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Degradation of cationized low density lipoprotein and regulation of cholesterol metabolism in homozygous familial hypercholesterolemia fibroblasts

(cell surface receptors/adsorptive endocytosis/lysosomes/3-hydroxy-3-methylglutaryl CoA reductase)

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ABSTRACT **Cultured fibroblasts derived from patients with homozygous familial hypercholesterolemia, which lack functional low density lipoprotein (LDL) receptors, fail to bind, take up, or degrade the lipoprotein with high affinity; therefore LDL-cholesterol is not made available for suppression of cholesterol synthesis or activation of cholesteryl ester formation. When LDL was given a positive charge by reaction with *N,N*-dimethyl-1,3-propanediamine (cationized LDL), the rate of degradation of the lipoprotein was increased by more than 100-fold in the homozygous familial hypercholesterolemia fibroblasts. Degradation of cationized LDL was inhibited by chloroquine, suggesting that it occurred in cellular lysosomes. Although the cationized LDL entered the cell through a mechanism independent of the LDL receptor, the cholesterol liberated from the degradation of the lipoprotein became available for suppression of cholesterol synthesis and stimulation of cholesteryl ester formation in the homozygous familial hypercholesterolemia fibroblasts. The rate of degradation of albumin by fibroblasts was also increased by more than 100-fold when this protein was coupled to *N,N*-dimethyl-1,3-propanediamine. The ability to deliver a protein to lysosomes by giving it a strong positive charge may have potential relevance not only to familial hypercholesterolemia, but also to inborn errors of metabolism that involve deficiencies in lysosomal enzymes.**

Normal human fibroblasts acquire cholesterol through the adsorptive endocytosis of plasma low density lipoprotein (LDL) (reviewed in refs. 1 and 2). The initial step in this uptake process is the high affinity binding of LDL to a specific cell surface receptor (3–6). The internalized LDL is delivered to lysosomes, where its protein and cholesteryl ester components are hydrolyzed (4, 7, 8). The free cholesterol that is generated enters the nonlysosomal cellular compartment where it is used by the cells for membrane synthesis and where it exerts three regulatory actions: (*i*) it suppresses 3-hydroxy-3-methylglutaryl coenzyme A reductase [EC 1.1.1.34; mevalonate:NADP$^+$ oxidoreductase (CoA-acylating)] (HMG CoA reductase), reducing cholesterol synthesis; (*ii*) it activates an acyl-CoA:cholesterol acyltransferase (EC 2.3.1.26) that catalyzes the reesterification of the LDL-derived cholesterol; and (*iii*) it suppresses the synthesis of LDL receptor molecules themselves, thus limiting LDL uptake and preventing an overaccumulation of cholesterol by the cell (reviewed in refs. 1 and 2).

In fibroblasts from patients with the homozygous form of familial hypercholesterolemia (FH), the LDL receptor is nonfunctional (3–6), LDL is not bound with high affinity, and the lipoprotein's cholesterol is not available either to be used by the cell or to regulate cholesterol metabolism (reviewed in refs. 1, 2, and 9). The FH homozygote cells can regulate their cholesterol synthesis and cholesteryl ester formation in a normal manner, however, if cholesterol is added to the culture medium in an ethanol solution, a form in which it can cross the cell membrane without a requirement for the LDL receptor (10).

The latter data raised the possibility that LDL itself might be able to regulate cholesterol metabolism in the FH homozygote cells if a way could be found to enhance the cellular uptake of LDL and its subsequent delivery to lysosomes through a mechanism that did not require the LDL receptor. For this purpose, we have taken advantage of the observation (11) that ferritin that had been rendered cationic by reaction with *N,N*-dimethyl-1,3-propanediamine bound to widespread sites on the negatively charged plasma membranes of various cell types (11, 12). Accordingly, we prepared cationized LDL by coupling the lipoprotein to *N,N*-dimethyl-1,3-propanediamine, which converts free carboxyl groups of the protein into positively charged tertiary amine derivatives (11). As a control, we prepared acetylated LDL by reacting the free amino groups of the lipoprotein with acetic anhydride, which increased the net negative charge of the lipoprotein particle. The present results show that the cationized LDL preparation, but not the acetylated LDL, acquires the ability to be taken up and degraded by the FH homozygote cells and that cationized LDL effectively regulates cholesterol metabolism in these cells.

