Lipoprotein–Heparin–Fibronectin–Denatured Collagen Complexes Enhance Cholesteryl Ester Accumulation in Macrophages

DOMENICK J. FALCONE, NOE MATED, HELEN SHIO, C. RICHARD MINICK, and STANLEY D. FOWLER

Department of Pathology, Cornell University Medical College, and Laboratory of Biochemical Cytology, The Rockefeller University, New York 10021. Dr. Fowler's current address is Department of Pathology, School of Medicine, University of South Carolina, Columbia, South Carolina 29208.

ABSTRACT The sequestration of low-density lipoprotein (LDL) by components of the vascular extracellular matrix has long been recognized as a contributing factor to lipid accumulation during atherogenesis. The effects, however, that components of the extracellular matrix might have on LDL catabolism by scavenger cells have been little investigated. For these purposes we have prepared insoluble complexes of LDL, heparin, fibronectin, and denatured collagen (gelatin) and examined their effects on lipid accumulation, LDL uptake and degradation, and cholesteryl ester synthesis in mouse peritoneal macrophages. The results of these experiments have demonstrated that the cholesteryl ester content of macrophages incubated with a particular suspension of LDL, heparin, fibronectin, and collagen complexes is four- to fivefold that of cells incubated with LDL alone. The uptake of complexes containing ^{125}I-LDL is rapid; however, in contrast to either endocytosed ^{125}I-LDL or ^{125}I-acetyl LDL, the degradation of complex-derived LDL is impaired. In addition, the uptake of complex-derived LDL stimulates the incorporation of [^{14}C]oleic acid into cholesteryl oleate, however, the stimulation was a small fraction of that observed in cells incubated with acetyl LDL. Ultrastructurally, macrophages incubated with LDL, heparin, fibronectin, and collagen complexes did not contain many lipid droplets, but rather their cytoplasm is filled with phagosomes containing material similar in appearance to LDL–matrix complexes. These results indicate that components of the extracellular matrix can alter the catabolism of LDL by scavenger cells, suggesting that they may play a role in cellular lipid accumulation in the atherosclerotic lesion.

Recent evidence suggests that some lipid-laden (foam) cells observed in atherosclerotic lesions are derived from macrophages (1–5). The mechanism by which these cells accumulate lipid is unclear. When peritoneal macrophages (6), monocyte-derived macrophages (7, 8), and Kupffer cells (6) are assayed for receptor-mediated uptake of low-density lipoprotein (LDL),[1] these cells demonstrate few LDL receptors. Furthermore, macrophages fail to accumulate lipid when incubated in medium containing high concentrations of LDL (6–8). However, if LDL is altered by acetylation (6, 9), acetoacetylation (10), maleylation (6), or malondialdehyde derivatization (8, 11), there is a dramatic increase in the uptake of the modified LDL by macrophages leading to lipid accumulation. Enhanced endocytosis of LDL also resulted when macrophages were incubated in medium containing dextran sulfate (12). Other experiments have demonstrated that LDL preincubated with endothelial monolayers (13) and lipoproteins isolated from the arterial wall (14) are modified in an undefined manner resulting in increased uptake and degradation by macrophages. Finally, β-very low-density lipoprotein isolated from hypercholesterolemic sera is also taken up with high affinity and degraded by macrophages leading to lipid overloading (15, 16). Together, the results of these experiments suggest that native LDL could be modified in the blood

[1] *Abbreviations used in this paper*: DME, Dulbecco's modified Eagle's medium; DPBS, Dulbecco's phosphate-buffered saline; LDL, low-density lipoprotein; LDL-Hp-Fn, low-density lipoprotein–heparin–fibronectin complex; LDL-Hp-Fn-G, low density lipoprotein–heparin–fibronectin–gelatin complex.

THE JOURNAL OF CELL BIOLOGY · VOLUME 99 OCTOBER 1984 1266–1274

or interstitial space leading to enhanced uptake of the modified LDL and subsequent lipid accumulation.

Alternatively, inasmuch as a large fraction of LDL that infiltrates the vascular wall remains extracellular (17–19), the possibility that components of the extracellular matrix may affect the catabolism of LDL by macrophages should also be considered. In this regard, it has been demonstrated that components of the extracellular matrix can influence a variety of cellular functions, including endocytosis (20–22). For example, it has been repeatedly demonstrated that the uptake of particles coated with denatured collagen by macrophages was mediated by fibronectin with heparin as a cofactor (23–25). Fibronectin, a large glycoprotein, is involved in reticuloendothelial function, cell spreading and adherence, the organization of connective tissue, and wound healing (21, 26–29). It has been suggested that fibronectin may bind to exposed collagen and fibrin at sites of tissue injury. The binding of fibronectin may act to concentrate monocytes at these sites via cell surface fibronectin receptors (30), thereby promoting the phagocytosis of tissue debris and fibrin (27, 30). Furthermore, fibronectin is a prominent component of atherosclerotic lesions (31) and has been shown to accumulate in response to injury of small vessels (32).

In this report, we describe the preparation of insoluble complexes of LDL, heparin, fibronectin, and denatured collagen, and their effects on lipid accumulation in macrophages. Specifically, we have measured cholesterol, cholesteryl esters, and triacylglycerol accumulation, the synthesis of cholesteryl esters and triacylglycerols, and the uptake and breakdown of complex-derived LDL. The results demonstrate that macrophages incubated with these complexes accumulate cholesteryl esters. This accumulation appears to result from increased cholesteryl ester synthesis and the accumulation of undegraded lipoprotein.

MATERIALS AND METHODS

Lipoproteins

We isolated human LDL (d = 1.019–1.063 g/ml) from fresh human plasma by ultracentrifugation (33). We dialyzed isolated LDL against 150 mM NaCl–0.3 mM EDTA, pH 7.4, at 4°C for 24 h. LDL was determined to be free of other lipoproteins or proteins by electrophoresis in agar–agarose gels (34). LDL was acetylated (acetyl LDL) with repeated additions of acetic anhydride as described by Goldstein et al. (6), and dialyzed as above. Acetyl LDL migrated to a more cathodic position than native LDL when electrophoresed in agar–agarose gels. Both LDL and acetyl LDL were iodinated by the method described by Bilheimer et al. (35). Both labeled and unlabeled LDL and acetyl LDL preparations co-migrated after electrophoresis in agar–agarose gels. Precipitable (trichloroacetic acid-insoluble) radioactivity was >99.9% in both cases.

