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Binding and Degradation of Low Density Lipoproteins by Cultured Human Fibroblasts

COMPARISON OF CELLS FROM A NORMAL SUBJECT AND FROM A PATIENT WITH HOMOZYGOUS FAMILIAL HYPERCHOLESTEROLEMIA*

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JOSEPH L. GOLDSTEIN‡ AND MICHAEL S. BROWN

From the Divisions of Medical Genetics and Gastroenterology-Liver, Department of Internal Medicine, University of Texas Southwestern Medical School, Dallas, Texas 75235

SUMMARY

^{125}I-Labeled low density lipoproteins were found to associate with monolayers of cultured normal fibroblasts by two processes—one of high affinity and one of low affinity. The high affinity association appeared to represent binding of the low density lipoprotein to specific receptor sites on the cell surface. This binding process exhibited saturation kinetics at low concentrations of the lipoprotein and competition by related molecules such as very low density lipoproteins. In addition, this process was stimulated by the presence of calcium in the culture medium and could be destroyed by limited treatment of the cells with pronase. The other process, designated low affinity uptake, may represent nonspecific endocytosis since the uptake was proportional to the lipoprotein concentration in the medium with no apparent saturation and because it showed no competition by very low density lipoproteins, no stimulation by calcium, and no destruction by pronase treatment. The ^{125}I-labeled low density lipoproteins associated with normal cells by either the high or low affinity process were degraded by proteolysis to trichloroacetic acid-soluble material, most of which was ^{125}I-tyrosine.

In normal cells, binding of low density lipoproteins to the high affinity membrane receptor sites appears to serve two functions: (*a*) it results in suppression of the synthesis of 3-hydroxy-3-methylglutaryl coenzyme A reductase, the rate-controlling enzyme in cholesterol biosynthesis, and (*b*) it facilitates the degradation of low density lipoproteins when they are present in the culture medium at low concentrations (i.e. high affinity degradation). Cultured cells from subjects with the homozygous form of familial hypercholesterolemia, which were found to lack the high affinity binding process, were resistant to suppression of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity by low density lipoproteins and were also deficient in high affinity degradation. On the other hand, these mutant cells showed a normal low affinity uptake of low density lipoproteins and were able to degrade these lipoproteins when they were present in the culture medium at high concentrations. The possibility is raised, therefore, that a prerequisite for the regulation of cholesterogenesis in cultured fibroblasts is the initial binding of low density lipoproteins to the high affinity surface receptor sites and that a defect in this process represents the primary genetic abnormality in the disorder familial hypercholesterolemia.

In cultured human fibroblasts, the rate of cholesterol synthesis is controlled by the content of low density lipoproteins in the culture medium (1–4). In recent studies, we have shown that LDL[1] suppresses cholesterol synthesis by inhibiting the synthesis of 3-hydroxy-3-methylglutaryl coenzyme A reductase, the rate-controlling enzyme in the cholesterol biosynthetic pathway (1–4). In order to produce this inhibition, LDL must bind to specific high affinity receptor sites on the cell membrane (5). In cultured fibroblasts from subjects with the homozygous form of familial hypercholesterolemia, this high affinity binding of LDL is deficient (5). As a consequence of this genetic defect, LDL fails to suppress HMG-CoA reductase activity and cholesterol is overproduced (2).

In the course of studying the binding of ^{125}I-LDL to normal fibroblasts, it was noted that the bound material was degraded to a product that was dialyzable and could no longer be precipitated with trichloroacetic acid (5). The present studies were designed to characterize this degradative process in more detail and to examine its relationship to the binding process in normal and hypercholesterolemic cells.[2] The data demonstrate that at

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[1] The abbreviations used are: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LDL, low density lipoproteins; VLDL, very low density lipoproteins; HDL, high density lipoproteins; Tricine, *N*-tris(hydroxymethyl)methylglycine.

[2] For the sake of convenience, the term "hypercholesterolemic

low LDL concentrations high affinity binding is rate-limiting in the process by which cultured fibroblasts degrade this lipoprotein. In the hypercholesterolemic cells, the defect in high affinity binding results in a reduced ability to degrade LDL. This defect may be causally related to the high serum levels of LDL that are characteristic of familial hypercholesterolemia.

EXPERIMENTAL PROCEDURE

Materials—^{125}I-Sodium (carrier free in 0.05 N NaOH) was obtained from Schwarz-Mann. ^{125}I-Labeled bovine serum albumin (29 Ci per mmole) was purchased from New England Nuclear Corp. Tricine and bovine albumin stock solution (Cat. No. 904-10) were purchased from Sigma Chemical Co. 3-Iodo-L-tyrosine was obtained from Aldrich Chemical Co. Topical thrombin (bovine origin) was purchased from Parke, Davis, and Co. H_2O_2 (30% solution) was obtained from Matheson. Pronase from *Streptomyces griseus*, B grade (45,000 units per g) was obtained from Calbiochem. A monospecific goat antiserum to human LDL was obtained from Hyland Laboratories. Silica Gel G (without gypsum) and cellulose (polygram cel 300) coated thin layer chromatography sheets with plastic backs were obtained from Brinkmann Industries. Eagle's minimum essential medium (Cat. No. F-11), penicillin (10,000 units per ml)-streptomycin (10 mg per ml) solution, trypsin-EDTA solution (1X), nonessential amino acids solution (100 X), and Dulbecco's phosphate-buffered saline (0.9% NaCl) (Cat. No. 419) were purchased from GIBCO. Petri tissue culture dishes (60 mm diameter) and flasks (75 cm^2, 250 ml) were obtained from Falcon Plastics. Fetal calf serum was obtained from Flow Laboratories.

Cells—The fibroblasts used in these studies were established from explants of human skin as previously described (1–4). In all experiments the normal cell line was derived from the foreskin of a healthy newborn. Previous studies have shown that the regulation of HMG-CoA reductase activity and cholesterol synthesis by LDL and the binding of LDL to these cells are similar to that of fibroblasts derived from adults and children with normal lipid levels (3, 5). Unless otherwise stated, the hypercholesterolemic cell line was obtained from J.P., a 12-year-old female subject with the homozygous form of familial hypercholesterolemia (2). All cell lines were grown in monolayer and were utilized after 5 to 25 passages. No cells were used after the 25th passage unless indicated. Cell lines were maintained in logarithmic growth in a humidified CO_2 incubator at 37° in 75-cm^2 flasks containing 10 ml of Eagle's minimum essential medium supplemented with penicillin (100 units per ml); streptomycin (100 μg per ml); 20 mM Tricine, pH 7.4; 24 mM $NaHCO_3$; 1% (v/v) nonessential amino acids; and 10% (v/v) fetal calf serum. For all experiments, cells from the stock flasks were dissociated with 0.05% trypsin-0.05% EDTA and were seeded (Day 0) at a concentration of 2×10^5 cells per dish into 60-mm dishes containing 3 ml of the above growth medium with 10% fetal calf serum. On Day 3 the medium was replaced with fresh growth medium containing 10% fetal calf serum. On Day 6 when the cells were confluent (average cell density 7 to 9×10^5 cells per dish), the medium was removed, the cellular monolayer was washed once with 2 ml of Dulbecco's phosphate-buffered saline (0.9% NaCl), following which 2 ml of fresh medium containing 5% (v/v) lipoprotein-deficient human serum (final concentration of protein, 2.5 mg per ml) were added.