METHODS

Cells. Cultured fibroblasts were derived from skin biopsies obtained from a normal subject (D.S.) and a patient (M.C.) with the receptor-negative form of homozygous FH (13). Cells were grown in monolayer as described (13). All experiments were done in a similar format: confluent monolayers of cells from stock flasks were dissociated with 0.05% trypsin/0.02% EDTA solution and were seeded (day 0) at a concentration of 1×10^5 cells per dish into 60 × 15 mm petri dishes containing 3 ml of growth medium with 10% fetal calf serum (13). On day 3 the medium was replaced with 3 ml of fresh growth medium containing 10% fetal calf serum. On day 5, each monolayer was washed with 3 ml of phosphate-buffered saline, after which 2 ml of fresh medium containing 5% (vol/vol) human lipoprotein-deficient serum (LPDS) was added)final protein concentration, 2.5 mg/ml). All experiments were initiated on day 7 after the cells had been incubated for 48 hr in the presence of LPDS.

Lipoproteins. LDL (density 1.019–1.063 g/ml) and LPDS (density > 1.215 g/ml) were obtained from human plasma and prepared by differential ultracentrifugation (14). In the LDL used in the present studies, the ratio of total cholesterol to protein content on a weight basis was 1.6:1, and 70% of the total cholesterol was esterified. Lipoprotein electrophoresis was

Abbreviations: FH, familial hypercholesterolemia; LDL, low density lipoprotein; acetyl-LDL, LDL modified by acetylation with acetic anhydride; cationized LDL, LDL modified by coupling to *N,N*-dimethyl-1,3-propanediamine; HMG CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase; LPDS, lipoprotein-deficient serum.

Table 1. Hydrolysis of modified LDL preparations labeled with [^{3}H]cholesteryl linoleate

Addition to medium	[^{3}H]Cholesterol formed (pmol/mg of protein)					
	Normal cells			FH homozygote cells		
	(−) Chloroquine (a)	(+) Chloroquine (b)	(a − b)	(−) Chloroquine (a)	(+) Chloroquine (b)	(a − b)
Native LDL	510	22	488	52	8	44
Acetyl-LDL	110	16	94	70	14	56
Cationized LDL	970	170	800	870	360	510

On day 7 the medium was replaced with 2 ml of growth medium containing 2% LPDS, either no chloroquine or 75 μM chloroquine as indicated, and 5 μg of protein per ml of preparations of LDL that had been labeled with [^{3}H]cholesteryl linoleate. After incubation for 6 hr at 37°, the cellular content of free [^{3}H]cholesterol was determined. Each value represents the average of duplicate incubations.

performed in barbital buffer at pH 8.6 in agarose gel (15).

Chemical Modification of LDL. LDL was acetylated (acetyl-LDL) by the method of Fraenkel-Conrat (16). In a typical preparation, 1 ml of 0.15 M NaCl containing 16 mg of LDL protein was added to 1 ml of a saturated solution of sodium acetate with continuous stirring in an ice-water bath. Next, acetic anhydride was added in multiple small aliquots (2 μl) over a period of 1 hr with continuous stirring. After the addition of a total mass of acetic anhydride equal to 1.5 times the mass of protein used, the mixture was stirred for an additional 30 min without further additions. The reaction solution was then dialyzed for 24 hr at 4° against 12 liters of buffer containing 0.15 M NaCl and 0.3 mM EDTA, pH, 7.4. LDL was cationized at room temperature as described (11). In a typical preparation, 1 ml of 0.15 M NaCl containing 16 mg of LDL protein was added to 1 ml of 2 M *N,N*-dimethyl-1,3-propanediamine (Eastman) adjusted to pH 6.5 with HCl. Next, 100 mg of 1-ethyl-3(3-dimethylaminopropyl)carbodiimide·HCl (Pierce) was added, and the pH was maintained at 6.5 by additions of 0.2 M HCl with continuous stirring until the reaction had gone to completion, as indicated by no measurable change in pH (about 2–3 hr). The number of moles of the dimethylpropanediamine present in the reaction mixture was more than 10-fold greater than the total number of moles of glutamic acid and aspartic acid in the LDL preparation (17). The reaction mixture was left stoppered overnight at 4° and then dialyzed for 24 hr at 4° against 12 liters of buffer containing 0.15 M NaCl and 0.3 mM EDTA, pH 7.4. All of the modified LDL preparations formed optically clear solutions in 0.15 M NaCl at concentrations up to 16 mg of protein per ml.

