METABOLISM OF CATIONIZED LIPOPROTEINS BY HUMAN FIBROBLASTS

Biochemical and Morphologic Correlations

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ABSTRACT

Human plasma low density lipoprotein (LDL) that had been rendered polycationic by coupling with *N,N*-dimethyl-1,3-propanediamine (DMPA) was shown by electron microscopy to bind in clusters to the surface of human fibroblasts. The clusters resembled those formed by polycationic ferritin (DMPA-ferritin), a visual probe that binds to anionic sites on the plasma membrane. Biochemical studies with ^{125}I-labeled DMPA-LDL showed that the membrane-bound lipoprotein was internalized and hydrolyzed in lysosomes. The turnover time for cell-bound ^{125}I-DMPA-LDL, i.e., the time in which the amount of ^{125}I-DMPA-LDL degraded was equal to the steady-state cellular content of the lipoprotein, was about 50 h. Because the DMPA-LDL gained access to fibroblasts by binding nonspecifically to anionic sites on the cell surface rather than by binding to the physiologic LDL receptor, its uptake failed to be regulated under conditions in which the uptake of native LDL was reduced by feedback suppression of the LDL receptor. As a result, unlike the case with native LDL, the DMPA-LDL accumulated progressively within the cell, and this led to a massive increase in the cellular content of both free and esterified cholesterol. Studies with ^{14}C-oleate showed that at least 20% of the accumulated cholesteryl esters represented cholesterol that had been esterified within the cell. After 4 days of incubation with 10 μg/ml of DMPA-LDL, fibroblasts had accumulated so much cholesteryl ester that neutral lipid droplets were visible at the light microscope level with Oil Red O staining. By electron microscopy, these intracellular lipid droplets were observed to lack a tripartite limiting membrane. The ability to cause the overaccumulation of cholesteryl esters within cells by using DMPA-LDL provides a model system for study of the pathologic consequences at the cellular level of massive deposition of cholesteryl ester.

Normal human fibroblasts regulate their cholesterol content by controlling the rate of uptake of low density lipoprotein (LDL), the major cholesterol-rich lipoprotein in human plasma (10, 12, 21). Control of this uptake process is achieved through regulation of the activity of a cell surface

receptor that is required to bind the lipoprotein before its internalization by adsorptive endocytosis (9, 28). When fibroblasts are deprived of exogenous cholesterol, cellular cholesterol stores become depleted, and the number of LDL receptors is increased. On the other hand, when cellular cholesterol stores are expanded by exposure of the cells to an exogenous source of cholesterol, the number of receptors is reduced by more than 90% (9, 28). This cholesterol-mediated feedback suppression of LDL receptor activity serves to limit the uptake of LDL and thus to prevent the overaccumulation of cholesterol by the cell (6, 9, 28).

In order for the cholesterol of the internalized LDL to be utilized by the cell for membrane synthesis, the cholesteryl esters of the lipoprotein, which constitute about 70% of its total cholesterol content (37), must first be hydrolyzed in lysosomes (5, 14, 27). The resultant lipoprotein-derived free cholesterol then becomes available to regulate three events in cellular sterol metabolism: (*a*) it suppresses endogenous cholesterol synthesis through a reduction in the activity of the rate-controlling enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase) (3); (*b*) it activates a microsomal acyl-CoA:cholesterol acyltransferase that serves to reesterify any excess free cholesterol that is derived from LDL so that it can be stored by the cell as cholesteryl esters (4, 25); and (*c*) it reduces the number of LDL receptors as mentioned above.

Because of the efficiency of these control mechanisms, it has so far been impossible to induce experimentally an overaccumulation of cholesteryl esters in human fibroblasts by incubating them with plasma LDL even when the lipoprotein is added at extremely high levels, i.e., at concentrations more than 30-fold above the level required to saturate the LDL receptors (6, 22). Recently, however, a method has been devised by which the uptake of LDL can be enhanced in human fibroblasts through a mechanism that does not involve the LDL receptor (2). This method involves the covalent coupling of the tertiary amine *N,N*-dimethyl-1,3-propanediamine (DMPA) primarily to the free carboxyl groups of the protein component of LDL, thus resulting in a net increase of two units of positive charge per carboxyl group modified and changing the net charge of LDL from negative to positive (2). The conclusion that this polycationic LDL (DMPA-LDL) entered fibroblasts by a mechanism that bypassed the LDL receptor was supported by the finding that rapid uptake and degradation of this modified LDL occurred in cells derived from a subject with the homozygous form of familial hypercholesterolemia (FH) (2). These mutant cells have been shown previously to possess a complete deficiency of functional cell surface LDL receptors as defined by both biochemical and ultrastructural criteria (1, 7, 11, 13, 19, 20). By analogy to the known behavior of polycationic ferritin (DMPA-ferritin) (15, 29), which binds to widespread negatively charged sites on plasma membranes (39), we have postulated that DMPA-LDL binds to similar nonspecific anionic sites on the cell surface and that this membrane-bound lipoprotein is then taken up by endocytosis and delivered to lysosomes (2). Since the hydrolysis of both the protein and cholesteryl ester components of DMPA-LDL was inhibited by the lysosomal inhibitory agent chloroquine, it was concluded that the degradation of this modified lipoprotein occurred in lysosomes (2, 24, 27).

The current studies were undertaken with the following aims: (*a*) to characterize the chemical and physical properties of DMPA-LDL; (*b*) to determine by means of electron microscopy whether the DMPA-LDL does, in fact, bind to the plasma membrane; and (*c*) to determine whether human fibroblasts can be induced to accumulate nonphysiologic amounts of cholesteryl esters when incubated with a modified lipoprotein whose uptake bypasses the LDL receptor and hence is not susceptible to normal feedback regulation.

MATERIALS AND METHODS

Materials

DMPA was obtained from Eastman Kodak Co. (Rochester, N. Y.). Ethylenediamine and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride were products of Aldrich Chemical Co., Inc. (Milwaukee, Wis.) and Pierce Chemical Co. (Rockford, Ill.), respectively. Cationized ferritin was obtained from Miles Laboratories, Inc. (Elkhart, Ind.). Native ferritin and Harris hematoxylin stain were purchased from Polysciences, Inc. (Warrington, Penn.). Glutaraldehyde was obtained from Electron Microscopy Sciences (Fort Washington, Penn.). Pronase from *Streptomyces griseus*, B grade (45,000 U/g) was obtained from Calbiochem (San Diego, Calif.). Bio-gel A 15 M was obtained from Bio-Rad Laboratories (Richmond, Calif.). Bovine serum albumin (catalog no. A4378) and chloroquine diphosphate were obtained from Sigma Chemical Co. (St. Louis, Mo.). Oil Red O was purchased from Chroma-Gesellschaft Schmid and Co. NCS Tissue Solubilizer, [U-^{14}C]ethylenediamine hydrochloride (7.95 mCi/

mmol) and [1-^{14}C]oleic acid (58 mCi/mmol) were obtained from Amersham/Searle Corp. (Arlington Heights, Ill.). Sodium ^{125}I-iodide (carrier free in 0.05 N NaOH) was purchased from Schwarz/Mann Div., Becton, Dickinson & Co. (Orangeburg, N. Y.). Aquasol counting fluid was purchased from New England Nuclear (Boston, Mass.). Fetal calf serum was purchased from Flow Laboratories, Inc. (Rockville, Md.). Assay reagents for measuring triglycerides were obtained from Boehringer-Mannheim Biochemicals (Indianapolis, Ind.) (catalog no. 15989). Tissue culture supplies, thin-layer chromatographic materials, and reagents for assays were obtained from sources as previously reported (3, 8).

Cell Culture

Cultured fibroblasts were derived from skin biopsies obtained from a normal subject (D. S.) and a patient with the receptor-negative form of homozygous FH (M. C.) (26). Cells were grown in monolayer and used between the 5th and 20th passages. Stock cultures were maintained in a humidified incubator (5% CO_2) at 37°C in 250-ml flasks containing 10 ml of growth medium consisting of Eagle's minimum essential medium (Grand Island Biological Co., Grand Island, N. Y., catalog no. F-11) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), 20 mM Tricine, pH 7.4, 24 mM $NaHCO_3$, 1% (vol/vol) nonessential amino acids, and 10% (vol/vol) fetal calf serum. Unless otherwise noted, all experiments were done in a similar format: On day 0, confluent monolayers of cells from stock flasks were dissociated with 0.05% trypsin-0.02% EDTA solution, and 1×10^5 cells were seeded into each 60 × 15-mm Petri dish containing 3 ml of growth medium with 10% fetal calf serum. 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 was added (final protein concentration, 2.5 mg/ml). Experiments were initiated on day 7 after the cells had been incubated for 48 h in the presence of lipoprotein-deficient serum.

