content of cholesteryl [¹⁴C]oleate as described under "Experimental Procedure."

of acyl-CoA:cholesterol acyltransferase (as measured in cell-free extracts) (Table III). In this experiment it was noted that acyltransferase activity *in vitro* was considerably higher than esterification activity *in vivo* in the cells grown without LDL. This has been a consistent finding in all of our experiments

TABLE III

Enhancement of LDL-mediated stimulation of cholesteryl ester formation in Cholesteryl Ester Storage Disease fibroblasts after incubation with conditioned medium, as measured in vivo and in vitro

Cell monolayers were prepared and conditioned medium was obtained as described under "Experimental Procedure." On Day 7 of cell growth, each monolayer of the indicated strain received 2 ml of conditioned medium obtained from the indicated cell strain. After incubation at 37° for 24 hours, half of the dishes in each group received 200 μg of protein/ml of LDL and the incubations were continued at 37° for 7 hours. For the *in vivo* assay, each dish was pulse-labeled during the last 2 hours with 0.1 mM [^{14}C]oleate-albumin (11,000 cpm/nmol), after which the cells were harvested for determination of the cellular content of cholesteryl [^{14}C]oleate as described under "Experimental Procedure." For the *in vitro* assay, the cells from each group were harvested by scraping, pooled together, homogenized with a Dounce homogenizer, and acyl-CoA:cholesterol acyltransferase activity was measured in aliquots of the pooled cell-free extracts as described under "Experimental Procedure." Each value in the *in vivo* assay represents the average of duplicate incubations; each value in the *in vitro* assay represents the average of duplicate assays of extracts pooled from eight dishes of cells. CESD, Cholesteryl Ester Storage Disease; FH, Familial Hypercholesterolemia

Cells assayed	Cell strain used to condition medium	LDL	[^{14}C]Oleate → cholesteryl [^{14}C]oleate (*in vivo*)	Acyl-CoA: cholesterol acyltransferase activity (*in vitro*)
			pmol·hr^{-1}·mg protein^{-1}	
CESD	CESD	−	20 (160)[a]	200 (170)[a]
		+	180	370
CESD	FH homozygote	−	30 (580)	160 (300)
		+	610	460
Normal	Normal	−	30 (1970)	200 (900)
		+	2000	1100

[a] The figures in parentheses represent the LDL-dependent stimulation of cholesteryl ester formation (activity after incubation in the presence of LDL minus activity after incubation in the absence of LDL).

carried out with a variety of different cells strains. The reason for the apparent increase in acyltransferase activity when LDL-deprived cells are ruptured is not yet clear. In the experiment of Table III the same increase was present irrespective of the cell strain or conditioned medium used.

Evidence that the corrective factor in the conditioned medium from the Familial Hypercholesterolemia homozygote cells was in fact the acid lipase enzyme, itself, is provided by the data in Fig. 9 and Table IV. First, the data in Fig. 9 show that the conditioned medium contained cholesteryl ester hydrolase activity and that this activity was progressively inactivated when the conditioned medium was heated at 56°. The amount of thermal inactivation of the cholesteryl ester hydrolase activity was matched by a proportional decline in the ability of the conditioned medium to restore the LDL-mediated stimulation of cholesteryl ester formation (Fig. 9). Second, the data in Table IV show that when the Cholesteryl Ester Storage Disease cells were incubated with conditioned medium from a Familial Hypercholesterolemia homozygote they did in fact acquire cholesteryl ester hydrolase activity. Although this acquired activity was still about 10-fold below the activity present in normal or Familial Hypercholesterolemia homozygote cells, it was nevertheless apparently sufficient to restore the ability of the cells to hydrolyze the