Lipoproteins—Human LDL (d 1.019 to 1.063 g per ml), HDL (d 1.063 to 1.215 g per ml) and lipoprotein-deficient serum ($d > 1.215$ g per ml) were prepared from single 500-ml units of blood collected in 0.1% EDTA from healthy subjects who had been fasted for 15 hours. VLDL ($d < 1.006$ g per ml) was isolated from the blood of a patient with endogenous hypertriglyceridemia associated with partial lipodystrophy (fasting plasma lipid levels: cholesterol, 180 mg per dl; triglyceride, 790 mg per dl). Lipoproteins were fractionated by sequential flotation in a Beckman preparative ultracentrifuge at 214,000 × g (average) and 4–10° for 16 to 24 hours according to standard techniques (6) using solid KBr for density adjustment (7). Isolated fractions were dialyzed at least 36 hours at 4° against 3 changes of at least 50 volumes of buffer containing 0.15 M NaCl and 0.3 mM EDTA, pH 7.4. Following dialysis, the lipoprotein-deficient fraction was defibrinated with thrombin as previously described (4), sterilized by Millipore filtration, and stored at 4°. Each isolated lipoprotein fraction migrated as a homogenous peak on lipoprotein electrophoresis (8) and maintained full biologic activity for at least 3 months.

^{125}I-LDL—^{125}I-LDL was prepared by Dr. David W. Bilheimer of the University of Texas using a modification (9) of the iodine monochloride method of MacFarlane (10). Several preparations were used, all of which were dialyzed extensively into 0.15 M NaCl and 0.3 mM EDTA, pH 7.4, and contained less than 1 atom of iodine for each LDL molecule. The final specific activities varied between 400 and 600 cpm per ng of protein. In all preparations, more than 97% of the radioactivity was precipitated by incubation with 15% trichloroacetic acid at 90°, at least 91% was precipitated by a monospecific goat antiserum to human LDL, and less than 2% was extractable into chloroform-methanol (11). When prepared by this method, the ^{125}I-LDL was able to suppress HMG-CoA reductase activity of normal fibroblasts in a manner similar to that of native LDL (5). The ^{125}I-LDL was stored at 4° and retained full immunologic and cell binding activity for at least 1 month. For binding experiments the ^{125}I-LDL was diluted with native LDL to give the final specific activity indicated in the legend to each figure or table.

^{125}I-LDL Binding to Intact Fibroblasts—Cells were grown in dishes as described above, and on Day 7 after 24 hours of growth in medium containing lipoprotein-deficient serum, the medium was removed from each dish and replaced with 2 ml of Medium A, consisting of Eagle's minimum essential medium containing penicillin and streptomycin; 20 mM Tricine-Cl, pH 7.4; and 5 mg of lipoprotein-deficient serum, and the indicated amounts of ^{125}I-LDL protein and native LDL protein. After incubation on a rotatory shaker (80 oscillations per min) in air at 37°, the medium was removed and all subsequent operations were carried out at 4° in a cold room. Each monolayer was washed 3 times in rapid succession with 3 ml of buffer containing 50 mM Tris-Cl, pH 7.4; 0.15 M NaCl; and 6 mg of bovine serum albumin, after which a further 3 ml of the same buffer was added and the monolayer was incubated for 2 min. This latter step was repeated once, each monolayer was washed finally with 3 ml of buffer containing 50 mM Tris-Cl, pH 7.4 and 0.15 M NaCl, and the cells were removed from the dish by dissolution in 1 ml of 0.1 N NaOH. Aliquots of 500 μl were removed from each dish for scintillation counting in a gamma counter and 50-μl aliquots were taken for measurement of protein concentration (12). In all figures, each point represents the value obtained from a single dish and is expressed as the counts per min of ^{125}I bound per mg of total cell protein. Each dish contained 350 to 500 μg of total cell protein. Duplicate determinations of ^{125}I-LDL binding varied by less than 10% of the mean values.

Degradation of ^{125}I-LDL to Acid-soluble Material—Cell monolayers were prepared and incubated with ^{125}I-LDL exactly as described for the binding experiments. After incubation, the medium was removed and the entire cell-free 2 ml were added to 0.5 ml of 50% (w/v) trichloroacetic acid, incubated for 30 min at 4°, and the precipitate was removed by centrifugation. To a 1-ml aliquot of the supernatant were added 10 μl of 40% (w/v) KI and 40 μl of 30% H_2O_2. After incubation at room temperature for 5 min, the mixture was extracted with 2 ml of $CHCl_3$ to remove free iodine (see below), after which 0.5 ml of the aqueous fraction was removed for scintillation counting in a gamma counter. In all figures each point represents the value obtained from a single dish and is expressed as the counts per min of ^{125}I-labeled acid-soluble material formed per mg of total cell protein. Duplicate determinations of ^{125}I-LDL degradation varied by less than 5% of the mean values.

HMG-CoA Reductase Assay—Cell extracts for measurement of HMG-CoA reductase activity were prepared by a nonionic detergent as previously reported (4). Aliquots (20 to 100 μg of soluble protein) were incubated for 120 min at 37° in a final volume of 0.2 ml containing 0.1 M potassium-phosphate, pH 7.5; 20 mM glucose 6-phosphate; 2.5 mM TPN; 0.7 unit glucose 6-phosphate dehydrogenase; 4 mM dithiothreitol; and 3×10^{-5} M DL-[3-^{14}C]HMG-CoA (8.47 Ci per mole). The [^{14}C]mevalonate formed was isolated by thin layer chromatography and counted with an internal standard of [^{3}H]mevalonate to correct for incomplete recovery (13).

cells" is used to designate cell lines derived from subjects with the homozygous form of familial hypercholesterolemia.

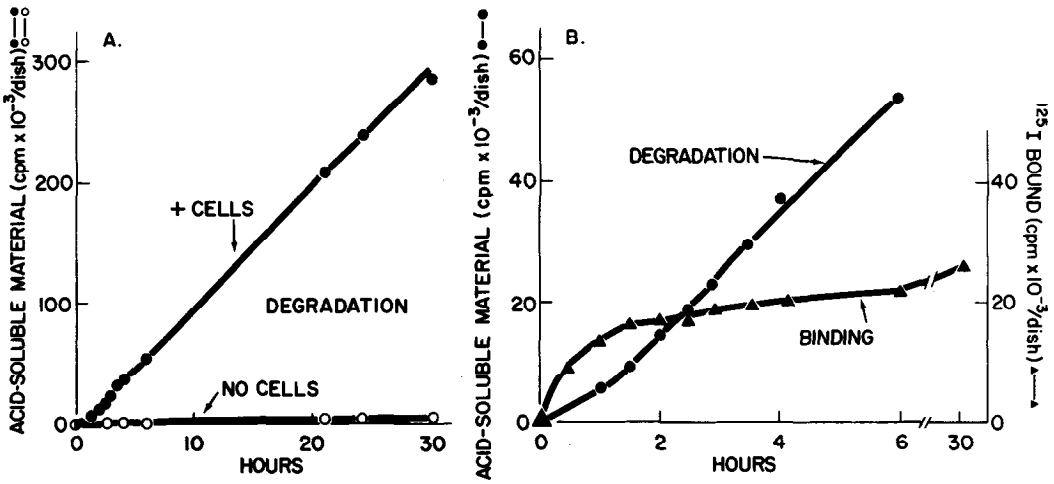

FIG. 1. Time course of degradation of ^{125}I-LDL to acid-soluble material by intact fibroblasts and its relation to binding. Cell monolayers were prepared in dishes as described under "Experimental Procedure." At zero time the medium was replaced with 2 ml of Medium A containing 5 μg per ml of ^{125}I-LDL (260 cpm per ng) and the cells were incubated at 37°. As a control for spontaneous degradation of the ^{125}I-LDL, dishes without cells were incubated under identical conditions. At each indicated time, the medium from a single dish was removed, its content of acid-soluble material was measured (●, ○), and the amount of ^{125}I bound to the cells was determined (▲). The cellular protein content of each dish averaged 0.4 mg (range, 0.37 to 0.42 mg).