Fibronectin

We isolated fibronectin from outdated human plasma as described by Lorand and Gotoh (36) with minor modifications. A fibrinogen–fibronectin-rich fraction, isolated by ammonium sulfate precipitation, was defibrinated by heat denaturation. The crude fibronectin fraction was chromatographed on DEAE–Sephacel (Pharmacia Fine Chemicals, Piscataway, NJ) and eluted with a linear NaCl gradient (0–300 mM) in 50 mM Tris–HCl buffer containing 1 mM EDTA, pH 7.5. The isolated fibronectin was concentrated by ultrafiltration and was dialyzed against phosphate-buffered saline, pH 7.5, and stored at −70°C. The fibronectin isolated by this procedure eluted as a single peak from a column of gelatin–Sepharose (Pharmacia Fine Chemicals) (37, 38).

The isolated fibronectin was labeled in terminal amino groups and lysine amino groups by reductive alkylation with [^{14}C]formaldehyde (New England Nuclear, Boston, MA) followed by sodium cyanoborohydride (39). Fibronectin was also labeled in available sulfhydryl groups by carboxymethylation with iodo-[^{14}C]acetic acid (New England Nuclear) (40).

LDL Complexes with Heparin, Fibronectin, and Denatured Collagen

We made complexes of LDL with heparin, fibronectin, and denatured collagen according to procedures described by Srinivasan et al. (41) and Mawhinney et al. (42) for preparation of LDL-heparin complexes. The specific protocols are as follows:

LDL-HEPARIN-FIBRONECTIN (LDL-Hp-Fn): LDL (355 μg protein) was diluted to 3.0 ml with distilled H_2O, and 100 μl of heparin (Sigma Chemical Co., St. Louis, MO) (1 μg/μl) was added. The mixture became tubid after the addition of 500 μl of $CaCl_2$ (0.5 M). Fibronectin (653 μg) was then added and total volumes were adjusted to 3.8 ml. We left the preparation overnight in the cold. We centrifuged insoluble complexes (1000 g for 20 min), and resuspended the pellets in Dulbecco's modified Eagle's medium (DME) before addition to cell monolayers.

LDL-HEPARIN-FIBRONECTIN-GELATIN (LDL-Hp-Fn-G): We followed essentially the same procedure as above except that the additions were carried out in 5.0% gelatin instead of distilled water. The resultant complexes were solid at 4°C, and it was necessary to warm the mixture to 37°C for 5–10 min before pelleting the complexes (2000 g, 20 min).

Isolation of Mouse Peritoneal Cells

We obtained resident peritoneal macrophages from female Nelson–Collins strain mice (Rockefeller University) as described by Edelson and Cohn (43). Peritoneal cells were harvested in DME and were collected by centrifugation at 400 g for 10 min. Contaminating red blood cells were removed by lysis in NH_4Cl–Tris buffer. The cell suspension was centrifuged as above and the cell pellet was resuspended in DME containing 20% heat-inactivated fetal calf serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml). Mouse peritoneal cells were aliquoted into plastic Petri dishes (35 × 10 mm or 60 × 15 mm) and incubated in a humidified CO_2 (5%) incubator at 37°C for 2 h. Dishes were then washed twice with DME and monolayers were incubated with DME containing 10% fetal calf serum, penicillin, and streptomycin overnight. We always performed experiments the next day (day 0).

Incorporation of [^{14}C]Oleic Acid into Cholesteryl Esters and Triacylglycerols by Macrophages

To each dish an [^{14}C]oleic acid–albumin mixture, prepared as described by Goldstein et al. (44), was added to give a final concentration of 100 μM oleate/20 μM albumin. After a 6-h incubation in humidified CO_2 incubator at 37°C, the cultured cells were washed twice with Dulbecco's phosphate-buffered saline (DPBS). Lipid extraction in situ was then performed as described below. An internal standard ([^{3}H]cholesterol, 14,000 cpm/dish) and carrier lipids were added to the combined extracts from each dish. The extract was evaporated under N_2 and dissolved in 100 μl of dichloromethane/methanol (2:1). Lipid classes were separated by thin-layer chromatography and scraped into vials. Each vial received 10 ml of Aquasol II (New England Nuclear) and was counted. The remaining cell protein was dissolved in 0.2 N NaOH for at least 1 h and aliquots were removed for protein determinations. Esterification was expressed as nanomoles of [^{14}C]oleic acid incorporated per milligram of cell protein.

LDL Uptake, Cell Content, and Degradation

^{125}I-LDL or ^{125}I-LDL incorporated into complexes was incubated with macrophage monolayers for 5 and 24 h. LDL uptake, cell content and degradation were determined as described by Goldstein and Brown (45). Media (1 ml) was removed and 50% trichloroacetic acid added to give a final concentration of 10%. After 30 min of incubation at 4°C, denatured protein was pelleted and supernatant collected, to which 10 μl of 40% KI and 40 μl of 30% H_2O_2 were added. Free iodine was extracted with $CHCl_3$. Monolayers were treated with (0.5%) trypsin (Flow Laboratories, Inc., McLean, VA) for a total of 30 min and washed twice, and cell protein was digested with 0.2 N NaOH. Aliquots were removed for counting and protein determinations. In that the various lipoprotein preparations (LDL, acetyl LDL, and complex-derived LDL) exhibited different properties, all data were corrected by including control dishes without cells. Uptake, cell content, and degradation were expressed as micrograms of LDL per milligrams of cell protein.

Analytical Procedures

LIPIDS. Lipids were extracted from lipoproteins by the procedure of Folch et al. (46). They were separated into individual classes by thin-layer chroma-

tography and quantitated by scanning fluorometry after rhodamine 6G staining as previously described (47).

Lipids were extracted from macrophage cultures by the in situ procedure described by Hara and Radin (48). Each dish was treated with 2 ml of hexane/isopropyl alcohol (3:2) for 30 min. The organic solvent was removed and replaced with 1 ml of fresh solvent for a second 10-min extraction. The combined extracts were evaporated under N_2 and dissolved in 100 μl of dichloromethane/methanol (2:1) for thin-layer chromatography. The remaining protein precipitate in the dishes was dissolved in 0.2 N NaOH for 1 h and aliquots were then used for protein determinations.