Labeling of LDL Preparations with ^{125}I and [^{3}H]Cholesteryl Linoleate. Native LDL, acetyl-LDL, and cationized LDL were labeled with ^{125}I as described (3), yielding specific activities of 260, 550, and 640 cpm/ng of protein, respectively. For each preparation, > 98% of the ^{125}I radioactivity was precipitable with 10% trichloroacetic acid and < 2% of the ^{125}I radioactivity was extractable into chloroform:methanol (2:1). Native LDL, acetyl-LDL, and cationized LDL were labeled with [^{3}H]cholesteryl linoleate as previously described (8), yielding specific activities of 61×10^3, 24×10^3, and 38×10^3 cpm/nmol of total cholesteryl linoleate, respectively.

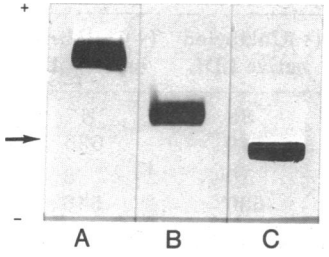

FIG. 1. Electrophoretic mobility of modified LDL preparations. Forty micrograms of protein of each LDL preparation were subjected to electrophoresis in agarose at pH 8.6, after which the gel was stained with Fat Red 7B. The arrow indicates the point of application of the sample. (A) Acetyl-LDL; (B) native LDL; (C) cationized LDL.

Chemical Modification and Labeling of Albumin. Crystallized bovine albumin (Sigma, Cat. no. A4378) was labeled with ^{125}I as described for LDL (3). Albumin and ^{125}I-albumin were cationized as described above.

Assays. Measurements of the cell surface binding (5) and total cellular content of ^{125}I-LDL (4), proteolytic degradation of ^{125}I-LDL (4), hydrolysis of [^{3}H]cholesteryl linoleate-labeled LDL (8), incorporation of [^{14}C]oleate into cellular cholesteryl [^{14}C]oleate (18), and HMG CoA reductase activity (14) were done exactly as described in the reference articles. The content of protein was determined by a modification of the method of Lowry *et al.* (19), with bovine serum albumin as a standard.

RESULTS

Electrophoresis of the modified LDL preparations in agarose gel at pH 8.6 indicated that the acetyl-LDL had an enhanced negative charge as compared with native LDL, whereas the cationized LDL migrated slowly toward the cathode (Fig. 1). On Ouchterlony immunodiffusion plates, the acetyl-LDL was precipitated by an antibody to LDL, but the cationized LDL showed no precipitin band. The size and configuration of the cationized LDL and acetyl-LDL preparations were similar to that of native LDL, as revealed by electron microscopy after negative staining of the lipoproteins with uranyl acetate.* Moreover, cationized LDL and native LDL had identical elution profiles when subjected to chromatography in 1 M NaCl on 4% agarose gel.* A detailed description of the chemical and physical properties of the modified LDL preparations will be reported elsewhere.*

As previously demonstrated (4), FH homozygote fibroblasts had a markedly reduced ability to degrade ^{125}I-LDL to trichloroacetic acid-soluble material when compared with normal fibroblasts (Fig. 2A). This failure of degradation by the mutant cells is due to a lack of cellular uptake of the lipoprotein, which results from the absence of LDL receptors (3–6). Whereas the FH homozygote cells degraded less than 30 ng/mg of ^{125}I-LDL in 18 hr, they degraded 3000 ng/mg of cationized ^{125}I-LDL in the same interval, an amount that was similar to the amount of cationized ^{125}I-LDL degraded in the normal cells (Fig. 2C). Degradation of both ^{125}I-LDL and cationized ^{125}I-LDL was

* S. K. Basu, R. G. W. Anderson, M. S. Brown and J. L. Goldstein, manuscript in preparation.