RESULTS

When ^{125}I-LDL was incubated with monolayers of intact fibroblasts, the LDL was degraded to material that was soluble in 10% trichloroacetic acid (Fig. 1). To eliminate contamination by the small amount of free iodide that persisted in the ^{125}I-LDL preparation despite extensive dialysis, the acid soluble material formed by the cells was oxidized with hydrogen peroxide and then extracted with chloroform. Control experiments showed that under these conditions greater than 95% of free iodide added as ^{131}I-sodium was extracted into the chloroform phase.

When this extraction procedure was applied to the trichloroacetic acid-soluble material formed by the cells, a constant fraction of the radioactivity (about 65%) remained in the water phase at every time point.[3] The formation of this iodide-free, acid-soluble degradative product of LDL, hereafter referred to as acid-soluble material, was absolutely dependent on the presence of cells and was linear with time up to at least 30 hours (Fig. 1*A*). At this point, 12% of the total radioactivity in the medium had been converted to acid-soluble material.

To determine the relation between the degradative process and the previously described binding of ^{125}I-LDL to fibroblasts (5), the time courses of binding and degradation were compared (Fig. 1*B*). Whereas the amount of ^{125}I bound to the cells reached a maximum at 2 hours and remained constant for the next 28 hours, the rate of formation of acid-soluble material proceeded in a linear fashion over the entire 30-hour period after an initial lag phase. The 2-hour lag phase corresponded to the time required to establish maximal binding (Fig. 1*B*).

Despite the accumulation of acid-soluble material in the medium, the ^{125}I bound to the cells remained precipitable by trichloroacetic acid (Table I). About 50% of this bound material was precipitable by a monospecific antibody to LDL after the cells were solubilized in 1% sodium deoxycholate.

To demonstrate directly the conversion of bound ^{125}I-LDL to acid-soluble material, cells which had been preincubated at 4° with ^{125}I-LDL were transferred to medium without ^{125}I-LDL and were incubated at either 4 or 37° (Fig. 2). At the beginning of the second incubation, all the radioactivity bound to the cells was precipitable with trichloroacetic acid. At 37° nearly all of this bound radioactivity was released to the medium within 3 hours and approximately 65% had been converted to acid-soluble material. The remaining 35% of the released material was precipitable both by trichloroacetic acid and by a specific antibody to LDL. In contrast, at 4° only about 35% of the ^{125}I bound to the cells was released and all of this was precipitable by trichloroacetic acid (Fig. 2). Thus, while the release of presumably intact LDL from the cell occurred independent of temperature, the conversion to acid-soluble material was temperature-dependent.

Although the release of ^{125}I-LDL from the cell at 37° was associated with partial degradation of the LDL molecule, the binding sites on the cell did not appear to be altered. Table II demon-

TABLE I

Distribution of acid-soluble material after incubation of normal fibroblasts with ^{125}I-LDL

Cell monolayers were prepared in dishes as described under "Experimental Procedure." At the beginning of the experiment, the medium was replaced with 2 ml of Medium A containing 5 μg per ml of ^{125}I-LDL (200 cpm per ng) and the cells were incubated at 37°. At the indicated time, the medium was removed and its content of acid-soluble material was measured. The cell monolayers were washed 6 times as described under "Experimental Procedure" and the cells from each dish were scraped with a rubber policeman into 2 ml of Medium A. Aliquots of 0.1 ml were removed for determination of total ^{125}I radioactivity bound to the cells and the remaining 1.9 ml of the cell extract were processed for measurement of its content of acid-soluble material. Each value represents the mean of duplicate dishes.

Time of incubation	Total ^{125}I bound to cells	Acid-soluble radioactivity	
		Medium	Cells
hr	*cpm × 10⁻³/dish*	*cpm × 10⁻³/dish*	
3	24.7	50.0	2.6
6	21.7	108.0	3.5
24	15.8	347.6	2.4

[3] The 35% of the trichloroacetic acid-soluble fraction of radioactivity that was extractable into chloroform appeared to represent largely free iodide formed by the cells at a rate proportional to the overall production of acid-soluble material. This product was not studied further.

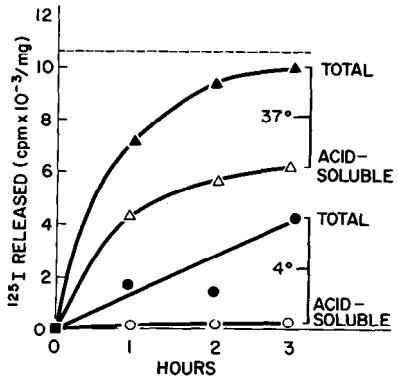

FIG. 2. Release of bound ^{125}I from normal fibroblasts at 4° and 37°. Cell monolayers were prepared in dishes as described under "Experimental Procedure." At the beginning of the experiment the medium was replaced with 2 ml of Medium A containing 5 μg per ml of ^{125}I-LDL (220 cpm per ng) and the cells were incubated at 4° for 3 hours. At this point (zero time), the medium was removed and the cell monolayers were washed 6 times as described under "Experimental Procedure," after which 2 ml of Medium A without LDL were added. The monolayers were then further incubated at either 4° (●, ○) or 37° (▲, △). At the indicated time, the medium from each dish was removed, its content of acid-soluble material was measured (○, △), and the amount of ^{125}I-LDL remaining bound to the cells was determined. The total ^{125}I radioactivity released to the medium (●, ▲) was calculated by subtraction of the amount remaining bound to the cells at each time point from the total bound to the cells at zero time, indicated by the *dashed line* at the top of the figure (– – –).

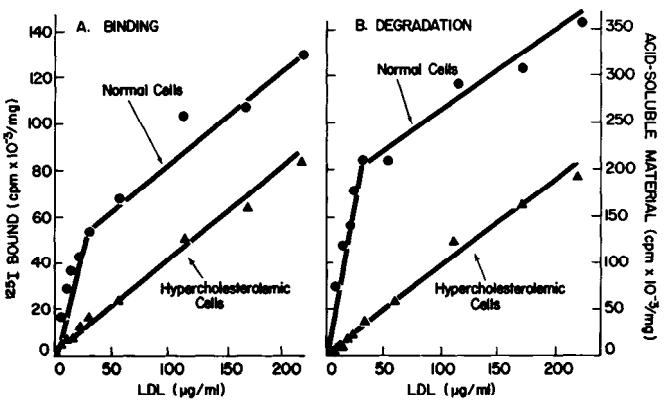

FIG. 3. Relation of the concentration of ^{125}I-LDL to the extent of binding and the rate of degradation in normal and hypercholesterolemic cells. Cell monolayers were prepared in dishes as described under "Experimental Procedure." At the beginning of the experiment, the medium was replaced with 2 ml of Medium A containing the indicated concentrations of ^{125}I-LDL (49 cpm per ng). After incubation at 37° for 6 hours, the medium from each dish was removed, its content of acid-soluble material was measured (*B*), and the amount of ^{125}I-LDL remaining bound to the cells was determined (*A*). ●, normal cells; ▲, hypercholesterolemic cells.