URONIC ACID: Uronic acid was assayed by the method of Blumenkrantz and Asboe-Hansen (49). Inasmuch as sulfated glycosaminoglycans have been determined to be ~35% uronic acid by weight (50), uronic acid content was converted to an estimated heparin content by dividing by 0.35.

PROTEINS: Proteins were assayed by the method of Lowry et al. (51), except for analysis of the LDL complexes. Because the presence of Ca^{++} in the supernatants interfered with the Lowry procedure, the method of Bradford (52) was employed. Bovine serum albumin served as standard.

Light and Electron Microscopy

Lipid deposits in macrophage monolayers fixed in 2.5% glutaraldehyde were revealed by staining cultures with oil red O dissolved in triethyl phosphate as described by Gomori (53). The preparations were then lightly counterstained with fast green. The cells were photographed with a Zeiss photomicroscope (Carl Zeiss, Inc., New York) using Kodachrome 64 color film (Eastman Kodak Co., Rochester, NY).

For electron microscopy, the cultured macrophages were fixed for 3 h in ice-cold 2.5% glutaraldehyde in 100 mM cacodylate buffer, pH 7.4. The fixed cells were then scraped from the dishes with a rubber policeman and pelleted by centrifugation for 5 min in a Beckman 152 microfuge (Beckman Instruments, Inc., Fullerton, CA). The pellets were diced into small pieces with a razor blade and postfixed 1.5 h in ice-cold 1% OsO_4 in 100 mM cacodylate buffer, pH 7.4. The specimens were stained *en bloc* with uranyl acetate (54), then dehydrated in graded ethanol, treated with propylene oxide, and embedded in Epon 812 (Ladd Research Industries, Inc., Burlington, VT). Silver sections were cut, doubly stained with uranyl acetate and lead citrate before viewing in a Philips EM-300 electron microscope (Philips Electronic Instruments, Inc., Mahwah, NJ) operated at 80 kV.

Statistical Analysis

Mean cellular content of cholesterol, cholesteryl esters, and triacylglycerols in macrophages incubated under various conditions were compared separately with the use of single factor analysis of variance (55). When indicated by a significant F statistic, subsequent multiple pairwise comparisons were performed with the use of multiple range testing (55). Mean cellular ^{125}I-LDL in macrophages incubated with either ^{125}I-LDL or ^{125}I-LDL-Hp-Fn were compared with the use of two-factor analysis of variance (55). When indicated by a significant F statistic, the individual comparisons at 5 and 24 h were performed with the use of independent Student's *t* tests (55).

RESULTS

Properties of LDL Complexes with Heparin, Fibronectin, and Denatured Collagen

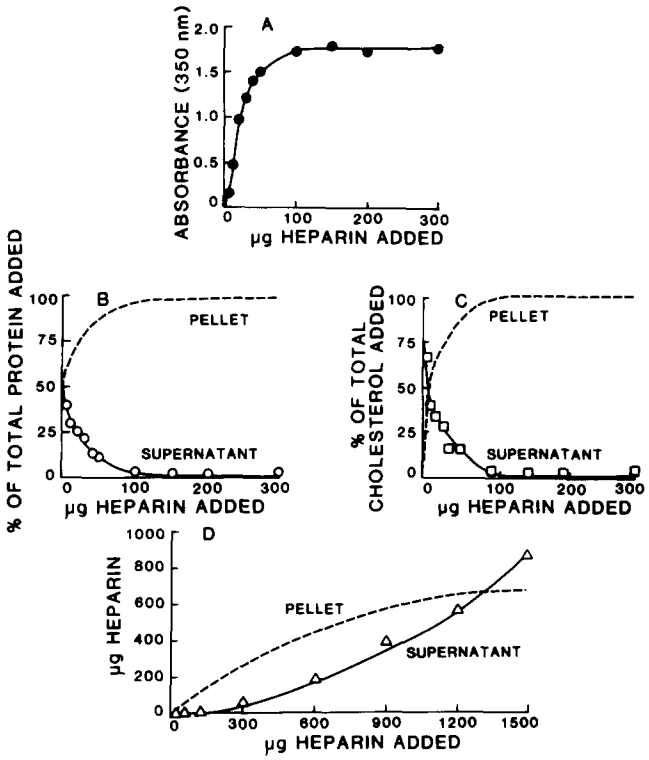

FIGURE 1 Characterization of LDL-heparin complexes. (*A*) Formation of LDL-heparin complexes. Heparin (5–300 μg) was added to 355 μg of LDL in H_2O (3.3 ml in total volume). The mixture became turbid with the addition of 500 μl of $CaCl_2$ (0.5 m). After gentle mixing, the preparations were allowed to stand undisturbed for 1 h at 4°C. Turbidity was measured as absorbance at 350 nm. (*B* and *C*) Precipitation of LDL as LDL-heparin complexes. Complexes were prepared as described above and allowed to stand undisturbed overnight at 4°C. The complexes were then pelleted by centrifugation (1,000 *g*, 20 min), and aliquots of the supernatants were removed for protein (*B*) and cholesterol (C) determination. Protein or cholesterol content of the pellet was calculated as content measured in the supernatant subtracted from the amount of LDL added to each tube (*dashed line*). (*D*) Heparin content of LDL-heparin complexes. Heparin (30–1,500 μg) was added to 2,130 μg of LDL protein in H_2O (3.5 ml in total volume). $CaCl_2$ (0.5 M) was added to the mixture to give a final concentration of 65 mM. The preparations were gently mixed, left standing overnight at 4°C, and pelleted as above. Aliquots of supernatants were assayed for uronic acid. Total heparin bound in the pellet (*dashed line*) was calculated as heparin assayed in the supernatant (heparin = uronic acid/0.35) subtracted from the amount of heparin added to each tube.