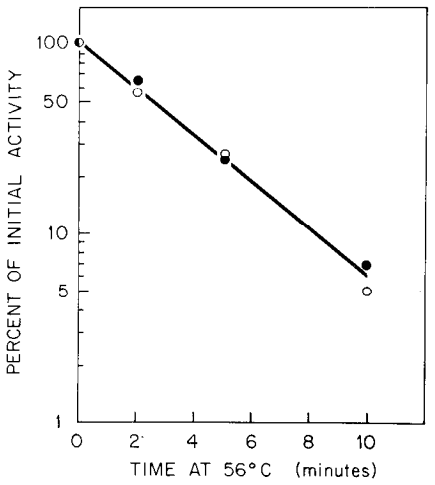

FIG. 9. Heat inactivation of the cholesteryl ester hydrolase activity of conditioned medium and simultaneous destruction of its ability to correct the defective LDL-mediated stimulation of cholesteryl ester formation in Cholesteryl Ester Storage Disease fibroblasts. Monolayers of Cholesteryl Ester Storage Disease cells were prepared and conditioned medium from Familial Hypercholesterolemia homozygote cells was obtained as described under "Experimental Procedure." The conditioned medium was heated at 56° for the indicated time, after which aliquots were removed and simultaneously assayed for cholesteryl ester hydrolase activity (○) and for their ability to correct the defective LDL-mediated stimulation of cholesteryl ester formation in Cholesteryl Ester Storage Disease cells (●). Cholesteryl ester hydrolase activity of the conditioned medium was determined under standard assay conditions at pH 4.3 as described for the hydrolysis of [^{3}H]cholesteryl linoleate by cell-free extracts under "Experimental Procedure" except that 100 μl of conditioned medium was used instead of a cell-free extract, and the incubations were conducted for 1 hour in a volume of 200 μl containing 86 pmol of [^{3}H]cholesteryl linoleate (16,835 cpm/pmol). The ability of the conditioned medium to restore LDL-mediated stimulation of cholesteryl ester formation in Cholesteryl Ester Storage Disease cells was measured by incubating the monolayers for 16 hours at 37° with 2 ml of the heated conditioned medium. Following this incubation, 200 μg of protein/ml of LDL was added to each dish and 5 hours later the cells were pulse labeled for 2 hours with 0.1 mM [^{14}C]oleate-albumin (9600 cpm/nmol). The cells were then harvested for determination of the cellular content of cholesteryl [^{14}C]oleate as described under "Experimental Procedure." For both assays, the activity of the heated conditioned medium is expressed as a percentage of the activity using unheated medium. These initial values were: cholesteryl ester hydrolase activity, 1.2 pmol/hour/2 ml of medium (○); and cholesterol-esterifying activity, 671 pmol of [^{14}C]oleate incorporated/hour into cholesteryl [^{14}C]oleate per mg of cell protein (●).

cholesteryl esters of the incoming LDL and to allow them to respond to LDL by an enhancement of cholesterol esterification.

The experiment in Fig. 10 was designed to determine whether the acquisition of cholesteryl ester hydrolase activity from conditioned medium would lessen the accumulation of cholesteryl esters in Wolman Syndrome fibroblasts. At the beginning of this experiment the Wolman Syndrome cells were growing logarithmically in 10% fetal calf serum containing lipoproteins and had accumulated an amount of cholesteryl esters that was 10 to 20 times greater than that of normal fibroblasts grown under comparable conditions. On the other hand, the free cholesterol content of these mutant cells was similar to that which we have previously observed in normal fibroblasts growing under similar conditions (9, 27). When the Wolman Syndrome cells were switched to fresh medium containing no LDL, the total mass of cholesteryl esters in each

TABLE IV

Uptake of cholesteryl ester hydrolase activity by Cholesteryl Ester Storage Disease fibroblasts after incubation with conditioned medium

Monolayers of CESD (Cholesteryl Ester Storage Disease) cells were prepared and conditioned medium was obtained as described under "Experimental Procedure." On Day 7, each monolayer received 3 ml of conditioned medium obtained from either CESD cells or FH (Familial Hypercholesterolemia) homozygote cells as indicated. After incubation at 37° for 24 hours, cell monolayers were harvested, the cells from three dishes were pooled together, and cell-free extracts were prepared as described under "Experimental Procedure." Aliquots of each extract (300 to 400 μg of protein) were incubated with 100 pmol of [^{3}H]cholesteryl linoleate (18,900 cpm/pmol) in a final volume of 200 μl at pH 4.3 for 3.5 hours at 37° and the formation of free [^{3}H]cholesterol was determined as described under "Experimental Procedure." Each value represents the cholesteryl ester hydrolase activity in cells from three pooled dishes. In the same experiment, the FH homozygote fibroblasts contained a cholesteryl ester hydrolase activity of 61 pmol of [^{3}H]cholesterol formed/mg of protein.

Cell strain used to condition medium	Cholesteryl ester hydrolase activity in cells
	pmol [^{3}H]cholesterol formed/mg protein
CESD	0.56, 1.4, 0.35 (mean = 0.77)
FH homozygote	5.9, 5.5, 5.6 (mean = 5.7)

dish declined only slightly (Fig. 10*A*), but since the cells were increasing in number during the experiment and not accumulating additional cholesteryl esters the content of esterified cholesterol per mg of cell protein declined (Fig. 10*C*). In the dishes that received conditioned medium a slow but definite decline in the total cholesteryl ester content per dish occurred (Fig. 10*A*) so that by the end of 72 hours there was approximately one-half as much esterified cholesterol in the cells that had received conditioned medium as compared with the cells receiving fresh medium. This decrease in cholesteryl esters occurred without a corresponding increase in the free cholesterol content of the cells (Fig. 10*G*), indicating that the free cholesterol liberated from the hydrolysis of the previously stored cholesteryl esters had been excreted by the cells.