Lipoproteins

Human very low density lipoprotein (VLDL, density <1.006 g/ml), LDL, (density 1.019-1.063 g/ml), and lipoprotein-deficient serum (density >1.215 g/ml) were obtained from the plasma of healthy subjects and prepared by differential ultracentrifugation (3). Calf lipoprotein-deficient serum was prepared as previously described (8). Unless otherwise noted, the concentrations of the lipoproteins are expressed in terms of their protein content. The mass ratio of cholesterol to protein in LDL was 1.6:1, as determined by gas-liquid chromatography (6). ^{125}I-labeled LDL (sp act, 200–400 cpm/ng protein) was prepared as previously described (7). Cationized LDL (DMPA-LDL) was synthesized by a previously described method (2) that was based on the procedure originally described by Danon et al. for the cationization of ferritin (15). DMPA-LDL was labeled with ^{125}I as previously described (2).

Biochemical Assays

Measurements of the total cellular binding of ^{125}I-LDL (20), the proteolytic degradation of ^{125}I-LDL (20), and the incorporation of ^{14}C-oleate into cellular lipids (25) in intact fibroblast monolayers were carried out as described in the referenced articles. 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 (6). The content of triglycerides in fibroblasts was determined according to the method of Schmidt and von Dahl (36), using the Boehringer-Mannheim triglyceride assay reagents. To extract triglycerides quantitatively from cells, each washed cell pellet (suspended in 0.2 ml water) was taken up in 5 ml of chloroform: methanol (2:1) (17), and the mixture was sonicated for 45 s and then extracted overnight at 37°C on a rotating platform.

Protein Estimation

The protein content of cell extracts and lipoproteins was determined by the method of Lowry et al., using bovine serum albumin as standard (32). Proteins coupled to DMPA gave a color reaction in the Lowry assay that was higher than could be accounted for by the known protein content of the sample. To correct for this DMPA effect, the extent of the increase in color reaction for DMPA-LDL was determined as follows: The cholesterol content and the "Lowry protein" content of a sample of ^{125}I-labeled LDL were measured by gas-liquid chromatography and the Lowry method, respectively, and the specific radioactivity of the LDL (cpm/μg protein) was calculated. This ^{125}I-LDL preparation was then divided into three aliquots: one aliquot was reacted with DMPA under the standard cationization reaction conditions and then dialyzed, the second aliquot was not treated with DMPA but was dialyzed, and the third aliquot was neither treated nor dialyzed. All three preparations were then assayed for their content of cholesterol, ^{125}I-radioactivity, and Lowry protein. As shown in Table I, all three preparations had a similar ratio of cholesterol content to ^{125}I-radioactivity content (column *a*). On the other hand, the ratio of cholesterol content to Lowry protein content (column *b*) and the ratio of ^{125}I-radioactivity content to Lowry protein content (column *c*) were both about 1.5-fold lower in the DMPA-LDL preparation than in the native LDL preparations. These data indicated that the Lowry protein value for the DMPA-

TABLE I
Cholesterol: Lowry Protein Ratio in Preparations of Native LDL and Cationized LDL

Treatment of LDL	(a) Cholesterol/^{125}I	(b) Cholesterol/Lowry protein	(c) ^{125}I/Lowry protein
	μg/cpm	*μg/μg*	*cpm/μg*
None	0.121	1.60	13.1
Dialyzed	0.117	1.57	13.7
Reacted with DMPA, then dialyzed	0.123	1.07	8.7

A preparation of ^{125}I-LDL (13.1×10^3 cpm/mg protein) was analyzed for its content of cholesterol, ^{125}I-radioactivity, and Lowry protein as described under Materials and Methods. One aliquot (20 mg protein) from the ^{125}I-LDL preparation was treated with DMPA as described under Materials and Methods. A second aliquot of the same ^{125}I-LDL preparation was treated similarly except that no carbodiimide or DMPA was added. Both LDL preparations were dialyzed for 18 h at 4°C against 6 liters of 0.15 M NaCl, pH 6. After dialysis, the content of cholesterol, ^{125}I-radioactivity, and Lowry protein were measured. Lowry protein refers to the apparent protein content as measured by the Lowry reaction using bovine serum albumin as a standard. The values represent the ratios of cholesterol: ^{125}I-radioactivity; cholesterol: Lowry protein; and ^{125}I-radioactivity: Lowry protein for each sample. Each measurement represents the mean of duplicate determinations.

LDL was falsely elevated by a factor of 1.5. A similar 1.5-fold elevated ratio of Lowry protein to cholesterol was consistently found in three other preparations of DMPA-LDL. Accordingly, in the subsequent determination of protein content of cationized protein preparations, the value obtained by the Lowry protein method was divided by 1.5 to give the true protein content.

Binding of Cationized Ferritin to the Cell Surface of Fibroblasts

On day 7 of cell growth, monolayers of fibroblasts were washed once with 3 ml of phosphate-buffered saline, after which 2 ml of 0.1 M sodium phosphate, pH 7.3 containing 320 μg/ml of cationized ferritin was added to each dish. After exposure to the cationized ferritin for 10–20 s at room temperature, each monolayer was washed three times with 3 ml of phosphate-buffered saline and fixed *in situ* at room temperature for 30 min with 3% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.3

Binding of Cationized LDL to the Cell Surface of Fibroblasts

On day 7 of cell growth, monolayers of fibroblasts were chilled to 4°C for 30 min, after which the medium from each dish was removed and replaced with 2 ml of ice-cold growth medium containing 5% human lipoprotein-deficient serum and either native LDL or DMPA-LDL at a concentration corresponding to 9.4 μg/ml of LDL-protein. After incubation at 4°C for 1 h, each monolayer was washed extensively with an albumin-containing buffer (19). Each monolayer then received 2 ml of ice-cold growth medium containing 5% human lipoprotein-deficient serum and 5 mg/ml of native ferritin. After incubation at 4°C for 1 h, each monolayer was washed five times with 3 ml of ice-cold phosphate-buffered saline and fixed *in situ* at 4°C for 30 min with 3% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.3.

Electron Microscopy

For the experiments showing the cell surface binding sites for cationized ferritin and DMPA-LDL (Figs. 2 and 3), the fixed cells were washed in the original petri dish with phosphate-buffered saline and postfixed with 2% OsO_4 in 0.1 M sodium phosphate buffer, pH 7.3. Cells were dehydrated through a graded series of ethanol solutions, washed several times with 100% ethanol, embedded in Epon (33), and prepared for thin sectioning as previously described (1).

For the experiments showing the intracellular distribution of lipid in cells that had been incubated with DMPA-LDL (Fig. 8), the monolayers were fixed *in situ* for 30 min with 3% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.3, scraped from the dish with a rubber policeman, and centrifuged in a Beckman microfuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) (5 min, 4°C, 12,000 rpm). The cell pellets were then postfixed with 2% OsO_4 in 0.1 M sodium phosphate buffer, pH 7.3, dehydrated, and embedded in Araldite.

All material for electron microscopy was sectioned on a Sorvall MT 2-B ultramicrotome (DuPont Instruments, Sorvall Operations, Newtown, Conn.), stained with uranyl acetate and lead citrate, and viewed with either a Philips 200 or a Philips 300 electron microscope.

Oil Red O Staining of Fibroblasts

Fibroblast monolayers were grown on glass cover slips contained in Petri dishes. Cells were initially seeded on day 0 at a concentration of 1×10^5 cells per dish as described above. On day 4 of cell growth, each dish received 2 ml of growth medium containing 5% human lipoprotein-deficient serum and one of the following additions: none; native LDL at a concentration corresponding to 10 μg/ml of LDL protein; or DMPA-LDL at a concentration corresponding to 10 μg/ml of LDL-protein. This growth medium was replaced with 2 ml of an identical medium on day 5. On day 6, each monolayer was washed three times with 3 ml of phosphate-buffered saline, after which was added 3 ml of fresh growth medium containing 5% human lipoprotein-deficient se-

rum. After incubation in the absence of lipoproteins for 24 h, on day 7 the cover slips were removed, fixed with 6% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.3, washed with a solution containing 4.5% sucrose and 0.1 M sodium phosphate, pH 7.3, and then stained with Oil Red O (34). The cover slips were counter-stained with double strength Harris hematoxylin stain for 10 min.