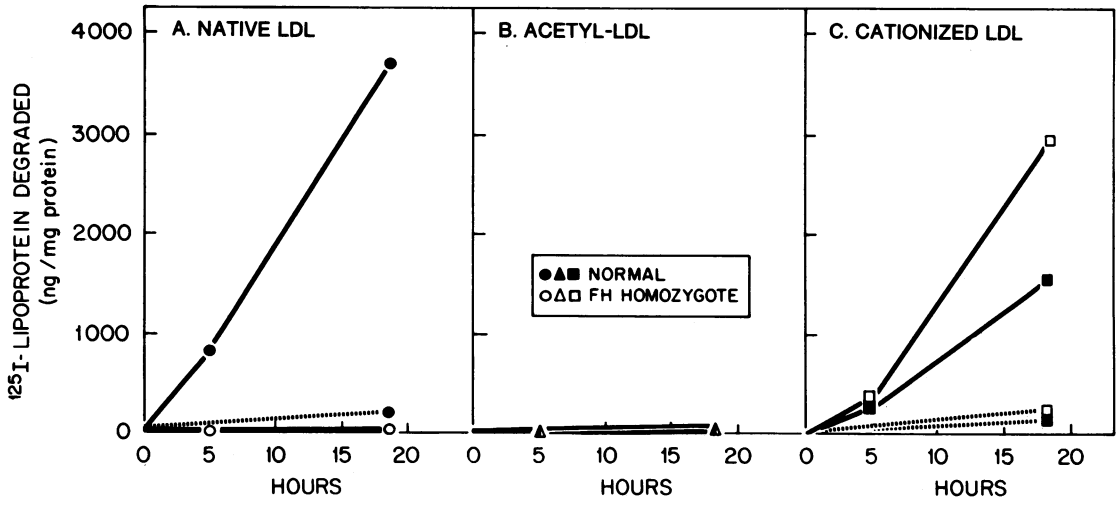

FIG. 2. Proteolytic degradation of modified ^{125}I-LDL preparations in monolayers of normal (closed symbols) and FH homozygote (open symbols) fibroblasts. On day 7 the medium was replaced with 2 ml of growth medium containing 2% LPDS and 5 μg of protein per ml of ^{125}I-labeled lipoprotein as indicated. After incubation for the indicated time at 37°, the content of trichloroacetic acid-soluble ^{125}I-labeled degradative products in the culture medium was determined. Each value represents the average of duplicate incubations. All values are corrected for the small amount of trichloroacetic acid-soluble ^{125}I radioactivity that was present in medium incubated with the appropriate ^{125}I-labeled lipoprotein preparation in the absence of cells (4). The dashed lines show the values for the proteolytic degradation over 18 hr in the presence of 75 μM chloroquine. The total cellular content of ^{125}I-LDL at 5 hr in the normal and FH homozygote cells was 343 and 26 ng/mg of protein, respectively. For acetyl-^{125}I-LDL, these values at 5 hr were 33 and 45 ng/mg of protein, respectively. For both ^{125}I-LDL and acetyl-^{125}I-LDL, the total cellular content of ^{125}I-lipoprotein at 18 hr was the same as at 5 hr. The cellular content of cationized ^{125}I-LDL could not be measured (see *text*).

more than 90% inhibited by chloroquine (Fig. 2A and C), an agent that inhibits the lysosomal hydrolysis of LDL by intact fibroblasts without inhibiting its cellular uptake (7, 8). Since the cationized ^{125}I-LDL had a strong tendency to adhere to the plastic culture dish in the absence of cells, the extent of its binding and uptake by the cells could not be determined.

Acetylation of LDL eliminated its ability to compete with ^{125}I-LDL for binding to fibroblasts (data not shown), indicating that acetylation had destroyed the ability of the lipoprotein to bind to the LDL RECEPTOR/ Consistent with this finding was the observation that acetyl-^{125}I-LDL was not taken up or degraded by either the normal or FH homozygote cells (Fig. 2B).