An even more striking result was obtained when the growth medium contained LDL. Under these conditions in the absence of conditioned medium, the total cholesteryl ester content per dish (Fig. 10*B*) rose in proportion to the number of cells so that the cellular content of esterified cholesterol expressed in terms of milligrams of cell protein was constant after a slight initial drop (Fig. 10*D*). In the dishes that received conditioned medium the LDL-mediated accumulation of cholesteryl esters was prevented. In addition, the hydrolysis of the previously stored cholesteryl esters produced an absolute decline in

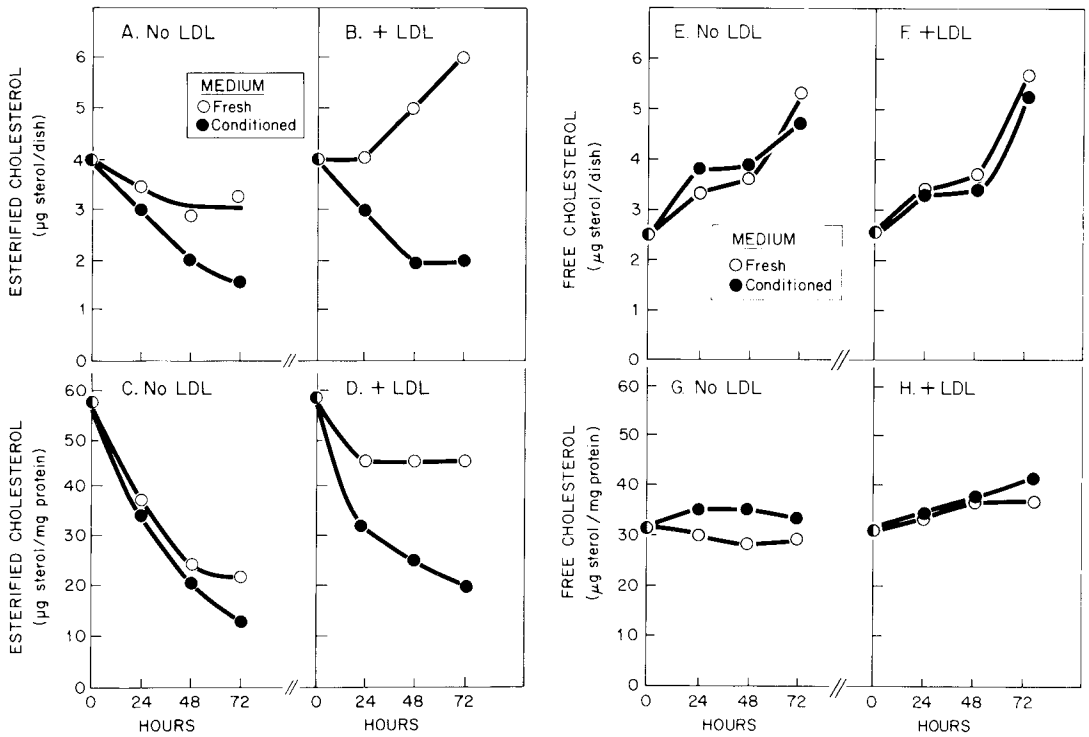

FIG. 10. Reduction in the cellular content of cholesteryl esters in Wolman Syndrome fibroblasts by incubation with conditioned medium. Monolayers of Wolman Syndrome cells were prepared as described under "Experimental Procedure." On Day 3 of cell growth, the medium was replaced with 3 ml of fresh medium containing 10% fetal calf serum. On Day 4 (zero time), the medium was replaced with 2 ml of fresh medium containing 10% fetal calf lipoprotein-deficient serum (○) or conditioned medium containing 10% fetal calf lipoprotein-deficient serum that had been previously incubated for 24 hours with Familial Hypercholesterolemia homozygote cells (●). Dishes also received at zero time either no LDL (*A*, *C*, *E*, *G*) or 10 μg of protein/ml of LDL (*B*, *D*, *F*, *H*). Every 24 hours, the medium was removed and replaced with a new aliquot of medium of identical composition. At the indicated interval, duplicate or triplicate dishes were harvested and the cellular content of free and esterified cholesterol was determined as described under "Experimental Procedure." *Panels A to D* show the esterified cholesterol content and *Panels E to H* show the free cholesterol content. In the upper panels the data are expressed as micrograms of sterol per dish, and in the lower panels these numbers have been divided by the total cell protein content per dish.

cellular cholesteryl ester content despite the presence of LDL (Fig. 10*B*). Again, this decline in cholesteryl ester content occurred in the absence of a significant rise in the cellular content of free cholesterol (Fig. 10*H*).