RESULTS

Chemical and Physical Characteristics of DMPA-LDL

Previous studies have shown that DMPA-LDL exhibits a net positive charge at pH 8.6 and therefore migrates toward the cathode, in the direction opposite from that of native LDL, when subjected to electrophoresis in agarose gel (2). This change in charge has been interpreted to indicate that the DMPA molecule was incorporated into LDL under the standard reaction conditions. To quantitate the number of DMPA residues incorporated into each mole of LDL-protein under these conditions, we have employed an indirect approach using [^{14}C]ethylenediamine, a commercially available radiolabeled compound, that, like DMPA, forms amide bonds primarily with the free carboxyl groups of proteins in the presence of a carbodiimide reagent (30). Accordingly, as shown in Table II, a preparation of native LDL was divided into two aliquots, one of which was treated with DMPA under the standard reaction conditions. Both the untreated and DMPA-treated preparations of LDL were then dialyzed, after which each was reacted with [^{14}C]ethylenediamine in the presence of the carbodiimide reagent. After a repeat dialysis, the amount of [^{14}C]ethylenediamine coupled to each LDL preparation was measured. In the LDL that had never been exposed to DMPA, 290 mol of [^{14}C]ethylenediamine were incorporated per mole of LDL-protein (assuming a molecular weight of 500,000 for LDL-protein [31]). On the other hand, in the LDL preparation that had been reacted previously with DMPA, only 38 mol of [^{14}C]ethylenediamine could subsequently be incorporated per mole of LDL-protein. The difference between these two numbers, 252 mol/mol of LDL-protein, represents the apparent number of sites that had reacted with DMPA and hence were blocked from subsequent reaction with [^{14}C]ethylenediamine. Since LDL has been reported to contain about 360 mol of glutamic and aspartic acid residues per mole of LDL-protein (35), the data in Table II suggest that ~70% of these negatively charged amino acids were coupled to DMPA under the standard reaction conditions. These results are similar to those of Danon and coworkers who were able to link DMPA to 28–60% of the total titratable carboxylic acid groups on ferritin under reaction conditions similar to those of the current study (15).

When subjected to gel filtration on a column containing 4% agarose gel, DMPA-LDL and na-

TABLE II

Determination of Available Sites on Plasma LDL for Carbodiimide-Mediated Cationization

LDL preparation	[^{14}C]Ethylenediamine incorporated into LDL	
	cpm/mg LDL-protein	*mol/mol LDL**
Untreated (A)	23,000	290
DMPA-treated (B)	2,980	38
(A) − (B)	20,020	252

One aliquot of ^{125}I-LDL (20 mg protein, sp act, 13.5 × 10^3 cpm/mg protein) was treated with DMPA as described under Materials and Methods. Another aliquot of the same ^{125}I-LDL preparation was treated similarly except that no carbodiimide or DMPA was added. Both LDL preparations were then dialyzed for 18 h at 4°C against 6 liters of 0.15 M NaCl adjusted to pH 7, after which 400-μl aliquots of the untreated and DMPA-treated LDL preparations (each containing about 4 mg protein) were mixed with 200 μl of 1.6 M [^{14}C]ethylenediamine-HCl (4 × 10^4 cpm/μmol) at pH 6.0. Next, 25 mg of 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide HCl was added to each tube. Each reaction mixture was incubated at 24°C for 2 h and then at 4°C for 24 h, after which it was dialyzed sequentially at 4°C against 6 liters of 0.15 M NaCl for 5 h and 6 liters of buffer containing 20 mM Tris-chloride, pH 7.4, 0.15 M NaCl, 0.01% EDTA for 18 h. 1 ml of the final dialysate contained no detectable ^{14}C- or ^{125}I-radioactivity. Each of the dialyzed LDL preparations was then analyzed for its content of ^{14}C- and ^{125}I-radioactivity. The ^{14}C-radioactivity was determined by counting an aliquot (50 μl) in 10 ml of Aquasol counting fluid containing 0.2% NCS Tissue Solubilizer in a Packard scintillation spectrometer (Packard Instrument Co., Inc., Downer's Grove, Ill.). The ^{125}I-radioactivity was determined by counting an aliquot (50 μl) in a well-type gamma counter. Under these conditions, 14.3% of ^{125}I-radioactivity was detected in the ^{14}C channel of the Packard scintillation counter. Results were corrected for this spillover. About 95% of the ^{14}C-radioactivity in the dialyzed LDL preparations was precipitable by 10% trichloroacetic acid and about 3% of the ^{14}C-radioactivity was extractable into chloroform:methanol (2:1).

* Assuming a molecular weight of LDL-protein of 500,000 (31).

tive LDL exhibited similar elution patterns (Fig. 1), indicating that the native and DMPA-coupled lipoprotein particles were similar in size. To prevent the DMPA-LDL from sticking to the agarose itself, it was necessary to conduct this gel filtration experiment in the presence of a high concentration of salt.

The DMPA-LDL and native LDL had similar densities as determined by isopycnic density centrifugation in cesium chloride (data not shown). The two lipoproteins also had a similar ratio of esterified cholesterol to free cholesterol (2.3:1) and a similar ratio of total cholesterol to protein (1.6:1) after appropriate correction for the effect of DMPA on the Lowry protein determinations (see Materials and Methods and Table I). The DMPA-LDL retained its spherical shape as determined by electron microscopy after negative staining with uranyl acetate. The mean ± 1 SD diameter of the DMPA-LDL particles was 243 ± 32 Å,

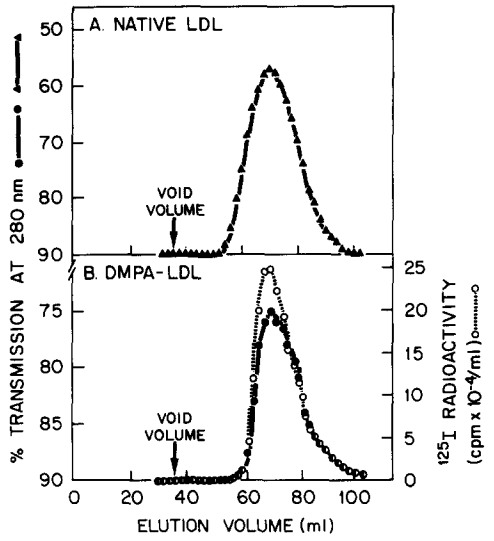

FIGURE 1 Chromatography of native LDL (A) and DMPA-LDL (B) on Bio-Gel A 15 M. Native LDL (21.4 mg protein) or ^{125}I-DMPA-LDL (16.2 mg protein containing 2.7 × 10^6 cpm) in a total volume of 1.8 ml of buffer A (50 mM Tris-chloride, pH 7.4, and 1 M NaCl) was applied to a column packed with Bio-Gel A 15 M (bed dimensions, 1.6 × 65 cm), and the column was eluted with buffer A at 4°C. The percentage of transmission at 280 nm was measured using an LKB Uvicord Absorptiometer (LKB Instruments, Inc., Rockville, Md.), and the mean value for each 2 ml of fraction was plotted (▲,●). The content of ^{125}I-radioactivity (○) was determined by counting a 50-μl aliquot of each fraction. The void volume of the column was determined from the elution position of human plasma VLDL.

a value that was slightly smaller than the mean diameter of a control preparation of native LDL particles obtained from a different subject and studied simultaneously (283 ± 22). However, the value for the DMPA-LDL was within the range of reported molecular diameters for normal LDL particles (37).[1]

Binding of DMPA-LDL to the Plasma Membrane

To determine the native distribution of negative charges on the surface of the normal fibroblasts that were used in these studies, cell monolayers were exposed for 10 s to cationized ferritin, a visual probe that has been used in other cell types to localize membrane anionic sites (15, 29). Fig. 2 shows that the cationized ferritin was unevenly distributed on the cell surface. Over most of the plasma membrane, clumps or piles of ferritin were separated by regions of membrane that bound no ferritin. The cationized ferritin was found over "coated regions" of the plasma membrane (16, 18, 38) as well as over noncoated regions. An identical pattern of cationized ferritin binding was observed in the FH homozygote fibroblasts.