Hydrolysis of the cholesteryl ester component of the modified LDL preparations paralleled the hydrolysis of the protein component. Thus, normal cells hydrolyzed the cholesteryl esters of native LDL more rapidly than those of acetyl LDL, whereas FH homozygote cells were not able to hydrolyze effectively either LDL preparation (Table 1). On the other hand, [^{3}H] cholesteryl linoleate bound to cationized LDL was hydrolyzed by both cell strains. In all cases, the hydrolysis process was inhibited by the lysosomal inhibitor, chloroquine (Table 1).

Inasmuch as the cationized LDL was degraded equally by the normal and FH homozygote cells, it appeared that this modified lipoprotein was entering the cells by some mechanism other than one involving the LDL receptor. To confirm this conclusion, we incubated fibroblasts with 25-hydroxycholesterol plus cholesterol for 48 hr, during which time LDL receptor activity in the normal cells is markedly suppressed (20). Table 2 shows that under these conditions the rate of degradation of ^{125}I-LDL was reduced by more than 90% in the normal cells. On the other hand, prior incubation with sterols did not affect the degradation of cationized ^{125}I-LDL in either the normal or FH homozygote cells. The data in Table 2 also show that whereas excess unlabeled native LDL competed with ^{125}I-LDL for degradation [due to its ability to compete for binding to the

Table 2. Dissociation of the degradation of cationized LDL from the LDL receptor-mediated degradation of native LDL

Prior treatment of cells	Lipoprotein added to medium	^{125}I-Lipoprotein degraded (ng of acid-soluble material formed/mg of protein)			
		Normal cells		FH homozygote cells	
		(−)Unlabeled native LDL	(+)Unlabeled native LDL	(−)Unlabeled native LDL	(+)Unlabeled native LDL
Ethanol	^{125}I-LDL	1150	88	17	4.4
	Cationized ^{125}I-LDL	785	540	636	619
25-Hydroxycholesterol + cholesterol in ethanol	^{125}I-LDL	85	10	8	0.5
	Cationized ^{125}I-LDL	830	680	546	540

On day 5 the medium was replaced with 2 ml of growth medium containing 10% LPDS and either 10 μl of ethanol alone or 10 μl of ethanol containing 1.2 μg of 25-hydroxycholesterol plus 24 μg of cholesterol. After 48 hr at 37° (day 7), each dish was washed with 2 ml of phosphate-buffered saline, after which was added 2 ml of medium containing 2% LPDS and 5 μg of protein per ml of the indicated ^{125}I-labeled lipoprotein with or without 250 μg of protein per ml of unlabeled native LDL as indicated. After incubation for 5 hr at 37°, the content of trichloroacetic acid-soluble ^{125}I-labeled degradative products in the culture medium was determined. Each value represents the mean of triplicate incubations.

Table 3. Stimulation of cholesteryl ester formation by modified LDL preparations

Addition to medium	[^{14}C]Oleate incorporated into cholesteryl [^{14}C]oleate (pmol·hr^{-1}·mg of protein^{-1})			
	Normal cells		FH homozygote cells	
	(−)Chloroquine	(+)Chloroquine	(−)Chloroquine	(+)Chloroquine
None	31	20	21	22
Native LDL	836	12	17	19
Cationized LDL	1943	46	1781	38
25-Hydroxycholesterol + cholesterol	890	692	1448	1932

On day 7 the medium was replaced with 2 ml of growth medium containing 2% LPDS, either no chloroquine or 75 μM chloroquine as indicated, and the indicated addition (10 μg of protein per ml of native LDL, 10 μg of protein per ml of cationized LDL, or 1 μg/ml of 25-hydroxycholesterol plus 15 μg/ml of cholesterol added in 10 μl of ethanol). After incubation at 37° for 17 hr, each monolayer was pulse-labeled for 2 hr with 0.1 mM [^{14}C]oleate-albumin (10,454 cpm/nmol), after which the cells were harvested for determination of the cellular content of cholesteryl [^{14}C]oleate. Each value represents the average of duplicate incubations.

LDL receptor (3, 21)], excess unlabeled native LDL did not effectively compete with cationized ^{125}I-LDL for the degradative process.