TABLE II

Ability of normal fibroblasts to bind ^{125}I-LDL after release of bound ^{125}I from cells

Cell monolayers were prepared in dishes as described under "Experimental Procedure." At the beginning of the experiment, the medium in 6 dishes was replaced with 2 ml of Medium A containing 25 μg per ml of ^{125}I-LDL (122 cpm per ng). After 3 hours at 37° (first incubation), all dishes were washed 6 times as described under "Experimental Procedure," and 2 dishes were harvested for determination of total ^{125}I radioactivity bound to the cells. The 4 remaining dishes were incubated at 37° for 2.5 hours with 2 ml of Medium A containing no LDL (second incubation). These 4 dishes were then washed 6 times and 2 dishes were harvested for determination of total ^{125}I radioactivity bound to the cells. The 2 remaining dishes were incubated at 37° for 3 hours with 2 ml of Medium A containing 25 μg per ml of ^{125}I-LDL (third incubation), after which they were washed 6 times and harvested for determination of total ^{125}I radioactivity bound to the cells. Each value in the table represents the mean of duplicate dishes.

Sequential incubation of cells	^{125}I-LDL concentration in medium	Time of incubation	^{125}I bound to cells
	μg/ml	*hr*	*cpm × 10^{-3}/mg protein*
First	25	3	129
Second	0	2.5	30
Third	25	3	176

strates that after release of bound LDL from the cell, the receptor sites were able to rebind the original amount of ^{125}I-LDL.

Fig. 3 compares the relation between the concentration of LDL and the extent of binding and the rate of degradation. In normal cells at concentrations of LDL below 25 μg per ml, the amount of binding rose sharply and linearly with increasing amounts of LDL; this component of the normal binding curve has been designated high affinity binding (Fig. 3*A*). At levels of LDL above 25 μg per ml, binding continued to increase but the slope of the line relating binding and LDL concentration was considerably less steep, suggesting that at these higher LDL levels a second process was taking place, designated low affinity binding. In cells from a subject with homozygous familial hypercholesterolemia, binding was linearly related to LDL concentration throughout the entire range. The slope of this line was identical with the second component of the normal binding curve (Fig. 3). These data are consistent with the previous conclusions derived from Scatchard plot analysis of ^{125}I-LDL binding (5) and suggest that the hypercholesterolemic cells lack the high affinity binding component. Remarkably similar curves were obtained when the rates of LDL degradation in normal and hypercholesterolemic cells were plotted as a function of LDL concentration (Fig. 3*B*). Thus, whereas normal cells appeared to have both low affinity and high affinity processes for degradation of LDL, the latter was deficient in the hypercholesterolemic cells. To date, we have examined cell lines from 5 unrelated subjects with the homozygous form of familial hypercholesterolemia and all 5 have shown a similar deficiency in high affinity binding and high affinity degradation.

To facilitate a comparison of the high affinity processes for binding and degradation in normal cells, the data were replotted using a "slope peeling" technique to subtract the contribution of the low affinity processes (Fig. 4). Both binding and degradation appeared to reach saturation at about 35 μg per ml. Double reciprocal plots of these data indicated that the apparent K_m values for high affinity binding and degradation were similar (about 10 μg per ml) (Fig. 4, *inset*).

Another method for evaluation of high affinity binding consists of determining the amount of radioactivity bound to the receptor in the absence and presence of an excess of nonradioactive material (14). Subtraction of the latter from the former gives the amount of displaceable or high affinity binding.[4] When this

[4] When the dilution in specific activity of the ^{125}I-LDL due to the addition of the excess native LDL is taken into account, it can be seen that the absolute amount of radioactive material bound and degraded by the cells in the presence of native LDL increased rather than decreased. However, since the purpose of these ex-

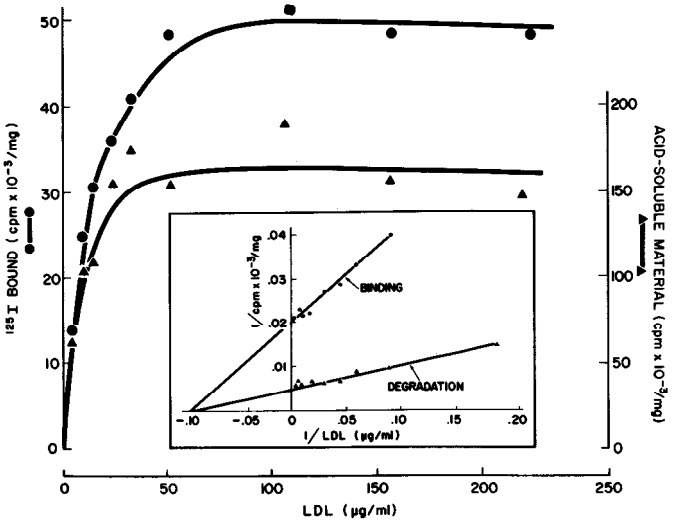

FIG. 4. Relation of high affinity binding and high affinity degradation of ^{125}I-LDL to the concentration of LDL in normal fibroblasts. At each LDL concentration the amounts of high affinity binding and high affinity degradation of ^{125}I-LDL were calculated from the experimental data in Fig. 3 by subtracting from the total binding or degradation the amounts contributed by the low affinity processes. The latter were derived by extrapolation from the slopes of the terminal linear portions of the appropriate concentration curves. For each microgram per ml of LDL, the calculated low affinity binding and low affinity degradation were 366 and 910 cpm per ng of cellular protein, respectively. The *inset* shows a double reciprocal plot of these high affinity data for binding and degradation. ●, high affinity binding; ▲, high affinity degradation.

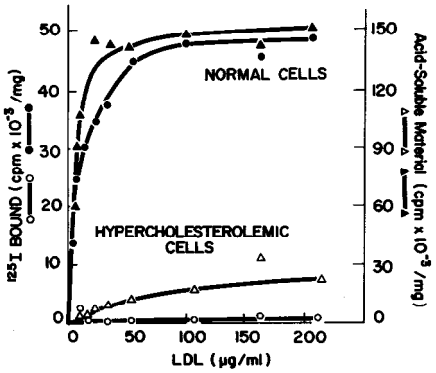

FIG. 5. High affinity binding and degradation of ^{125}I-LDL in normal and hypercholesterolemic fibroblasts. Cell monolayers were prepared in dishes as described under "Experimental Procedure." At the beginning of the experiment, the medium was replaced with 2 ml of Medium A containing the indicated concentrations of ^{125}I-LDL (49 cpm per ng) either in the absence or presence of 436 μg per ml of native LDL. After incubation at 37° for 6 hours, the medium from each dish was removed, its content of acid-soluble material was measured, and the amount of ^{125}I bound to the cells was determined. High affinity binding (●, ○) and degradation (▲, △) were calculated by subtracting the cpm bound or degraded in the presence of native LDL from that obtained in the absence of native LDL.[4] ▲, ●, normal cells; △, ○, hypercholesterolemic cells.

approach was applied to ^{125}I-LDL binding (Fig. 5), the normal cells showed saturation kinetics identical with that obtained by the "slope peeling" method used in Fig. 4. In contrast, the hypercholesterolemic cells had no high affinity binding (Fig. 5).

periments was to determine the amount of ^{125}I-LDL that could be specifically displaced by an excess of native LDL, no specific activity corrections were made.