DISCUSSION

The data in the current studies show: (*a*) that cholesteryl ester hydrolase activity is found in the culture medium of human fibroblasts; (*b*) that this enzyme activity can be taken up in an active form by cells with genetic deficiencies of lysosomal acid lipase; (*c*) that the acquisition of this enzyme activity by fibroblasts from patients with acid lipase deficiency allows these mutant cells to hydrolyze the cholesteryl esters that enter the cell in association with LDL; and (*d*) that the acquisition of this hydrolytic capacity enhances the ability of these cells to respond to LDL by suppression of 3-hydroxy-3-methylglutaryl-CoA reductase activity and stimulation of cellular cholesteryl ester formation.

Two lines of evidence indicate that the enhanced LDL responsiveness achieved by incubating Cholesteryl Ester Storage Disease or Wolman Syndrome cells with conditioned medium was due to the uptake of the acid lipase enzyme. First, heat inactivation curves showed that the ability of the conditioned medium to enhance LDL responsiveness was inactivated in parallel with its cholesteryl ester hydrolase activity. Second, whereas conditioned medium obtained from normal or Familial Hypercholesterolemia homozygote fibroblasts enhanced the LDL responsiveness of the acid lipase-deficient cells, conditioned medium obtained from either of the two acid lipase-deficient strains, which was devoid of detectable cholesteryl ester hydrolase activity, did not enhance LDL responsiveness.

Under normal circumstances in fibroblasts, the lysosomal acid lipase and the microsomal acyl-CoA:cholesterol acyltransferase function coordinately to control the compartmentalization of cholesteryl esters within the cell (reviewed in Ref. 16). When normal cells ingest LDL through the LDL receptor-mediated pathway, the acid lipase hydrolyzes the LDL-derived cholesteryl esters within the lysosome (9) and this allows the liberated cholesterol to leave the lysosome and enter the extralysosomal cellular compartment, where it activates the microsomal acyl-CoA:cholesterol acyltransferase, facilitating its own re-esterification (9, 23, 29). The net result is a transfer of cholesteryl esters from the lysosome into the extralysosomal cellular compartment (9, 27). In the acid lipase-deficient cells, LDL-derived cholesteryl esters are trapped within the lysosome and therefore free cholesterol is not generated in an amount sufficient to stimulate the acyltransferase in a normal fashion (9).[3, 4] However, when these mutant cells were exposed to LDL after incubation with an exogenous supply of cholesteryl ester hydrolase activity, there was both an hydrolysis of the LDL-derived cholesteryl esters and a secondary stimulation of the microsomal re-esterification process.

The free cholesterol produced from the conditioned medium-induced hydrolysis of LDL-cholesteryl esters in the Wolman Syndrome cells had two metabolic fates: some entered the extralysosomal cellular compartment for re-esterification by the acyltransferase and the remainder was excreted by the cell. As shown by the balance data of Fig. 10, when the Wolman Syndrome cells were incubated over a 3-day period with the conditioned medium, the overall long term result of the entire process was a decrease in the total cellular content of cholesteryl ester, whether or not LDL was present in the culture medium. This net lowering of cellular cholesterol was due to two factors. First, the conditioned medium had a greater effect on enhancing the rate of hydrolysis of both previously stored and LDL-derived lysosomal cholesteryl esters than it did on enhancing the rate of cholesterol re-esterification by the microsomal enzyme; hence, quantitatively more of the hydrolyzed free cholesterol was excreted from the cell than entered the extralysosomal cellular compartment for re-esterification. Second, it is likely that hydrolysis of LDL-cholesteryl esters elicited by the conditioned medium permitted sufficient free cholesterol to be generated to suppress the synthesis of LDL receptors (31), thus stopping LDL uptake and further reducing the accumulation of cholesteryl esters in lysosomes. These biochemical data are in agreement with the histochemical findings of Kyriakides *et al.* (32), who previously showed that exposure of Wolman Syndrome fibroblasts for 1 to 2 weeks to conditioned medium obtained from normal cells greatly diminished cellular lipid accumulation as demonstrated by Oil Red O staining.

An interesting aspect of the current study relates to the observation that the acquisition of only a small amount of acid lipase activity as measured in the *in vitro* cholesteryl ester hydrolase assay was sufficient to create a major improvement in cellular physiology as measured in intact cells. This would suggest that lysosomal acid lipase is present in vast excess in normal cells and that the restoration of only about 10% of normal activity is sufficient to allow cells to metabolize LDL-derived cholesteryl esters in a near-normal manner.

Acknowledgments—We thank Helen Bohmfalk, Jean Helgeson, and Marva Shaw for their excellent assistance.

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