In normal fibroblasts the receptor-mediated binding, uptake, and degradation of native LDL leads to a profound suppression of HMG CoA reductase activity (Fig. 3A). Such suppression by native LDL is not observed in FH homozygote cells (Fig. 3A). As predicted from the above data, the ability of acetyl-LDL to suppress HMG CoA reductase activity was reduced in normal cells (Fig. 3B). In contrast, cationized LDL suppressed HMG CoA reductase activity in both the normal and FH homozygote cells (Fig. 3C).

We have previously shown that suppression of HMG CoA reductase activity in fibroblasts by LDL-bound cholesterol is associated with a reciprocal increase in the activity of a microsomal acyl-CoA:cholesterol acyltransferase (22), which leads to an enhanced rate of incorporation of [^{14}C]oleate into cellular cholesteryl [^{14}C]oleate in intact cells (18). Table 3 shows that cationized LDL, unlike native LDL, led to a marked increase in the rate of cholesteryl ester formation in the FH homozygote fibroblasts. As previously reported for native LDL (8, 23), blocking the hydrolysis of the cholesteryl esters of cationized LDL with chloroquine reduced its activation of cholesteryl ester formation. Chloroquine did not inhibit the ability of a mixture of 25-hydroxycholesterol and cholesterol to stimulate this esterification reaction, indicating that this drug did not inhibit the acyltransferase enzyme itself (7).

To determine whether coupling with *N,N*-dimethyl-1,3-propanediamine would enhance the degradation of proteins other than LDL, we prepared cationized ^{125}I-albumin. Fig. 4 shows that the cationized ^{125}I-albumin was hydrolyzed more than 100 times faster than was ^{125}I-albumin in normal fibroblasts. As with cationized ^{125}I-LDL, the degradation of cationized ^{125}I-albumin was nearly completely inhibited by chloroquine.

The presence of free dimethylpropanediamine (150 μg/ml) or dimethylpropanediamine coupled to albumin (150 μg/ml), or to LDL (150 μg/ml) did not stimulate the degradation of ^{125}I-LDL in FH homozygote fibroblasts under conditions in which cationized ^{125}I-LDL was actively degraded.* These results would appear to rule out the possibility that dimethylpropanediamine–protein complexes were causing enhanced LDL uptake and degradation simply by stimulating bulk fluid pinocytosis.

DISCUSSION

A major goal in mammalian cellular physiology is the development of methods to enhance selectively the uptake of macromolecules by living cells and to deliver these molecules to specific cellular organelles. Although most cells take up macromolecules continuously by bulk phase pinocytosis, the efficiency of this process is extremely low. In an attempt to enhance the efficiency of uptake, recent studies have focused on the incorporation of macromolecules into phospholipid vesicles that can be taken up by cells by fusion or by endocytosis (24, 25).

The ability to enhance the cellular uptake of one class of

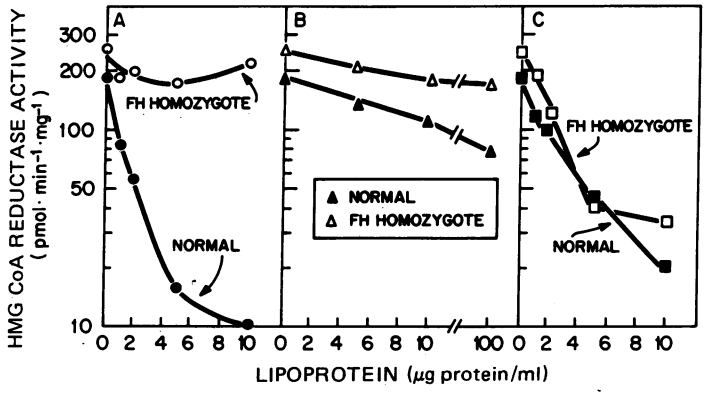

FIG. 3. Effect of modified LDL preparations on HMG CoA reductase activity of normal (closed symbols) and FH homozygote (open symbols) fibroblasts. On day 7 the medium was replaced with 2 ml of growth medium containing 5% LPDS and varying amounts of one of the following LDL preparations: A, (●, ○) native LDL; B, (▲, △) acetyl-LDL; C, (■, □) cationized LDL. After incubation for 24 hr at 37°, cells were harvested for measurement of HMG CoA reductase activity.