In order to study the binding of DMPA-LDL to the cell surface, the fibroblast monolayers were incubated at 4°C with either native LDL or DMPA-LDL, washed extensively, and then exposed to native ferritin (Fig. 3). In the cells previously incubated with native LDL, no ferritin was observed to be bound at the cell surface (Fig. 3*A* and *B*). On the other hand, in the cells that had been previously treated with DMPA-LDL, ferritin cores adhered to clusters of amorphous, electron-dense material located at intervals along the plasma membrane (Fig. 3*C–F*). These clusters, which most likely represent aggregates of DMPA-LDL, were frequently observed to extend outward from the membrane (Fig. 3*D*). The clustered dis-

[1] It should be noted that these measurements of LDL were performed after negative staining with uranyl acetate. Staining with uranyl acetate was found to be preferable to staining with phosphotungstate because the latter caused precipitation of the DMPA-LDL. When the two staining methods were compared directly using native LDL from three normal subjects, we found that the uranyl acetate method gave consistently larger diameters for the LDL particle (267 ± 27, 244 ± 27, and 263 ± 26 Å) as compared with the standard phosphotungstate method (212 ± 22, 207 ± 22, and 224 ± 36 Å, respectively).

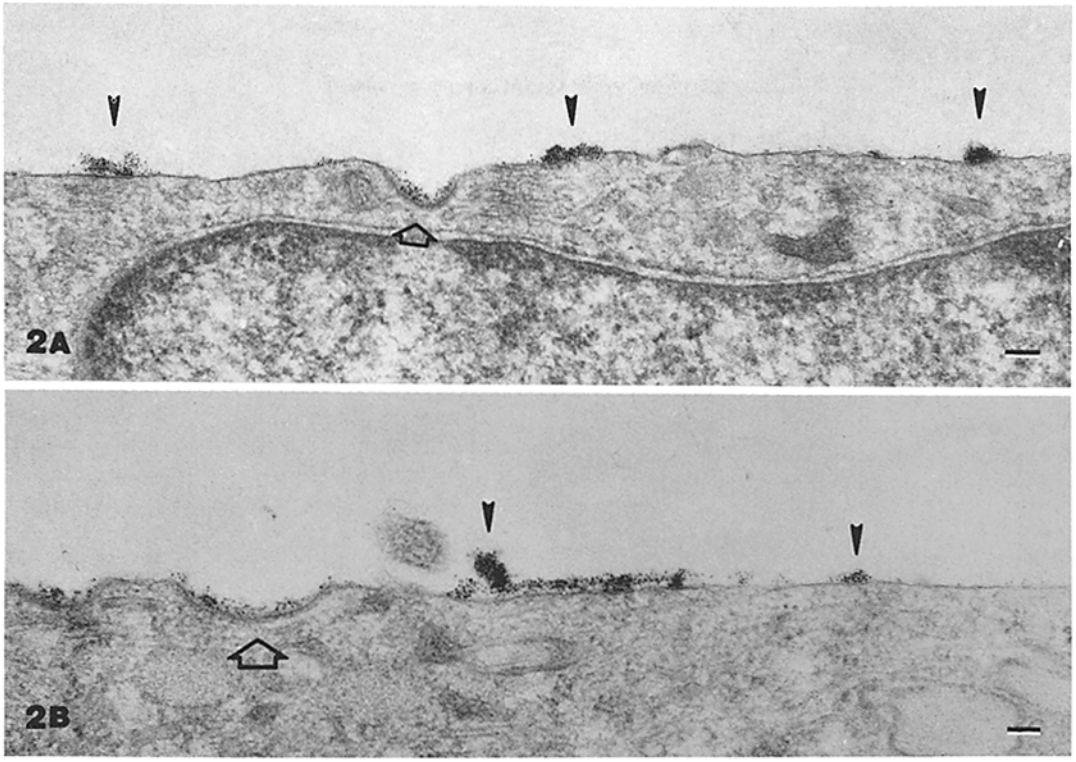

FIGURE 2 Representative electron micrographs showing the distribution of cationized ferritin bound to the surface of normal fibroblasts. Monolayers of cells were grown and incubated with 320 μg/ml of cationized ferritin for 10–20 s at room temperature as described under Materials and Methods. The cells were then fixed, embedded, and stained with uranyl acetate and lead citrate as described under Materials and Methods. The plasma membrane characteristically showed a patchy distribution of bound cationized ferritin (solid arrows) even when the cells were exposed to the probe for only 10 s. A more even distribution of bound cationized ferritin was observed over coated regions of the plasma membrane (open arrows). Note that the ferritin binding to noncoated regions of the plasma membrane was often several layers deep. Bars, 1000 Å. × 45,500.

tribution on the cell surface appeared to match the native distribution of anionic sites as determined by direct measurement of the binding of cationized ferritin (Fig. 2). In addition to being located randomly along the smooth surface of the plasma membrane (Fig. 3 *C* and *D*), particles of DMPA-LDL were observed to occur over coated regions and coated pits (Fig. 3 *E* and *F*). These coated regions have been noted to undergo endocytosis to form coated vesicles that then fuse with lysosomes (23). The coated regions have also been found to be the sites at which the high affinity receptors that bind native LDL are located (1, 23).

Uptake and Degradation of Cell-Bound DMPA-LDL

To follow biochemically the fate of DMPA-LDL, the modified lipoprotein was labeled with ^{125}I. When the ^{125}I-DMPA-LDL was incubated continuously with monolayers of fibroblasts, the total amount of lipoprotein associated with the cell reached a plateau within 5 h and did not change significantly over the ensuing 80 h (Fig. 4 A). On the other hand, during the interval between 5 and 80 h trichloracetic acid-soluble (non-iodide) ^{125}I-radioactivity was excreted continuously into the culture medium at a constant rate (Fig. 4 A). From the steady-state data between 5 and 80 h, it could be calculated (9, 19) that the turnover time for cell-bound ^{125}I-DMPA-LDL, i.e., the time in which the amount of ^{125}I-DMPA-LDL degraded was equal to the total cellular content of ^{125}I-DMPA-LDL, was about 50 h.