It is well recognized that LDL will form ionic bonds with sulfated glycosaminoglycans (41, 42). For the experiments described below, we prepared insoluble complexes of LDL-Hp-Fn-G. As shown in Fig. 1 *a*, LDL complexed with heparin upon addition of Ca^{++}. This is seen by the appearance of a turbid solution that is maximal at an approximate mass ratio of heparin to LDL protein of 1:3.5. When the complexes were allowed to stand undisturbed overnight in the cold, they aggregated and settled out of solution. Analysis of supernatants after pelleting of the insoluble complexes by centrifugation (Fig. 1, *B* and *C*) revealed the simultaneous disappearance of both LDL protein and cholesterol, indicating that intact lipoprotein was removed from solution by the addition of heparin and Ca^{++}. As shown in Fig. 1 *D*, nearly all the heparin added complexed to LDL until the mass ratio of 1:3.5 was attained. Then, we found unbound heparin remaining in the supernatants after removal of the complexes. In that the mass ratio of heparin to LDL in the pelleted complexes continued to increase at high concentrations of heparin, it appears that heparin molecules in addition to those required to precipitate LDL can bind to the complexes.

Aggregates of LDL-Hp-Fn could be produced by addition of purified fibronectin to a dilute suspension of LDL-heparin complexes. Fig. 2*A* shows the induction of turbidity upon addition of fibronectin. Because fibronectin added to a solution of LDL did not lead to an increase in absorbance until heparin was added, it appears that heparin binds to both LDL and fibronectin. To determine the amount of fibronectin bound, we prepared the same insoluble complexes with $^{14}CH_3$-NH-fibronectin. We found only a fraction of the added fibro-

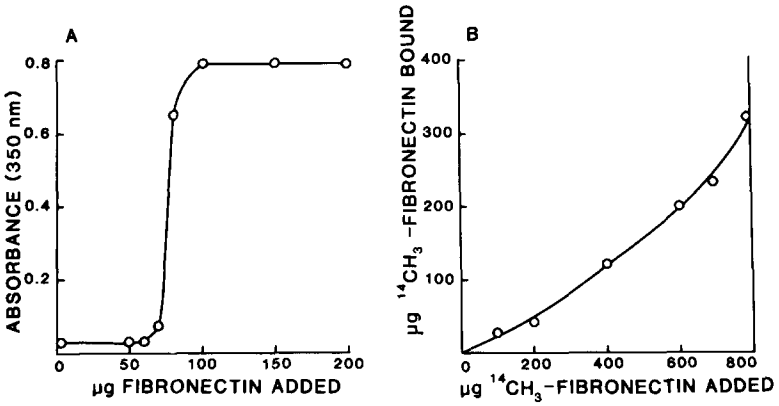

FIGURE 2 Binding of fibronectin to LDL–heparin complexes. (*A*) Formation of LDL-Hp-Fn. Heparin (20 μg) was added to 71 μg of LDL in 2.0 ml H_2O followed by 325 ml of $CaCl_2$ (0.5 M) and 50–200 μg of fibronectin. Total volumes were adjusted to 2,516 μl with H_2O and the mixture was gently vortexed. The preparations were allowed to stand undisturbed 1 h at 4°C and the turbidity to each then measured as absorbance at 350 nm. (*B*) Amount of fibronectin bound to LDL-heparin complexes. Heparin (100 μg) was added to 355 μg of LDL in 3.0 ml H_2O, followed by 500 μl of $CaCl_2$ (0.5 M). $^{14}CH_3$-fibronectin (100–800 μg) was then added and the mixture was gently vortexed. Total volumes were adjusted to 3.73 ml. The preparations were left standing overnight at 4°C, after which the complexes were pelleted by centrifugation (1,000 *g*, 20 min). Aliquots of the supernatants and pellets (the latter dissolved in 3.5 ml of 0.5 N NaOH) were counted for radioactivity. Recovery of radioactivity in supernatant plus pellet averaged 83.3 ± 9% (mean ± SD).

nectin bound in the complexes (Fig. 2*B*). Because the reductive alkylation of lysine amino groups used to prepare the $^{14}CH_3$-NH-fibronectin may have influenced the amount of binding observed, another procedure for labeling fibronectin was utilized. Under comparable experimental conditions (355 μg of LDL, 100 μg of heparin, 65 mM Ca^{++}, and 653 μg of derivatized fibronectin), thiol-acetylated fibronectin ($HOOC^{14}$-CH_2-S-fibronectin) and $^{14}CH_3$-NH-fibronectin were equally bound in the aggregates (28.0 vs. 26.1 μg bound/50 μg of LDL-heparin protein, respectively).

We also prepared LDL, heparin, and fibronectin complexes in 5% gelatin solution (LDL-Hp-Fn-G). The aggregates formed were more difficult to suspend after centrifugation. The suspensions consisted of large heterogeneous insoluble complexes reflecting, in part, the extremely strong noncovalent interaction between heparin, fibronectin, and denatured collagen (56).

We estimated the sizes of the complexes by filtration through filters with varying pore size. When prepared as a dilute suspension, complexes were retained by a 0.45-μm pore filter prewashed with albumin (1 mg/ml); whereas complexes were not retained by prewashed 1.2-μm pore filters. After filtration, if left undisturbed, complexes would aggregate. For the studies that follow, the various types of complexes were prepared by standardized procedures as described in the Materials and Methods section.

Lipid Accumulation in Cultured Macrophages Incubated with LDL-Hp-Fn or LDL-Hp-Fn-G

As illustrated in Fig. 3, cultured macrophages incubated 3 d with suspensions of LDL-Hp-Fn or LDL-Hp-Fn-G contained oil red O–positive droplets, indicating the accumulation of neutral lipids. The oil red O staining was present in all the cells of the cultures given the complexes. The staining of these cells was distinctly more intense than that observed for cells given LDL, but generally not as strong as that seen in the cultures given acetyl LDL.