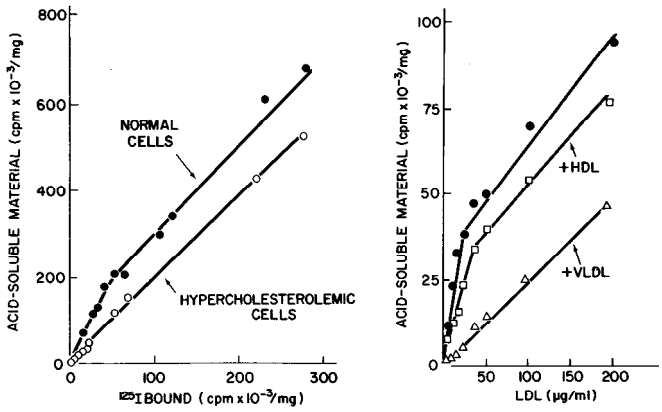

FIG. 6 (*left*). Relation of the extent of ^{125}I-LDL bound to the rate of degradation of ^{125}I-LDL in normal and hypercholesterolemic cells. Cell monolayers were prepared in dishes as described under "Experimental Procedure." At the beginning of the experiment, the medium was replaced with 2 ml of Medium A containing ^{125}I-LDL (49 cpm per ng) at concentrations ranging between 5.5 and 525 μg per ml. After incubation at 37° for 6 hours, the medium from each dish was removed, its content of acid-soluble material was measured, and the amount of ^{125}I bound to the cells was determined. ●, normal cells; ○, hypercholesterolemic cells.

FIG. 7 (*right*). Effect of VLDL and HDL on the rate of degradation of ^{125}I-LDL by normal cells. Cell monolayers were prepared in dishes as described under "Experimental Procedure." At the beginning of the experiment, the medium was replaced with 2 ml of Medium A containing the indicated concentrations of ^{125}I-LDL (21 cpm per ng) plus one of the following: △, VLDL, 200 μg per ml; □, HDL, 200 μg per ml; and ●, none. After incubation at 37° for 6 hours, the medium from each dish was removed for measurement of its content of acid-soluble material.

When this same method was applied to the degradation process, the normal cells again showed a saturation curve that was similar to the binding curve, while the hypercholesterolemic cells showed a markedly diminished amount of high affinity degradation.

The similarity in kinetics of binding and degradation of LDL in normal cells, together with the observation that high affinity binding and degradation are both deficient in the hypercholesterolemic cells, suggest that binding is rate-limiting for degradation of LDL by fibroblasts. Further support for this hypothesis is provided by the data in Fig. 6, which show that the rate of degradation in both normal and hypercholesterolemic cells is directly proportional to the amount of LDL bound. In normal cells the efficiency of degradation appeared to be somewhat greater for LDL bound at the high affinity site as compared to that bound at the low affinity site. This is reflected by the break in the normal curve, which represents the point at which the high affinity binding sites become saturated with LDL.

If high affinity binding of LDL is a prerequisite for high affinity degradation, then VLDL, which displaces LDL from its high affinity binding site (5), should also compete for degradation. On the other hand, HDL, which is much less effective in competing for binding (5), should also be less effective in inhibiting degradation. The experimental results described in Fig. 7 are consistent with these predictions. The data also show that the low affinity degradative process was not inhibited by any of the lipoproteins.

High affinity binding and degradation of LDL in normal cells were both strictly dependent on the calcium concentration in the incubation medium (Fig. 8). When ^{125}I-LDL was incubated with cells in isotonic buffer containing only Tris and NaCl, the addition of increasing amounts of calcium resulted in a 3-fold

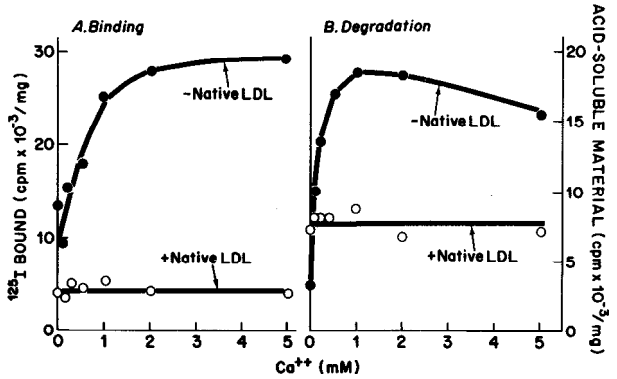

FIG. 8. Effect of Ca^{2+} on amount of ^{125}I-LDL bound and rate of degradation of ^{125}I-LDL in normal fibroblasts. Cell monolayers were prepared in dishes as described under "Experimental Procedure." At the beginning of the experiment, the medium was replaced with 2 ml of buffer containing 50 mM Tris-Cl (pH 7.5), 0.1 M NaCl, the indicated concentrations of $CaCl_2$, and 5 μg per ml of ^{125}I-LDL (120 cpm per ng) either in the absence or presence of 200 μg per ml of native LDL. After incubation at 37° for 4 hours, the buffer from each dish was removed, its content of acid-soluble material was measured, and the amount of ^{125}I bound to the cells was determined. High affinity binding and degradation (●) were calculated by subtracting the counts per min bound or degraded in the presence of native LDL (○) from that obtained in the absence of native LDL.

rise in binding with half-maximal effect achieved at 0.5 mM (Fig. 8*A*). In the same experiment, the addition of calcium stimulated degradation 6-fold with a half-maximal effect achieved at 0.2 mM (Fig. 8*B*). The data thus suggest that calcium may be acting at two different sites—one required for binding and the other required for degradation. At high levels of LDL, calcium had no effect on either binding or degradation (Fig. 8), suggesting that this ion is not required for the low affinity process. In identical experiments, magnesium at 2 mM was unable to replace calcium.

To determine whether modification of the cell surface could affect high affinity binding and degradation, normal and hypercholesterolemic cells were treated with pronase under conditions that are known to alter cell surface receptors without inhibiting cell growth (15). In normal cells, pronase treatment caused a 66% reduction in high affinity binding and a 55% reduction in the rate of LDL degradation (Table III). Since neither binding nor degradation was significantly reduced in the hypercholesterolemic cells (Table III), the data suggest that pronase was acting mainly on high affinity sites. This hypothesis was confirmed by the experiment shown in Fig. 9, in which pronase treatment of normal cells caused a reduction only in high affinity binding and had no effect on the slope of the low affinity process.

If, as suggested by our previous data, suppression of HMG-CoA reductase activity is mediated by binding of LDL to the high affinity sites (5), destruction of these sites with pronase should reduce the ability of normal cells to respond to LDL. Fig. 10 shows that after pronase treatment the normal cells did become resistant to LDL-mediated suppression of HMG-CoA reductase activity. On the other hand, the hypercholesterolemic cells, which lack the high affinity binding site and thus are genetically resistant to LDL-mediated suppression (5), showed no change in their LDL responsiveness.[5]

[5] An additional observation in these studies was that pronase itself reduced the HMG-CoA reductase activity of both the normal and the hypercholesterolemic cells. Since insulin is known to stimulate HMG-CoA reductase activity in both normal and hypercholesterolemic fibroblasts (4) and since the insulin receptor is destroyed by proteolytic enzymes (16), it is possible that the reduction in enzyme activity was due to degradation of the insulin receptor.

TABLE III

Effect of pronase treatment on ability of normal and hypercholesterolemic cells to bind and degrade ^{125}I-LDL

Cell monolayers were prepared in dishes as described under "Experimental Procedure." At the beginning of the experiment, the medium was replaced with 2 ml of Medium A from which the lipoprotein-deficient serum was omitted and which contained either no pronase or 6 μg of pronase (added as 3 μl of a 2 mg per ml solution). After incubation at 37° for 45 min without shaking, the medium was removed, the monolayers were washed once with 2 ml of Dulbecco's phosphate-buffered saline, and 2 ml of fresh Medium A containing 10 μg per ml of ^{125}I-LDL (160 cpm per ng) were added. After incubation at 37° for 6 hours, the medium was removed, its content of acid-soluble material was measured, and the amount of ^{125}I bound to cells was determined. Each value represents the mean of duplicate determinations. Subject A and Subject B are two different homozygotes with familial hypercholesterolemia.