The fate of the cell-bound ^{125}I-DMPA-LDL was

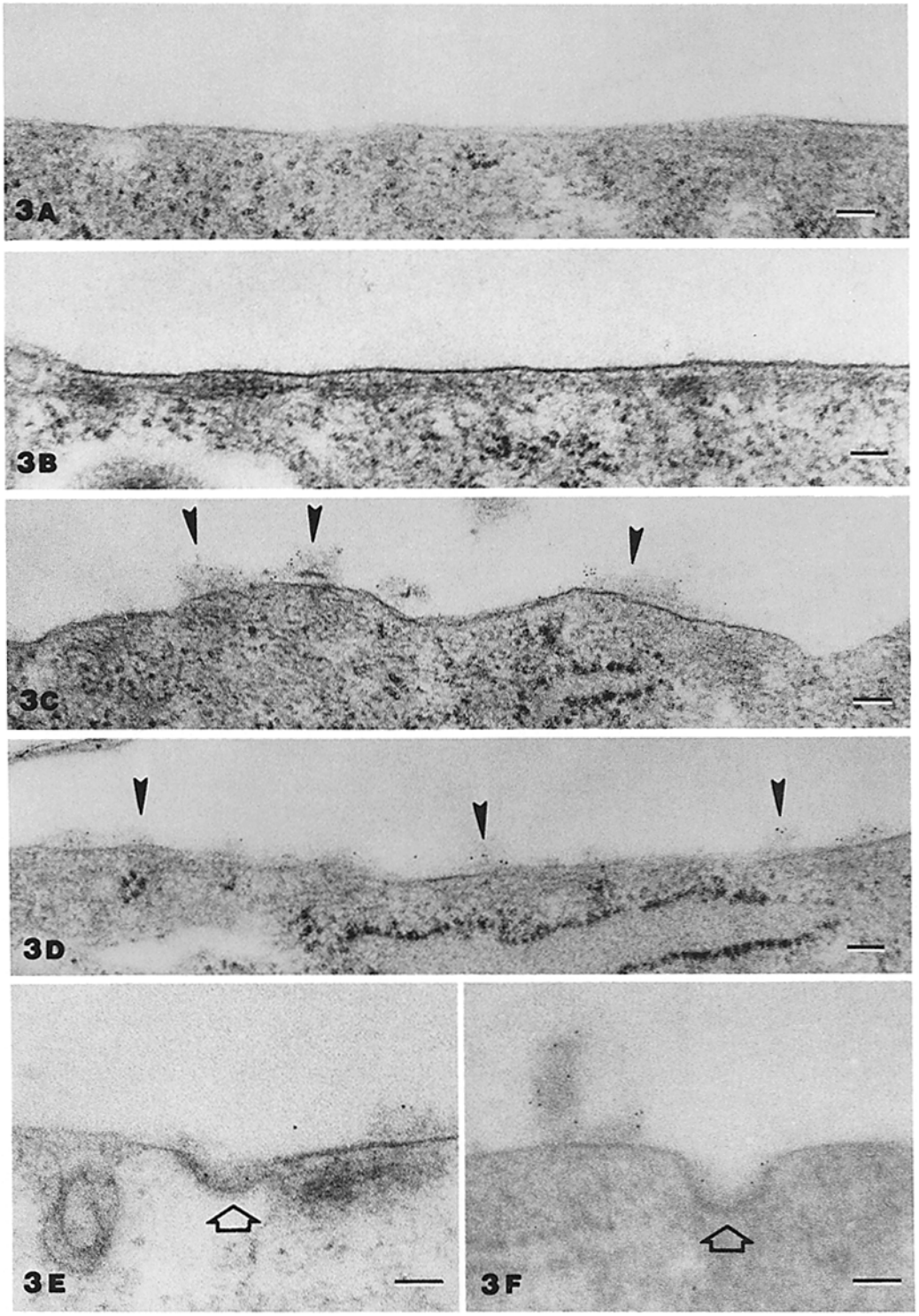

FIGURE 3 Localization of the binding of DMPA-LDL to the cell surface of normal fibroblasts. Monolayers of cells were grown and incubated for 30 min at 4°C with either native LDL (*A* and *B*) or DMPA-LDL (*C-F*) at a concentration corresponding to 9.4 μg/ml of LDL-protein, then washed extensively, and finally exposed to 5 mg/ml of native ferritin for 1 h at 4°C. The cells were then fixed, embedded, and stained with uranyl acetate and lead citrate as described under Materials and Methods. (*A* and *B*) Representative electron micrographs showing that native ferritin did not bind to the surface of cells that had been pretreated with native LDL. (*C* and *D*) Representative electron micrographs showing that native ferritin did bind to cells that had been pretreated with DMPA-LDL. The ferritin was observed in association with patches of electron-dense material (arrows) that most likely correspond to aggregations of DMPA-LDL. (*E*) High magnification of a ferritin-containing coated region of surface membrane (arrow) from a cell treated with DMPA-LDL and native ferritin. (*F*) A view similar to Fig. 3 *E* except that the section was unstained. Bars, 1,000 Å. (*A-D*) × 56,200; (*E* and *F*) × 72,000.

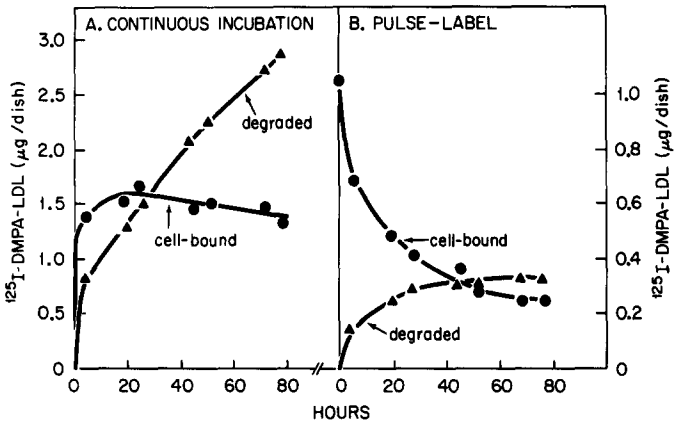

FIGURE 4 Turnover of cell-bound DMPA-LDL in FH homozygote fibroblasts. On day 7 of cell growth, dishes of cells were chilled for 30 min at 4°C. The medium in each dish was then replaced with 3 ml of growth medium containing 5% human lipoprotein-deficient serum and 2 μg protein/ml of ^{125}I-DMPA-LDL (498 cpm/ng), and the cells were incubated for 1 h at 4°C. After this incubation, one group of dishes (A) was warmed to 37°C and further incubated without removing the growth medium containing the ^{125}I-DMPA-LDL. A second group of dishes (B) was washed extensively (19) to remove unbound ^{125}I-DMPA-LDL, after which 3 ml of warm growth medium containing 5% human lipoprotein-deficient serum and no ^{125}I-DMPA-LDL was added to each dish and the cells were incubated at 37°C. At the indicated time, the medium from dishes in groups A and B was removed and its content of trichloroacetic acid-soluble ^{125}I-radioactivity was determined (▲). The monolayers were washed extensively (19) and the amount of bound ^{125}I-radioactivity was determined (●). Each value represents the average of duplicate incubations. The amount of total cellular protein per dish showed <10% variation in individual dishes throughout each experiment. The average values for exp A and B were 239 and 209 μg/dish, respectively.

also followed by exposing the fibroblast monolayers to the lipoprotein for 60 min at 4°C, washing the monolayers extensively, and then incubating the cells at 37°C in the absence of ^{125}I-DMPA-LDL. As shown in Fig. 4 B, under these conditions the cell-bound ^{125}I-radioactivity declined asymptotically over an 80-h period until about 80% of the radioactivity had left the cells. During this interval, approx. one-half of the ^{125}I-radioactivity that left the cell could be accounted for as trichloroacetic acid-soluble (non-iodide) material in the culture medium (Fig. 4 B), and the remainder was released into the medium in a form that was still precipitable with trichloroacetic acid. The experiments in Fig. 4 were conducted in cells from a FH homozygote in order to avoid any possible contribution by the LDL receptor to the observed effects. However, in other studies, quantitatively identical results were obtained with fibroblasts from normal subjects.

We have previously reported that the physiologic LDL receptor on the surface of normal fibroblasts can be destroyed by brief treatment of the monolayers with pronase or other proteolytic enzymes (19–21). As a result, such treatment reduces the rate at which normal fibroblasts are able to degrade ^{125}I-LDL (19). Table III shows that, in contrast to the inhibition of the degradation of native ^{125}I-LDL, brief treatment of fibroblast monolayers with pronase actually increased the rate of degradation of ^{125}I-DMPA-LDL. These data support the previous conclusion that the entry of DMPA-LDL into the cell does not involve the LDL receptor (2). To rule out the possibility that the enhanced degradation of ^{125}I-DMPA-LDL after pronase treatment was due to the action of traces of pronase that may have remained in the Petri dish after the proteolytic treatment of cells, the ^{125}I-labeled lipoproteins were incubated with the cells in the absence and presence of chloroquine, a drug that has previously been shown to inhibit the lysosomal degradation of LDL in monolayers of intact fibroblasts (5, 24, 27). Chloroquine inhibited the degradation of ^{125}I-DMPA-LDL both before and after treatment of the cells with pronase, indicating that in both case the degradation was occurring within lysosomes. In a separate control experiment, it was also shown that chloroquine at a concentration of 80 μM did not inhibit the degradation of ^{125}I-LDL or ^{125}I-DMPA-

TABLE III

Effect of Pronase Treatment on the Ability of Normal Fibroblasts to Degrade ^{125}I-LDL and ^{125}I-DMPA-LDL

Addition to medium	Prior treatment of cells	^{125}I-Lipoprotein degraded (−) Chloroquine	^{125}I-Lipoprotein degraded (+) Chloroquine
		ng/mg protein	
^{125}I-LDL	None	1,100	23
^{125}I-LDL	Pronase	300	27
^{125}I-DMPA-LDL	None	428	53
^{125}I-DMPA-LDL	Pronase	1,500	77

On day 7 of cell growth, each dish was washed once with 2 ml of phosphate-buffered saline and then incubated at 37°C for 40 min in 2 ml of growth medium containing no serum and either no pronase or 6 μg of pronase as indicated. Each dish was then washed twice with 2 ml of growth medium containing 10% human lipoprotein-deficient serum, after which was added 2 ml of growth medium containing 2% human lipoprotein-deficient serum, either no chloroquine or 75 μM chloroquine as indicated, and one of the following indicated additions: 5 μg protein/ml of ^{125}I-LDL (397 cpm/ng) or 5 μg protein/ml of ^{125}I-DMPA-LDL (527 cpm/ng). After incubation for 2.5 h at 37°C, 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.