Lipid analysis of the cultures confirmed the impressions given by the morphologic observations. However, analysis of cultures given LDL-Hp-Fn-G was complicated by the presence of LDL-Hp-Fn-G aggregates adhering to plastic and cells. The presence of adhering aggregates was indicated by the increased protein content of the cultures that received LDL-Hp-Fn-G compared with cultures given either LDL, acetyl LDL, or LDL-Hp-Fn, and extracellular material was evident

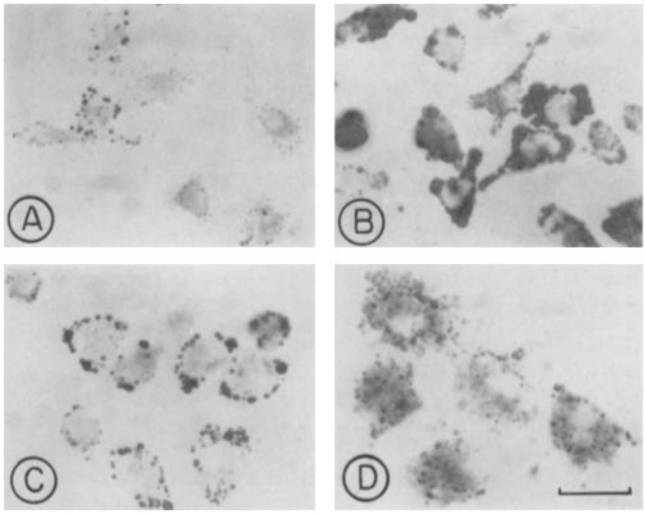

FIGURE 3 Lipid accumulation in culture macrophages incubated with LDL or LDL complexes. Appearance of macrophage cultures after incubation with 50 μg of LDL protein equivalent/ml of (*A*) LDL, (*B*) acetyl LDL, (*C*) LDL-Hp-Fn, or (*D*) LDL-Hp-Fn-G for 3 d. All preparations were twice-incubated for 15 min with 0.5% trypsin. They were then stained with oil red O to reveal lipid, and counterstained with fast green. Bar, 20 μm. × 476.

when the cultures receiving LDL-Hp-Fn-G were observed by microscopy. Extensive washing with DPBS was not sufficient to remove the adherent LDL-Hp-Fn-G but it could be removed by trypsin treatment of the cultures for 15–30 min (Table I). As shown in Table I, the intracellular cholesteryl ester content in macrophages varied significantly with incubation conditions ($P < 0.0001$). Pairwise comparisons of the data revealed that the cholesteryl ester content in cells receiving LDL-Hp-Fn was increased when compared with cells given LDL ($P < 0.001$). Macrophages incubated with acetyl LDL and LDL-Hp-Fn-G accumulated similar amounts of cholesteryl esters which were three to five times the content in cells given LDL ($P < 0.001$).

The cholesterol content of macrophages was not signficantly affected by the addition of acetyl LDL, LDL-Hp-Fn, or LDL-Hp-Fn-G. In contrast, the triacylglycerol content in cells varies significantly with incubation conditions ($P < 0.0025$). Pairwise comparisons of the data showed that only cells receiving acetyl LDL had an increased triacylglycerol content ($P < 0.05$). Under all conditions, the cellular lipid

TABLE I

Lipid Content of Cultured Macrophages Incubated 72 h with LDL, acetyl LDL, or LDL-Hp-Fn or LDL-Hp-Fn-G

Preparation	Cholesterol	Cholesteryl esters	Triacylglycerols
	μg of lipid/mg of cell protein		
LDL*	12.1 ± 2.3	3.7 ± 1.0	31.8 ± 3.6
Acetyl LDL	17.8 ± 7.1	18.9 ± 4.4	55.0 ± 16.1
LDL-Hp-Fn	14.5 ± 2.4	10.9 ± 1.5	28.0 ± 9.0
LDL-Hp-Fn-G	37.5 ± 4.2	37.3 ± 7.4	45.5 ± 2.7
+ 15-min trypsin treatment	17.6 ± 1.8	19.1 ± 4.7	39.7 ± 4.2
+ 30-min trypsin treatment	16.7 ± 1.0	14.7 ± 3.6	39.3 ± 2.9

Peritoneal cells (10^7) were dispensed into 60 × 15-mm plastic petri dishes, and monolayers of adherent cells were prepared as described in Materials and Methods section. On day 0, each culture received 2 ml of DME and 50 μg of LDL protein equivalent/ml of LDL, acetyl LDL, or suspensions of LDL-Hp-Fn or LDL-Hp-Fn-G. The medium and additives were replaced on days 1 and 2. On day 3, the cultures were washed three times with DPBS warmed to 37°C. Cultures receiving LDL-Hp-Fn-G were washed an additional three times with DPBS. Some cultures incubated with LDL-Hp-Fn-G were treated with 0.5% trypsin (2 ml) for 15 min. These dishes were either washed with DPBS or trypsinized again and then washed. The cultures were then assayed for their content of lipids and protein. The data represent mean ± standard deviation for five individual dishes, except for the trypsin-treated groups in which four individual dishes were analyzed.

* Lipid composition of LDL used for these studies: cholesterol, 16%; cholesteryl esters, 75%; triacylglycerols, 9%.

composition in percent was markedly different from the lipid composition of the LDL used for the incubations.

Effect of LDL-Hp-Fn and LDL-Hp-Fn-G on Lipid Synthesis in Cultured Macrophages

Cholesterol esterification in cultured mouse peritoneal macrophages is little affected when the cells are incubated with native LDL, but the uptake of chemically modified LDL is characteristically followed by a marked stimulation of cholesteryl ester synthesis (9, 11, 12, 14, 16). We measured the incorporation of [^{14}C]oleic acid into cholesteryl esters of cultured macrophages incubated with LDL, acetyl LDL, LDL-Hp-Fn, or LDL-Hp-Fn-G (Fig. 4). Incubation with LDL-Hp-Fn or LDL-Hp-Fn-G stimulated the macrophages to synthesize cholesteryl esters, compared with native LDL, but esterification in cells exposed to the complexes was only 1.5–3.0% of that which occurred in cells presented with acetyl LDL. Thus, the small increase in cholesterol esterification occurring in cells given LDL-Hp-Fn or LDL-Hp-Fn-G appears not to account for the cholesteryl ester mass accumulating in these cells after 3 d of incubation with the aggregates.

We also examined the incorporation of [^{14}C]oleic acid into triacylglycerols during a 5-h incubation of macrophage cultures with each of the four lipoprotein preparations used above. At all concentrations of lipoprotein employed, oleic acid incorporation into the triacylglycerols exceeded by 2–30 times the level of incorporation of label into cholesteryl esters, and, except for native LDL, incorporation of oleic acid into triacylglycerols appeared to be independent of the amount of lipoprotein present (data not shown). The latter results most likely reflect our observation that the rates of incorporation of oleic acid into the triacylglycerols were dependent on the concentration of oleic acid in the culture medium (data not shown). However, in the case of cholesteryl esters, under the conditions employed for the studies presented in Fig. 4, oleic acid incorporation into cholesteryl esters was not dependent on the oleic acid concentration in the medium.