Cell type	^{125}I bound to cells		Acid-soluble material in medium	
	−Pronase	+Pronase	−Pronase	+Pronase
	cpm × 10⁻³/mg protein		*cpm × 10⁻³/mg protein*	
Normal.....	51	17.5	122	55
Homozygote				
Subject A...	9.5	6.9	33	34
Subject B...	11.6	16.4	4.4	9.0

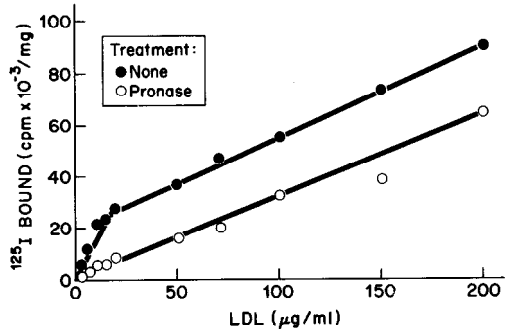

FIG. 9. Effect of pronase treatment on the ability of normal cells to bind ^{125}I-LDL. Cell monolayers were prepared in dishes as described under "Experimental Procedure." At the beginning of the experiment, the medium was replaced with 2 ml of Medium A from which the lipoprotein-deficient serum was omitted and which contained either no pronase (●) or 6 μg of pronase (added as 3 μl of a 2 mg per ml solution) (○). After incubation at 37° for 45 min without shaking, the medium was removed, the monolayers were washed once with 2 ml of Dulbecco's phosphate-buffered saline (0.9% NaCl), and 2 ml of fresh Medium A containing the indicated concentration of ^{125}I-LDL (77 cpm per ng) were added. After incubation at 37° for 3 hrs, the medium was removed and the amount of ^{125}I bound to the cells was determined.

The extent of high affinity binding and the rate of degradation of ^{125}I-LDL in normal fibroblasts were both significantly higher in actively dividing cells as compared with cells in the stationary phase of growth (Table IV). This finding correlates with the previous observation that when LDL is removed from the medium the relative rise in HMG-CoA reductase activity is greater in growing cells than in confluent cells (4). It was also observed that the extent of binding and the rate of degradation of ^{125}I-LDL were both about 2-fold greater in cells studied after the 14th

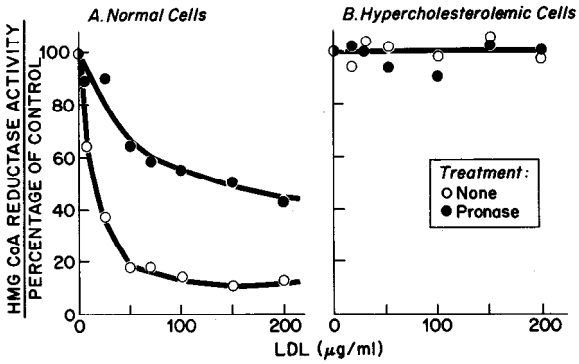

FIG. 10. Effect of pronase treatment on ability of normal and hypercholesterolemic cells to respond to LDL by suppression of HMG-CoA reductase activity. Cell monolayers were prepared in dishes as described under "Experimental Procedure." At the beginning of the experiment, the medium was replaced with 2 ml of Medium A from which the lipoprotein-deficient serum was omitted and which contained either no pronase (○) or 6 μg of pronase (added as 3 μl of a 2 mg per ml solution) (●). After incubation at 37° for 45 min without shaking, the medium was removed, the monolayers were washed once with 2 ml of Dulbecco's phosphate-buffered saline (0.9% NaCl), and 2 ml of fresh Medium A containing the indicated concentration of native LDL were added. After incubation at 37° for 5 hours in a humidified CO_2 incubator, the medium was removed and the cells were harvested for measurement of HMG-CoA reductase activity. Results are expressed as a percentage of the activity in the cells to which no LDL was added. These control activities (picomoles per min per mg of protein) were: *A:* ○, 57.2; ●, 18.2 and *B:* ○, 165; ●, 100.

TABLE IV

Effect of cell density on ^{125}I-LDL binding and degradation in normal fibroblasts

Cell monolayers were prepared in dishes as described under "Experimental Procedure" except that the addition of lipoprotein-deficient serum was made either on Day 1 (Group A) or on Day 6 (Group B). Twenty-four hours after the addition of lipoprotein-deficient serum, the medium was replaced with 2 ml of Medium A containing 5 μg per ml of ^{125}I-LDL (420 cpm per ng) either in the presence or absence of 200 μg per ml of native LDL. After incubation at 37° for 4.5 hours, the medium from each dish was removed, its content of acid-soluble material was measured and the amount of ^{125}I bound to the cells was determined. High affinity binding and degradation were calculated by subtracting the cpm bound or degraded in the presence of native LDL from that obtained in the absence of native LDL. Each value represents the mean of duplicate dishes.

Group	Days after transfer	Total cell protein	^{125}I bound to cells		Acid-soluble material in medium	
			Total	High affinity	Total	High affinity
		μg/dish	*cpm × 10⁻³/mg protein*		*cpm × 10⁻³/mg protein*	
A	2	124	232	180	680	570
B	7	436	50.4	37.4	159	119

transfer as compared with those studied after the 36th transfer (data not shown).

To characterize the low affinity uptake of LDL in more detail and to determine whether it could be attributed to endocytosis of macromolecules as is seen with albumin (17), uptake of ^{125}I-LDL and ^{125}I-albumin were compared. If both proteins were taken up by the nonspecific endocytosis of droplets of medium, then the volume of medium cleared by the uptake process should be the same for both proteins and should be independent of the

TABLE V

Volume of medium cleared of ^{125}I-labeled protein during uptake process

Cell monolayers were prepared in dishes as described under "Experimental Procedure." At the beginning of the experiment, the medium was replaced with 2 ml of either Eagle's minimum essential medium containing the indicated concentration of ^{125}I-labeled bovine serum albumin (714 cpm per ng at 10 μg per ml; 30 cpm per ng at 250 μg per ml) or 2 ml of Medium A containing ^{125}I-LDL (430 cpm per ng at 10 μg per ml; 17 cpm per ng at 250 μg per ml). After 3.5 hours at 37°, the cell monolayers were washed 6 times as described under "Experimental Procedure" and the amount of ^{125}I bound to the cell was determined. To calculate the volume of medium cleared during the uptake process, the ^{125}I bound (cpm per mg cell protein) was divided by the concentration of ^{125}I in the medium (cpm per μl). Each value represents the mean of duplicate determinations.

Cell type	^{125}I-labeled protein	Volume of medium cleared at	
		10 μg protein per ml	250 μg protein per ml
		μl/mg cell protein	
Normal.....	Bovine serum albumin	4.6	4.1
Normal.....	LDL	59.0	9.4
Homozygote.....	LDL	4.4	6.9

protein concentration. Table V demonstrates that at high protein concentrations the clearance volume was similar for both albumin and LDL in the normal and hypercholesterolemic cells. At low protein concentrations, the clearance volumes for albumin in the normal cells and LDL in the hypercholesterolemic cells remained equal to each other and unchanged from their respective values at the high protein concentrations. These data suggest that the low affinity uptake process observed in the normal cells at high LDL levels and in the hypercholesterolemic cells at all LDL levels was due to endocytosis. The data in Table V also demonstrate that at low LDL concentrations in the normal cells, the clearance volume was about 10-fold greater than that which could be attributed to endocytosis, confirming that this high affinity uptake process was the result of a different mechanism.