LDL by pronase itself when the lipoproteins were incubated directly with the proteolytic enzyme in vitro.

Pathologic Accumulation of Cholesteryl Esters in Fibroblasts Incubated with DMPA-LDL

In the experiment shown in Fig. 5, normal fibroblasts were incubated for 4 days in lipoprotein-deficient serum containing ^{14}C-oleate and supplemented with one of the following: no lipoproteins, native LDL, or DMPA-LDL. At the intervals indicated, groups of monolayers were harvested to determine the amount of ^{14}C-oleate that had been incorporated into ^{14}C-phospholipids, ^{14}C-triglycerides, and cholesteryl ^{14}C-oleate. The data show that fibroblasts incubated in the absence of lipoproteins actively synthesized triglycerides and phospholipids and that the presence of either native LDL or DMPA-LDL did not significantly affect these synthetic rates. On the other hand, as previously shown (25), cells incubated in the absence of lipoproteins incorporated only a trace amount of ^{14}C-oleate into cholesteryl ^{14}C-oleate. In the presence of native LDL, a significant amount of cholesteryl ^{14}C-oleate synthesis oc-

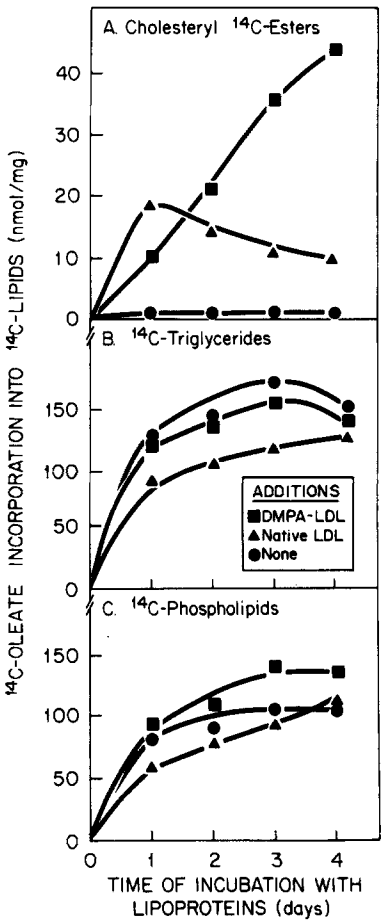

FIGURE 5 Incorporation of ^{14}C-oleate into cellular lipids in normal fibroblasts incubated with native LDL and DMPA-LDL. Cell monolayers were prepared as described under Materials and Methods. On day 1 of cell growth, each dish received 3 ml of growth medium containing 10% calf lipoprotein-deficient serum. On day 3 (zero time), each dish received 2 ml of growth medium containing 5% calf lipoprotein-deficient serum, 0.1 mM ^{14}C-oleate-albumin (10,000 cpm/nmol), and the indicated addition (●, none; ▲, 10 μg protein/ml of native LDL; or ■, 10 μg protein/ml of DMPA-LDL). Every 24 h throughout the experiment, the medium in each dish was replaced with fresh medium containing 0.1 mM ^{14}C-oleate-albumin and the indicated lipoprotein. At the indicated time, the cell monolayers in each group were harvested and their content of cholesteryl ^{14}C-oleate (A), ^{14}C-triglycerides (B), and ^{14}C-phospholipids (C) was determined by thin-layer chromatography as described in Materials and Methods. Each value represents the average of triplicate incubations.

curred on the first day. However, on subsequent days no further increase in the cellular content of cholesteryl ^{14}C-oleate occurred (Fig. 5 A). We have previously shown that this cessation in net cholesteryl ester synthesis, which occurs despite the continued presence of both LDL and ^{14}C-oleate in the culture medium, is due to the fact that the initial accumulation of cholesterol on the first day leads to a subsequent reduction in the number of LDL receptors (28). Thus, the rate of uptake of native LDL is markedly reduced after the first day. The accumulation of cholesterol derived from DMPA-LDL also produced a reduction in the number of LDL receptors (data not shown). However, since the cellular entry of DMPA-LDL does not decrease when the number of LDL receptors becomes suppressed (2), its uptake continued steadily for the 4 days of this experiment, and the cells continued to synthesize and accumulate cholesteryl ^{14}C-oleate in a linear fashion (Fig. 5 A).

Further evidence of the abnormal accumulation of cholesteryl ester in fibroblasts exposed to DMPA-LDL was obtained from measurements of the total cellular content of esterified cholesterol by gas-liquid chromatography (Fig. 6 B). This experiment also showed that, whereas native LDL had little effect on the cellular content of free cholesterol, DMPA-LDL caused a marked increase in the content of free cholesterol in the cell (Fig. 6 A).

The data in Table IV show that under conditions in which the cellular content of cholesteryl esters was 36-fold higher in cells grown in DMPA-LDL as compared with those grown in native LDL, the content of cellular triglyceride was the same under both circumstances.

The pathologic accumulation of cholesteryl esters that occurred in fibroblasts grown in DMPA-LDL could be detected morphologically at both the light microscope and electron microscope levels. In preliminary experiments, it was noted that when fibroblasts were incubated with DMPA-LDL, washed, and then stained immediately with Oil Red O, clumps of Oil Red O-positive material that presumably represented DMPA-LDL appeared on the surface of the cells, obscuring the visualization of lipid droplets within the cell. Accordingly, for the morphologic studies, cells were incubated for 48 h with DMPA-LDL, after which they were incubated for 24 h in lipoprotein-deficient serum to allow endocytosis of the surface-bound clusters of DMPA-LDL. Fig. 7 *A* and *B* show that cells incubated either in the absence of

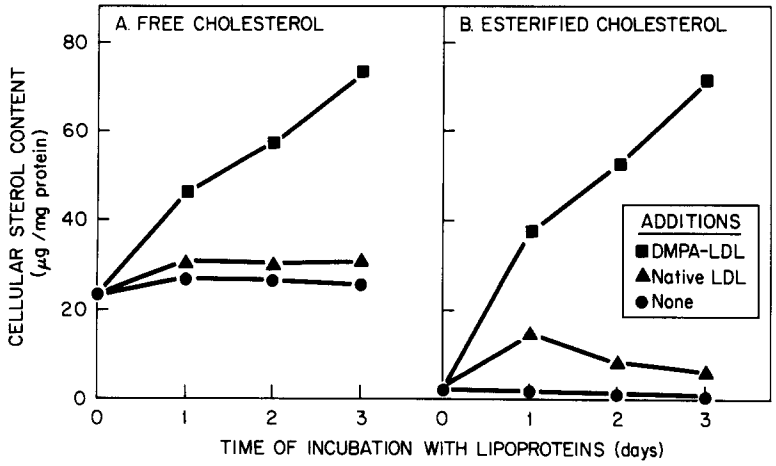

FIGURE 6 Accumulation of free (A) and esterified (B) cholesterol in normal fibroblasts incubated with native LDL and DMPA-LDL. Cell monolayers were prepared as described under Materials and Methods. On day 1 of cell growth, each dish received 3 ml of growth medium containing 10% calf lipoprotein-deficient serum. On day 3 (zero time), each dish received 2 ml of growth medium containing 5% calf lipoprotein-deficient serum and the indicated addition (●, none; ▲, 10 μg protein/ml of native LDL; or ■, 10 μg protein/ml of DMPA-LDL). Every 24 h throughout the experiment, the medium in each dish was replaced with fresh medium containing the indicated lipoprotein. At the indicated time, three cell monolayers in each group were washed, harvested, and pooled, and their content of free (A) and esterified cholesterol (B) was determined. Each value represents the average of duplicate samples (three monolayers per sample).