Uptake and Degradation of LDL-Hp-Fn and LDL-Hp-Fn-G

To further elucidate the mechanism of lipid accumulation by macrophages incubated with LDL-Hp-Fn or LDL-Hp-Fn-G, we studied the uptake and catabolism of the complexes using ^{125}I-labeled LDL as a tracer. As shown in Table II, cells endocytosed four times as much acetyl LDL and >10 times as much LDL-Hp-Fn-G as compared with LDL over a 5-h period. Endocytosis of LDL-Hp-Fn, however, was not enhanced. In dramatic contrast to degradation of acetyl-LDL, a small proportion (<3%) of endocytosed LDL-Hp-Fn-G was degraded. The degradation of LDL-Hp-Fn was also reduced when compared with LDL (97% vs. 89%) resulting in a twofold increase in cell content ($P < 0.001$).

Over the next 19 h, macrophages continued to take up acetyl LDL in what appeared to be a linear manner. As was the case at 5 h, almost all of the internalized acetyl LDL was degraded. In contrast, macrophages did not continue to internalize LDL-Hp-Fn-G, but by 24 h, cells did degrade a greater proportion (33%) of endocytosed complex than observed at 4 h. There was a small increase in undegraded cellular LDL in macrophages incubated with LDL-Hp-Fn as compared with LDL ($P < 0.01$).

In these experiments more complex was added to the cells than could be fully ingested; therefore, a condition of incomplete internalization may exist. To assess the degradation of LDL-Hp-Fn-G under conditions of complete internalization, we exposed cells to complex for 1 h after which we stripped uningested material from the cell surface and we determined degradation of complex-derived ^{125}I-LDL over the next 4 h (Table III). Under these conditions macrophages degraded

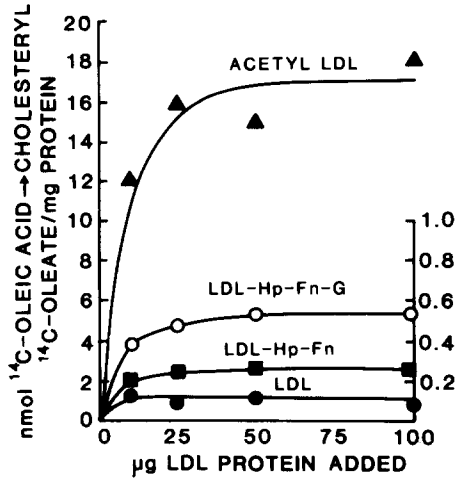

FIGURE 4 Cholesteryl ester synthesis in cultured macrophages incubated with various concentrations of LDL, acetyl LDL, LDL-Hp-Fn, or LDL-Hp-Fn-G. Peritoneal cells (4 × 10^6) were dispensed into 35-mm plastic petri dishes, and monolayers of adherent cells were prepared as described in Materials and Methods. On day 0, each culture received 1 ml of DME containing 100 nmol [^{14}C]oleate (9,404 cpm/nmol)–20 nmol albumin and appropriate quantities of either LDL, acetyl LDL, LDL-Hp-Fn or LDL-Hp-Fn-G. After incubation for 5 h at 37°C, the cellular content of cholesteryl [^{14}C]oleate was determined. Data represent the mean of determinations made on three separate cultures. Note difference in ordinate values for acetyl LDL (*left axis*) and LDL, LDL-Hp-Fn, LDL-Hp-Fn-G (*right axis*).

TABLE II

Uptake and Catabolism of ^{125}I-LDL, ^{125}I-Acetyl LDL, ^{125}I-LDL-Hp-Fn, and ^{125}I-LDL-Hp-Fn-G by Cultured Macrophages

Preparation	Incubation period	Uptake	Degraded	Cell content
	h		*μg of LDL/mg of cell protein*	
LDL	5	3.39 ± 0.53	3.24 ± 0.55	0.15 ± 0.04
Acetyl LDL	5	12.76 ± 1.29	11.80 ± 1.24	0.96 ± 0.13
LDL-Hp-Fn	5	3.25 ± 0.90	2.91 ± 0.87	0.34 ± 0.05
LDL-Hp-Fn-G	5	42.02 ± 5.36	1.16 ± 0.14	40.86 ± 5.37
LDL	24	5.99 ± 1.66	5.66 ± 1.68	0.33 ± 0.09
Acetyl LDL	24	57.37 ± 14.34	53.70 ± 14.32	3.68 ± 0.32
LDL-Hp-Fn	24	6.40 ± 1.41	5.88 ± 1.39	0.54 ± 0.09
LDL-Hp-Fn-G	24	45.57 ± 7.57	15.70 ± 6.31	29.87 ± 8.64

Mouse peritoneal cells (4 × 10^6) were dispensed into 35 × 10-mm plastic petri dishes and monolayers of adherent cells were prepared as described in Materials and Methods section. On day 0, each culture received 1 ml of DME and 50 μg of LDL protein equivalent/ml of ^{125}I-labeled LDL, acetyl LDL, LDL-Hp-Fn, or LDL-Hp-Fn-G. After a 5- or 24-h incubation, the culture medium was collected and assayed for trichloroacetic acid-soluble, noniodine activity. Trypsin (0.5%) was added to the dishes and incubated with the cells for 15 min. It was replaced with fresh trypsin and incubated for an additional 15 min. The cultures were washed with DPBS warmed to 37°C, and then assayed for content of ^{125}I and protein. The data represent the mean ± standard deviation for five individual dishes.

24% of the ingested material. Cells exposed to complex for the entire 5-h incubation period ingested slightly more complex; however, the fraction degraded was not significantly different from that observed in cells exposed to complex for 1 h. Therefore, presence of excess LDL-Hp-Fn-G was not responsible for the impaired degradation of complex-derived LDL observed in these studies.

Electron Microscopy

Macrophages cultured in the presence of LDL (Fig. 5*A*) appeared similar to that of control macrophages cultured in DME alone (not shown). Likewise, cells incubated in LDL-Hp-Fn (Fig. 5*C*) were very similar in appearance to the cells receiving LDL except for the apparent increase in cytoplasmic vacuoles containing flocculent material. In contrast to LDL and LDL-Hp-Fn, macrophages exposed to acetyl LDL (Fig. 5*B*) possessed numerous cytoplasmic lipid droplets and lysosomes containing whorls of membranous material. The cytoplasm of macrophages with LDL-Hp-Fn-G was packed with large phagocytic vacuoles (Fig. 5*D*). The content of the vacuoles (inset in Fig. 5*D*) is presumably the insoluble complex, which had a similar appearance in the extracellular medium (not shown).