To study further the acid-soluble material formed as a result of the high affinity degradation of ^{125}I-LDL by normal fibroblasts, the acid-soluble material produced after incubation of the cells with ^{125}I-LDL at 5 μg per ml was fractionated by Sephadex G-10 chromatography (Fig. 11). Nearly all of the acid-soluble material formed under these conditions was found in the inclusion volume of the column, confirming the earlier observation that most of the acid-soluble material was of a small enough molecular size to be dialyzable (5). Five discrete peaks were identified (Fig. 11*C*). The formation of the two major peaks (*II* and *V*) was dependent on the presence of cells and was approximately linear with time. On the other hand, the three minor peaks (*I*, *III*, and *IV*) were present in the ^{125}I-LDL preparation incubated without cells (Fig. 11*A*).

Peak *V*, which represented over 60% of the acid-soluble material, was eluted from the G-10 Sephadex column near the salt peak (Fig. 11*C*). Thin layer chromatography of this material in two different solvent systems showed that it had the same R_F value as 3-iodo-L-tyrosine (Fig. 12). Similar results were also obtained after ascending paper chromatography in collidine-NH_4OH-water. Although a precise characterization of Peaks *I* to *IV* has not been made, they would appear to represent small

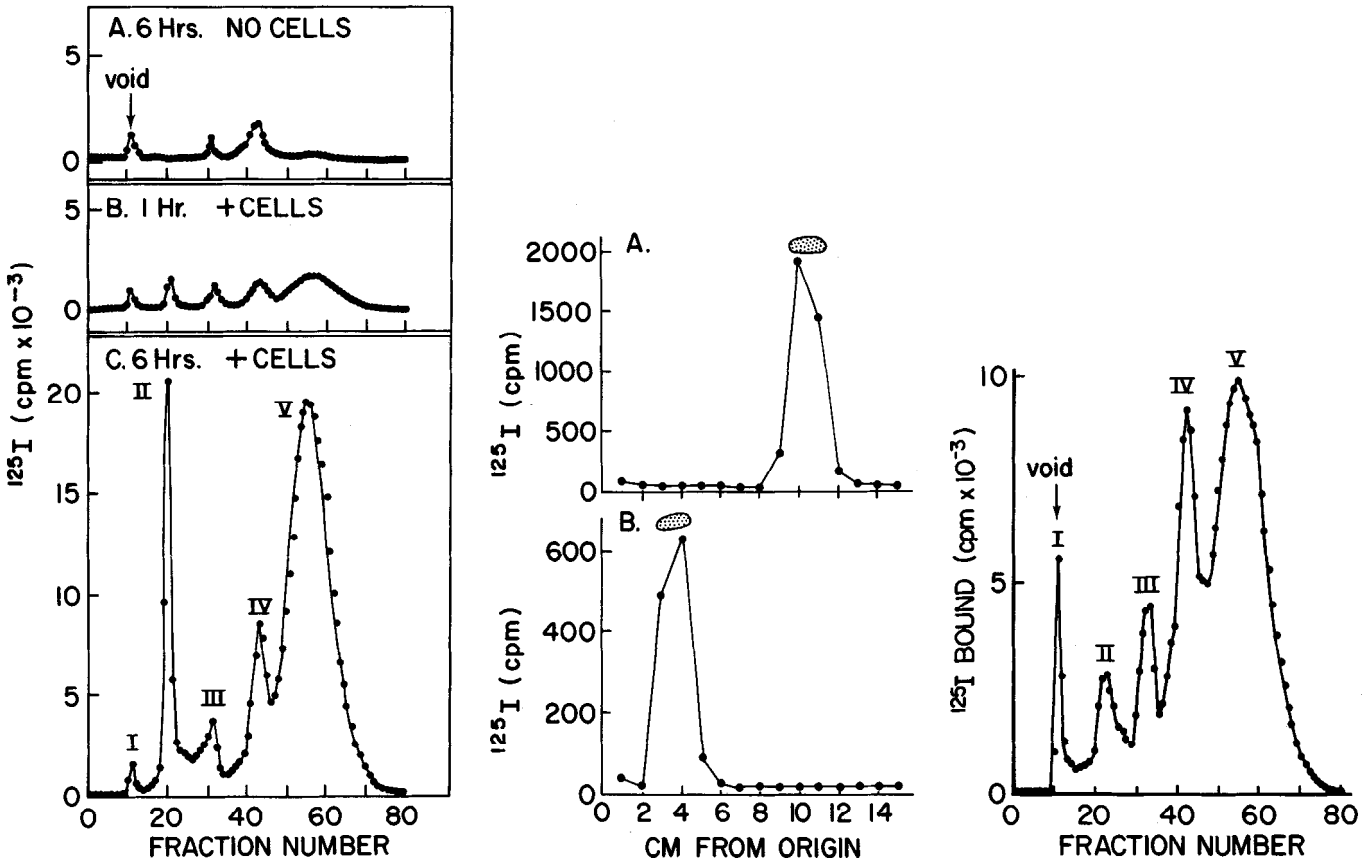

FIG. 11 (*left*). Sephadex G-10 chromatography of the ^{125}I-labeled acid-soluble material formed by the normal cells. Cell monolayers were prepared in dishes as described under "Experimental Procedure." At the beginning of the experiment, the medium in each dish was replaced with 2 ml of Medium A containing 5 μg per ml of ^{125}I-LDL (600 cpm per ng). One group of 10 dishes was incubated for 1 hour at 37° (*B*) and another group of 10 dishes was incubated for 6 hours at 37° (*C*). A control group of 10 dishes containing no cells was incubated for 6 hours at 37° under identical conditions (*A*). After incubations, the medium from each group of 10 dishes was pooled, lyophilized, and resuspended in 1.5 ml of distilled water. Each solution (containing approximately 49 × 10^6 cpm of ^{125}I) was then adjusted to 10% (w/v) trichloroacetic acid, incubated at 4° overnight, and the precipitate was removed by centrifugation. To each supernatant (2 ml) were added 20 μl of 40% KI (w/v) and 80 μl of 30% H_2O_2. After incubation at 24° for 5 min, each mixture was extracted with 4 ml of $CHCl_3$, after which the acid-soluble phase (1.5 ml) was removed for chromatography. The acid-soluble material (750 μl) from *A* (containing 15,749 cpm), *B* (containing 49,798 cpm), and *C* (containing 389,989 cpm) were applied sequentially to a Sephadex G-10 column (28 × 1 cm) equilibrated with 50 mM Tris-Cl, pH 7.5. The column was eluted with the same buffer at a flow rate of 10 ml per hour, and 0.7-ml fractions were collected and counted for radioactivity. The void volume of the column was determined using dextran blue.

FIG. 12 (*center*). Thin layer chromatography of Fraction V from Sephadex G-10 column. ^{125}I-labeled acid-soluble material was isolated from the medium of normal cells and chromatographed on a G-10 Sephadex column as described in the legend of Fig. 11 except that the column was equilibrated and eluted with distilled water. The 4 fractions of Peak *V* containing the largest amount of ^{125}I radioactivity were pooled, lyophilized, and resuspended in 0.2 ml of distilled water. Sixty microliters of authentic 3-iodo-L-tyrosine (6 mg per ml in 0.1 N HCl) were added and aliquots of the resulting mixture were applied to thin layer sheets as follows: *A*, cellulose sheets developed with 1-butanol-acetic acid-water (4:1:1); *B*, silica gel sheets developed with $CHCl_3$-methanol-NH_4OH (20:10:1). After the solvent had migrated 15 cm, the sheets were dried at 24°, sprayed with ninhydrin, and developed at 90° for 2 min to localize the 3-iodo-L-tyrosine spot. The sheets were then cut into 1-cm strips, which were placed directly into tubes for ^{125}I counting. The region containing authentic 3-iodo-L-tyrosine is indicated by the *shaded spot*.