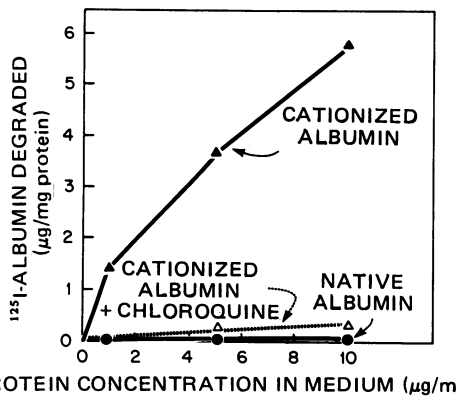

FIG. 4. Proteolytic degradation of ^{125}I-albumin and cationized ^{125}I-albumin in monolayers of normal fibroblasts. On day 7 the medium was replaced with 2 ml of growth medium containing the indicated concentration of either ^{125}I-albumin (1090 cpm/ng) (●) or cationized ^{125}I-albumin (1480 cpm/ng) (▲, △) in the presence (△) or absence (●, ▲) of 75 μM chloroquine. After incubation for 5 hr at 37°, the content of trichloroacetic acid-soluble ^{125}I-labeled degradative products in the culture medium was determined. Each value represents the average of duplicate incubations. All values are corrected for the small amount of trichloroacetic acid-soluble ^{125}I radioactivity that was present in medium incubated with the appropriate ^{125}I-labeled albumin preparation in the absence of cells.

macromolecules, i.e., plasma lipoproteins, is particularly relevant to familial hypercholesterolemia (FH), since this disorder results from the absence of a cell surface receptor that normally facilitates the cellular uptake and lysosomal degradation of plasma LDL (1–6). In the current studies, we have been able to enhance by more than 100-fold the rate of uptake and degradation of LDL in FH homozygote fibroblasts by an approach that involves the coupling of the lipoprotein to the tertiary amine, *N,N*-dimethyl-1,3-propanediamine. This modification endows the LDL with a net positive charge. By analogy with the behavior of ferritin that has been rendered cationic by the same reaction (11, 12), we believe that the cationized LDL binds to diffusely distributed, negatively charged sites on the plasma membrane. The bound cationized LDL is then presumably internalized by adsorptive endocytosis and delivered to lysosomes.

Four lines of evidence support the conclusion that the degradation of cationized LDL occurs in lysosomes and hence requires cellular uptake of the lipoprotein: (*i*) as previously observed for the lysosomal hydrolysis of native LDL (7, 8, 23), both the protein and cholesteryl ester components of cationized LDL are hydrolyzed in parallel; (*ii*) previous studies have demonstrated that the hydrolysis of both the protein and cholesteryl esters of LDL occurs in cell-free extracts only at acid pH (7, 8, 23); (*iii*) hydrolysis of both the protein and cholesteryl esters of cationized LDL is blocked by chloroquine, an agent that inhibits the lysosomal degradation of native LDL without inhibiting its cellular uptake (7, 23); and (*iv*) the free cholesterol that is released from the lysosomal degradation of cationized LDL, like the cholesterol derived from the lysosomal degradation of receptor-bound native LDL, suppresses HMG CoA reductase activity and stimulates cellular cholesterol esterification.

The enhanced uptake and degradation of cationized LDL does not depend on the structure of the lipoprotein *per se*, since cationized albumin is also degraded by fibroblasts at a similarly enhanced rate as compared with native albumin. That such enhanced uptake of dimethylpropanediamine-linked proteins is not simply a reflection of denaturation of the protein was indicated by the observation that irreversible denaturation of ^{125}I-LDL by heating for 20 min at 70° (26) decreased its uptake and degradation in normal cells and did not enhance its uptake and degradation by the FH homozygote cells.*

Inasmuch as the surface of mammalian cells usually bears a negative charge (27), the cationization reaction may have general usefulness as a means for enhancing the cellular uptake of macromolecules and their delivery to lysosomes.

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