DISCUSSION

Components of the extracellular matrix are thought to play an important role in atherogenesis (57). For example, the interaction between lipoproteins and the vascular extracellular matrix has been long recognized as important to lipid accumulation in atherosclerotic lesions (57). In this regard, the proportion of tightly bound (insoluble) LDL to extractable LDL is increased in atherosclerotic vessels (58, 59). The sequestration of LDL by the extracellular matrix may in part result from its interaction with sulfated glycosaminoglycans (41, 42, 60, 61) which have been demonstrated to co-distribute with collagen and fibronectin (62). Furthermore, components of the extracellular matrix have been demonstrated to influence cellular processes. Fibronectin, by nature of its multiple and diverse binding domains, promotes the endocytosis of gelatin-coated particles by macrophages (23–25), and has been suggested to play a role in phagocytosis of fibrin and components of the extracellular matrix by macrophages (27, 30). However, despite these findings, the effect of extracellular matrix on catabolism of LDL by arterial cells has been little considered (12, 14).

TABLE III

Effect of Uningested ^{125}I-LDL-Hp-Fn-G on the Catabolism of ^{125}I-LDL-Hp-Fn-G by Cultured Macrophages

Uningested complex	Uptake	Degraded	Cell content
	μg of LDL/mg of cell protein		
Absent	15.77 ± 3.01	3.70 ± 0.25	12.07 ± 2.93
Present	22.61 ± 2.76	6.82 ± 0.81	15.80 ± 2.11

Mouse peritoneal cells (2.5 × 10^6) were dispensed into 35 × 10-mm plastic petri dishes and monolayers of adherent cells were prepared as described in the Materials and Methods section. On the day of the experiment, each culture received 1 ml of DME and 50 μg of LDL protein equivalent of ^{125}I-LDL-Hp-Fn-G. After a 1-h incubation, the culture medium was removed from one set of dishes and uningested complex was removed by trypsinization (see Table II). Cultures then received 1 ml of DME and incubation continued for an additional 4 h. The culture medium from all dishes was then collected and assayed for trichloroacetic acid-soluble, noniodine activity. Cultures exposed to complex for the entire 5-h incubation were treated with trypsin (see Table II) and all cultures were assayed for content of ^{125}I and protein. The data represent the mean ± standard deviation for four individual dishes.

We investigated how components of the extracellular matrix might alter the catabolism of LDL by macrophages. For these purposes, we prepared insoluble complexes of LDL, heparin, fibronectin, and denatured collagen and tested their effects on lipid accumulation in macrophages. We have found that incubation of macrophages with a particulate suspension of LDL-Hp-Fn-G resulted in a dramatic increase in cytoplasmic oil red O-staining as compared with cells incubated with LDL alone. Subsequent lipid analysis indicated that cellular cholesteryl ester content in these cells was increased four- to fivefold. Further findings demonstrated that macrophages exhibited enhanced uptake of LDL sequestered in a matrix containing glycosaminoglycans, fibronectin, and collagen and that components of this matrix influence the catabolism of LDL leading to cholesteryl ester accumulation.

Early studies revealed that peritoneal macrophages readily phagocytosized large quantities of particulate complexes (0.5 μm) containing cholesteryl esters and albumin (63). The ingested cholesteryl esters were subsequently hydrolyzed by a lysosomal hydrolase and free cholesterol released into the medium. Later it was demonstrated that endocytosed LDL-cholesteryl esters are also hydrolyzed in the lysosome. The released cholesterol is either re-esterified (6, 9) to form drop-