FIG. 13 (*right*). Sephadex G-10 chromatography of the ^{125}I-labeled acid-soluble material formed by the hypercholesterolemic cells. One-cell monolayer was prepared in a Petri dish as described under "Experimental Procedure." At the beginning of the experiment, the medium in the dish was replaced with 2 ml of Medium A containing 240 μg per ml of ^{125}I-LDL (600 cpm per ng). After incubation for 6 hours at 37°, the medium was removed and adjusted to 10% trichloroacetic acid (w/v). After removal of the precipitate by centrifugation, the supernatant (2 ml) was treated with KI, H_2O_2, and chloroform as described in the legend of Fig. 11. The acid-soluble material (750 μl) (containing 192,450 cpm) was applied to the same column as that used in the experiments of Fig. 11 and eluted in an identical manner. The void volume of the column was determined using dextran blue.

peptides formed during the course of degradation of LDL to its constituent amino acids.

When the acid-soluble material formed by the hypercholesterolemic cells was fractionated on the same G-10 Sephadex column, 5 peaks were also observed (Fig. 13). Although the elution volume of each of these peaks could be correlated with the 5 peaks from the normal cells, the relative distribution of the radioactivity among the peaks was somewhat different. This may reflect a subtle difference in the products formed by the high affinity degradative process of the normal cells and the low affinity process of the hypercholesterolemic cells.

DISCUSSION

The studies presented in this paper demonstrate that the cultured human fibroblasts from normal subjects are able to bind and degrade LDL. LDL can be bound to the cell by two processes—one of high affinity and one of low affinity. That the high affinity process involves binding of LDL to specific receptor

sites on the cell surface is suggested by the following observations. First, the high affinity binding of LDL is saturable, shows competition with related molecules (such as VLDL), and the amount bound reaches a plateau with time. Second, although its extent is greater at 37°, high affinity LDL binding can also be demonstrated at 4° (5). Third, immunologically intact LDL can be dissociated from the cell at 4°. Fourth, treatments that act largely on the surface of the cell, such as limited pronase digestion (15) and addition of calcium to the medium, profoundly affect the high affinity process.

In contrast to the high affinity binding process that is observed at the low LDL levels, a different type of association predominates when LDL is present in the medium at levels above 25 μg per ml. This low affinity process is characterized by an apparent lack of saturability and competition and a resistance to treatment with either pronase or calcium. In addition, this low affinity process appears to be normal in the hypercholesterolemic cells despite the absence of high affinity receptor sites. Because this low affinity process lacks many of the above mentioned characteristics of surface binding, we believe that it may represent a nonspecific endocytosis of the LDL in solution in the culture medium (17).

Both the high and low affinity uptake processes for LDL appear to provide substrate for the proteolytic enzymes that degrade this lipoprotein. Evidence that high affinity binding is obligatory for high affinity degradation is provided by the following experiments. First, the LDL concentration curves for the extent of high affinity binding and the rate of high affinity degradation are identical. Second, VLDL, which competes with LDL for high affinity binding (5), also competes for high affinity degradation, whereas HDL, which does not effectively compete for high affinity binding (5), does not effectively compete for high affinity degradation. Third, both high affinity binding and degradation are stimulated by the presence of calcium in the medium, while neither process is affected by magnesium. Fourth, treatment of cells with pronase abolishes both high affinity binding and high affinity degradation. Fifth, in cells from subjects with homozygous familial hypercholesterolemia, the absence of high affinity binding is associated with the absence of high affinity degradation. Finally, in experiments soon to be published we have observed that in cells from 6 obligate heterozygotes with familial hypercholesterolemia (*i.e.* the parents of homozygotes), both the extent of high affinity binding and the maximal rate of high affinity degradation of LDL are reduced to values intermediate between those of 13 normal controls and 5 homozygotes (18).

Uptake of LDL by the low affinity process also results in degradation of the lipoprotein as indicated by (*a*) the experiments showing identical substrate concentration curves for binding and degradation at high levels of LDL and (*b*) the observation that the hypercholesterolemic cells have normal low affinity processes for both binding and degradation.

The degradative process in cultured cells appears to be similar regardless of whether LDL is bound to specific high affinity surface membrane receptors or whether it is nonspecifically taken up by the low affinity process. One hypothesis to account for these findings is that when LDL is associated with the cell by either process it becomes incorporated into endocytotic vesicles, where the proteolysis occurs. The LDL bound at high affinity surface receptor sites would be incorporated into such vesicles if the wall of the vesicle were formed by invagination of the portion of the plasma membrane containing the bound LDL. Low affinity incorporation of LDL into the same vesicle would result from nonspecific trapping of the lipoprotein contained in the medium that forms its interior. Once formed, the vesicles containing LDL incorporated by both mechanisms would then fuse with lysosomes containing proteolytic enzymes. Since it is generally believed that macromolecules ingested by endocytosis are degraded by such lysosomal enzymes (19), this mechanism would explain the nearly complete proteolytic degradation of LDL bound by either the high affinity or the low affinity processes in normal cells. Moreover, it would account for the observation that the hypercholesterolemic cells, which lack high affinity binding, can nevertheless degrade LDL by the low affinity process. Also consistent with this interpretation is the finding that the products of the low affinity degradation in the hypercholesterolemic cells appear to be qualitatively the same as those formed by the high affinity process in the normal cells.

Binding of LDL to the high affinity site, in addition to its role in facilitating LDL degradation, is also necessary for suppression of HMG-CoA reductase activity in normal cultured fibroblasts (5). Hence, selective destruction of the high affinity binding site with pronase reduces the ability of normal cells both to degrade LDL and to suppress HMG-CoA reductase activity when LDL is added to the medium, thus producing a phenotype similar to that of the hypercholesterolemic cells. The simplest hypothesis to explain the mechanism by which high affinity binding of LDL leads to suppression of HMG-CoA reductase is that the resultant degradation of LDL releases cholesterol and that cholesterol in turn suppresses enzyme activity. Indeed, previous data have shown that cholesterol administered to cells in a nonlipoprotein form is able to suppress enzyme activity in both the normal and hypercholesterolemic cells (4). However, this hypothesis is not sufficient to account for all the data since incubation of hypercholesterolemic cells with high concentrations of LDL leads to degradation of large amounts of lipoprotein by the low affinity process, yet HMG-CoA reductase activity is not suppressed. Thus, elucidation of the mechanism by which the LDL receptor regulates HMG-CoA reductase activity requires further study.

In summary, from the data presented in this and earlier papers (2–5), we believe that the primary genetic abnormality in familial hypercholesterolemia resides in a gene whose product is necessary for the production of a high affinity cell surface receptor for LDL. A defect in this LDL receptor results in deficient high affinity binding of LDL, and this appears to have at least two important consequences: (*a*) the cell cannot normally suppress HMG-CoA reductase activity in the presence of LDL and hence it overproduces cholesterol, and (*b*) the cell cannot degrade LDL when it is present extracellularly in low concentrations. Assuming that the familial hypercholesterolemia gene is expressed *in vivo* as it is *in vitro*, both of these secondary abnormalities may be important in producing and maintaining the high levels of serum LDL that are found in patients with this disorder (20).

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