TABLE IV

Failure of DMPA-LDL to Elevate the Triglyceride Content of Normal Human Fibroblasts

Addition to medium	Cholesterol content		Triglyceride content
	Free	Esterified	
	μg sterol/mg protein		*μg/mg protein*
Native LDL	32	1.0	19
DMPA-LDL	48	36	19

On day 0, 1.5×10^5 cells were seeded into each 100 × 20 mm Petri dish containing 7 ml of growth medium with 10% fetal calf serum. On day 3, each monolayer was washed with 5 ml of phosphate-buffered saline and the dishes were divided into two groups. In one group, each of 15 dishes received 7 ml of growth medium containing 5% calf lipoprotein-deficient serum and 6 μg protein/ml of native LDL. In the other group, each of 15 dishes received 7 ml of growth medium containing 5% calf lipoprotein-deficient serum and 6 μg protein/ml of DMPA-LDL. After incubation for 72 h at 37°C, the cell monolayers in each group were washed, harvested and pooled, and frozen at −20°C. The lipids of the thawed cells were extracted with chloroform:methanol (2:1). The content of free and esterified cholesterol and of triglyceride was determined as described in Materials and Methods. Each value represents the average of duplicate determinations.

lipoproteins or in the presence of native LDL did not accumulate significant lipid droplets that could be detected by staining with Oil Red O. On the other hand, in the same experiment cells incubated in the presence of DMPA-LDL contained large numbers of lipid droplets that were stained with Oil Red O (Fig. 7*C*). Although the number of such lipid droplets varied from cell to cell, each cell treated with DMPA-LDL exhibited at least some stainable lipid droplets.

Electron microscope examination of fibroblasts treated with DMPA-LDL showed that many cells had accumulated large numbers of neutral lipid droplets that lacked a tripartite limiting membrane (Fig. 8*A*). In those cells with large numbers of droplets, the lipid appeared to be associated with cytoplasmic areas that were devoid of ground substance (Fig. 8*B*). In contrast to the DMPA-LDL effect, fibroblasts treated with an equal amount of native LDL under the same conditions showed only an occasional lipid droplet and did not show areas devoid of ground substance.

DISCUSSION

Plasma LDL that had been rendered polycationic by covalent linkage with the tertiary amine DMPA has been shown by electron microscopy to bind in large amounts to widespread sites on the plasma membrane of human fibroblasts. The pattern of DMPA-LDL binding corresponded to the pattern of polycationic ferritin binding in the same cells, suggesting that the DMPA-LDL was binding to anionic sites on the cell surface (15, 29). Once DMPA-LDL had bound to the cell surface, it was taken up and hydrolyzed by the cell at a relatively slow rate so that an amount of DMPA-LDL equal to the steady-state content was degraded every 50 h (Fig. 4 A). The true turnover time for the cell-bound DMPA-LDL may actually be faster than 50 h since some of the ^{125}I-DMPA-LDL measured as being cell-bound might in fact have been bound to inert sites on the proteinaceous matrix between the cells and hence not susceptible to internalization and degradation. However, at the low concentration of DMPA-LDL used (2 μg/ml), binding to the culture dish in the absence of cells (2) was minimized. Moreover, the pulse-labeling experiment in Fig. 4*B* showed that at least 40% of the bound ^{125}I-DMPA-LDL was located at sites in which it was susceptible to cellular degradation and hence probably on or within the cell.

Compared with the specific receptor-mediated uptake and degradation process for native LDL, the nonspecific uptake process for DMPA-LDL is extremely inefficient. Thus, as previously shown, when ^{125}I-LDL binds to the LDL receptor, essentially all of the cell-bound ^{125}I-LDL is internalized within 12 min and degraded within 90 min (9, 18, 23) as contrasted with the 25–50 h required to clear the cell surface of ^{125}I-DMPA-LDL. However, since the amount of ^{125}I-DMPA-LDL that binds to the anionic sites is at least 100-fold greater than the amount ^{125}I-LDL that can bind to the limited number of specific cell surface LDL receptors (19), the absolute rate of internalization and degradation of DMPA-LDL can actually equal the maximal rate of internalization and degradation of native LDL.

With regard to cellular cholesterol metabolism, the most interesting aspect of the DMPA-LDL uptake process is that it is immune to the type of feedback regulation that governs the uptake of LDL by the physiologic LDL receptor mechanism. Thus, when fibroblasts accumulate sufficient cholesterol to satisfy their requirements for membrane synthesis, the cells suppress the number of LDL receptors, reducing the rate of LDL uptake and preventing an overaccumulation of cholesterol (9, 28). On the other hand, the uptake

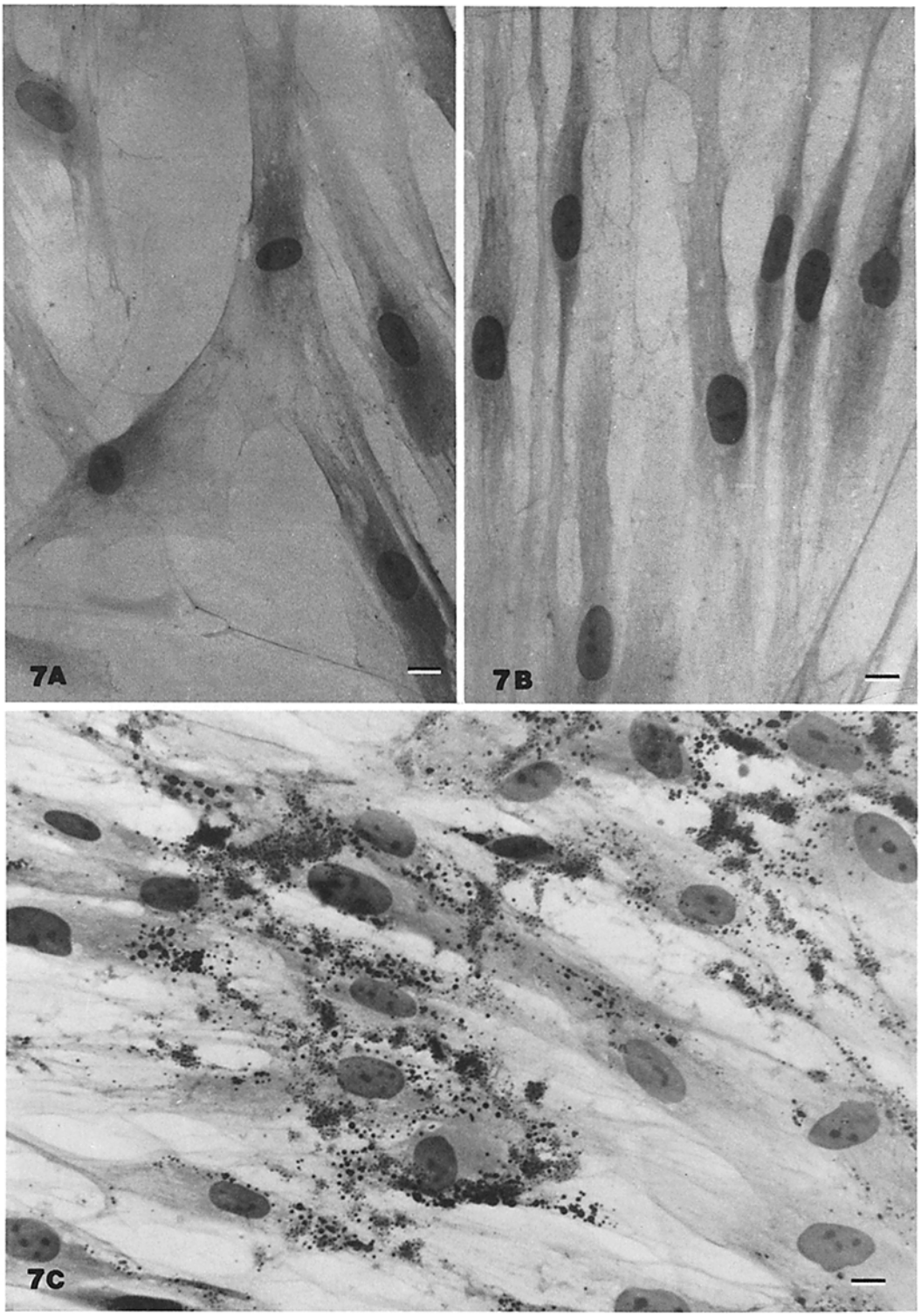

FIGURE 7 Monolayers of normal fibroblasts stained with Oil Red O to show accumulated cytoplasmic lipid and with hematoxylin to show nuclei. Cell monolayers were grown on glass cover slips and incubated with one of the following lipoprotein preparations as described under Materials and Methods: (*A*) none; (*B*) native LDL; or (*C*) DMPA-LDL. After these incubations, the cells were incubated for 24 h in lipoprotein-deficient serum without lipoproteins, after which they were stained with Oil Red O and counter-stained with Harris hematoxylin stain as described under Materials and Methods. The dark granules, which only accumulated in cells treated with DMPA-LDL (*C*), represent regions of Oil Red O staining. Bars (*A* and *C*), 10 μm; (*B*), 6.6 μm. (*A*) × 525; (*B*) × 700; (*C*) × 475.