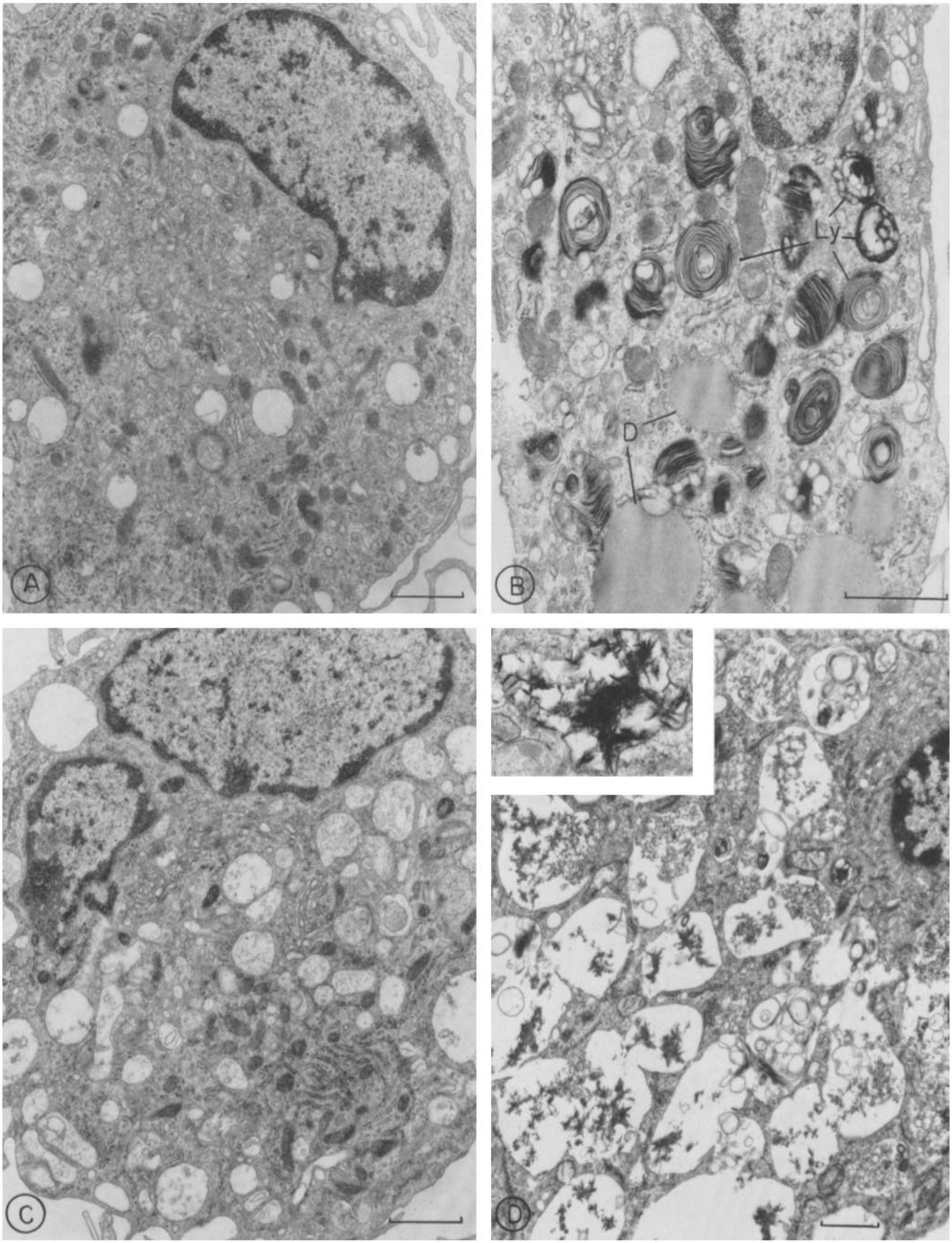

FIGURE 5 Appearance of cultured mouse peritoneal macrophages incubated with LDL, acetyl LDL, or LDL complexes for 3 d. All preparations were twice incubated for 15 min with 0.5% trypsin. (*A*) Macrophages incubated with native LDL. The cells' appearance is similar to macrophages cultured 3 d in DME alone (not shown). Some endocytic vacuoles are seen and, occasionally, cytoplasmic lipid droplets are found. (*B*) Macrophage incubated with acetyl LDL. Cytoplasmic lipid droplets (D) are numerous, and many lysosomelike structures (*Ly*) filled with myelinlike material are found. (*C*) Macrophage incubated with LDL-Hp-Fn. Numerous large vacuoles are seen containing flocculent material. (*D*) Macrophage incubated with LDL-Hp-Fn-G. The cytoplasm is filled with very large phagocytic vacuoles, most of which contain aggregates of electron-dense material similar in appearance to insoluble complexes found in the incubation medium. In some cases the aggregates appear as spicules (*inset*). Bar, 1 μm. (*A* and *C*) × 13,000; (*B*) × 19,000; (*D*) × 10,500; (*inset*) × 35,000.

lets, or excreted from the cell (64). The increased uptake of particulate cholesteryl ester-albumin coacervates or lipoprotein, as in the case of acetyl LDL, is characterized by a marked stimulation in cholesteryl ester synthesis (14). Based on the incorporation of [^{14}C]oleic acid into cholesteryl ester, the relatively small stimulation in cholesteryl ester synthesis observed in cells exposed to LDL-Hp-Fn-G does not appear to account for their increased cholesteryl ester content. Instead these results suggested that LDL-bound cholesteryl esters accumulated intracellularly thus indicating impaired lysosomal degradation. Experiments using ^{125}I-LDL incorporated into complexes as a tracer supported this interpretation by demonstrating that macrophages rapidly take up LDL-Hp-Fn-G but degradation of the internalized LDL was impaired. This is in contrast to particulate cholesteryl ester–albumin complexes which are ingested and readily degraded (14, 63).

Morphological observations confirm the conclusions reached from the biochemical studies. Cells stimulated to synthesize cholesteryl esters by exposure to acetyl LDL were shown to contain large lipid droplets by electron microscopy. In contrast, macrophages receiving LDL-Hp-Fn-G did not contain many lipid droplets. Instead, these cells contained large phagocytic vacuoles filled with material similar in appearance to the insoluble LDL complexes observed extracellularly.

The mechanism of enhanced uptake of LDL when incorporated into LDL-Hp-Fn-G complexes may simply reflect phagocytosis of insolubilized LDL. However, in that fibronectin (30) and heparin (65) receptors have been described for macrophages, specific adsorption of complexes to the cell surface may be important. In this regard, it is important to stress that the complexation of fibronectin, denatured collagen, and heparin is extremely strong and may have important biologic consequences (56). Indeed, fibronectin strongly stimulates the phagocytosis of gelatin-latex particles in the presence of heparin, whereas fibronectin alone is a weak stimulator and heparin alone has no effect (23, 25).

After uptake the observed impairment in LDL degradation may result from the failure of phagosome–lysosome fusion as reported for insoluble complexes of LDL and the sulfated polysaccharide–dextran sulfate (66). Many polyanions, including dextran sulfate (67), oxidized amylose (68), suramin, and poly-D-glutamic acid inhibit the fusion of phagosomes with lysosomes (69). Therefore, substances normally degraded by cells may escape digestion when coupled to a polyanion. For example, intracellular digestion of methotrexate–polylysine–heparin complexes was markedly inhibited as compared with complexes without heparin (70). Concerning the studies reported here, further experiments will be required to determine if LDL-Hp-Fn-G complexes are ingested intact, if heparin or other sulfated glycosaminoglycans modulate lysosomal degradative activity, and if there is specific adsorption of the complexes to the macrophages.

In summary, LDL when complexed to heparin, fibronectin, and denatured collagen is avidly taken up by macrophages. As compared with either LDL or acetyl LDL, the degradation of complex-derived LDL is impaired resulting in cholesteryl ester accumulation. Because substances similar to those used to form these complexes are present in the vascular wall, we believe these results indicate a potential role of extracellular matrix in foam cell development during altherogenesis.

The authors are indebted to Dr. Christian de Duve, Dr. David P. Hajjar, Dr. Annie Yau-Young, and Dr. Brian G. J. Salisbury for their valuable suggestions and assistance during the course of this study.

These studies were supported by research grants HL-18828, HL-07423, HL-18157, and HL-30055 from the National Heart, Lung and Blood Institute of the National Institutes of Health. Dr. Falcone is the recipient of an Andrew Mellon Teacher-Scientist Award and a National Institutes of Health New Investigator Award (HL-31099). Dr. Fowler was a recipient of an Otto G. Storm Established Investigatorship from the American Heart Association during the time these studies were performed.

A preliminary report of this work was presented at a meeting of the Federation of American Societies for Experimental Biology, Chicago, April 1983.

Received for publication 21 November 1983, and in revised form 4 May 1984.

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