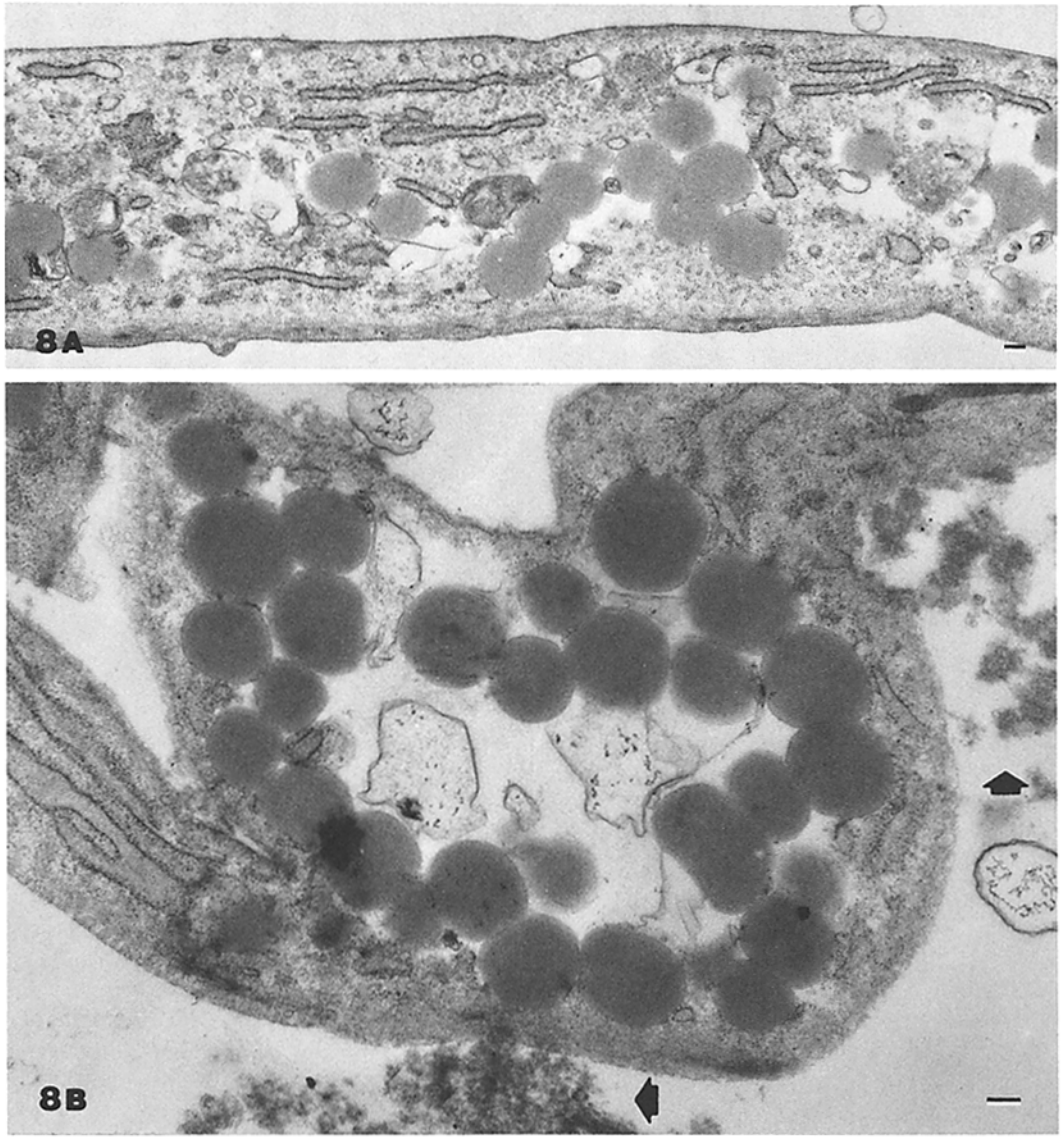

FIGURE 8 Two electron micrographs of fibroblasts treated with DMPA-LDL. Cell monolayers were grown, incubated with 10 μg protein/ml of DMPA-LDL for 2 days as described in the legend to Fig. 6, and then incubated for 24 h in lipoprotein-deficient serum without DMPA-LDL. The electron micrographs show that the cells have accumulated neutral lipid droplets that lack a tripartite limiting membrane. In some cells, the accumulation of lipid droplets appeared to have disrupted the normal cytoplasmic organization. Note the accumulation of electron-dense material (arrows) on the surfaces of DMPA-LDL treated cells; this material is most likely aggregates of DMPA-LDL. Bars, 2,000 Å. (*A*) × 14,000; (*B*) × 22,300.

process for DMPA-LDL is not reduced when LDL receptor activity is suppressed (2), and thus cells can be forced to accumulate pathologic amounts of cholesteryl esters.

On the basis of the experiment in Fig. 5 showing a continuous incorporation of ^{14}C-oleate into cholesteryl ^{14}C-oleate over 4 days in cells incubated with DMPA-LDL, it seems clear that a portion of the cellular cholesteryl esters accumulates as a result of esterification within the cell. The free

cholesterol substrate for this esterification reaction is derived from the lysosomal hydrolysis of the cholesteryl esters of DMPA-LDL, as indicated by the observation that mutant Cholesteryl Ester Storage Disease cells, which lack the lysosomal acid lipase enzyme (27), did not exhibit a normal enhancement in cholesteryl ester formation when incubated with DMPA-LDL (data not shown). These data are similar to those previously obtained in normal fibroblasts with the lysosomal enzyme inhibitor chloroquine (2).

The data in Fig. 4 A show that when ^{125}I-DMPA-LDL is incubated with fibroblasts, a dynamic steady state is achieved in which the cellular content of ^{125}I-DMPA-LDL remains constant over three days but in which the degradation of the lipoprotein continues at a linear rate. Despite an unchanging content of intact DMPA-LDL, total cholesteryl esters (Fig. 6 B) and cholesteryl esters newly synthesized from ^{14}C-oleate (Fig. 5 A) continue to accumulate in parallel with the degradation of the protein component of the DMPA-LDL (Fig. 4 A). Considered together, these data suggest that as the intact membrane-bound DMPA-LDL is transferred intracellularly, its protein component is hydrolyzed and the resultant radiolabeled degradative products (including mainly ^{125}I-monoiodotyrosine [20]) are excreted by the cell. On the other hand, the cholesteryl esters of the degraded DMPA-LDL continue to accumulate within the cell. Some of the accumulated cholesteryl esters are derived from cholesteryl esters that have been hydrolyzed and reesterified as discussed above, and some represent intact cholesteryl esters that have not been hydrolyzed. Since the experiments described in Figs. 5 and 6 were carried out under identical conditions, a comparison of these data allows a minimal estimate of the proportion of accumulated cholesteryl esters that is attributable to cholesterol that had been esterified within the cell. Thus, on day 3 the total cellular content of esterified cholesterol was 70 μg/mg protein (Fig. 6 B). On the other hand, Fig. 5 A shows that on day 3 ~36 nmol of ^{14}C-oleate had been incorporated into cholesteryl esters per mg protein. This latter value is equivalent to 14 μg of cholesterol that had been attached to ^{14}C-oleate per mg protein. Thus, out of a total accumulation of cholesteryl esters equal to 70 μg/mg, at least 14 μg/mg (or 20%) represented newly synthesized cholesteryl esters. This figure is likely to be a slight underestimate since the cell also attaches fatty acids other than oleate to cholesterol under these circumstances (5, 27). Of the ~80% of cholesteryl esters that had apparently not been hydrolyzed by the cell, a portion may reflect intact DMPA-LDL that was bound to the plasma membrane and that had not yet entered the cell. However, since the accumulation of cholesteryl esters was progressive with time whereas the amount of intact cell-bound ^{125}I-DMPA-LDL was constant (Fig. 4 A), it seems likely that much of the nonhydrolyzed cholesteryl esters represents DMPA-LDL that had entered the cell where its protein component had been hydrolyzed but where its cholesteryl ester component either was trapped within cellular lysosomes or had escaped from lysosomes to form lipid droplets.

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