Three Types of Naturally Occurring Modified Lipoproteins Induce Intracellular Lipid Accumulation Due to Lipoprotein Aggregation

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Low density lipoprotein (LDL) from patients with coronary atherosclerosis and diabetes mellitus as well as in vitro desialylated LDL, glycosylated LDL, and lipoprotein (a) caused a twofold to fourfold rise in cholesteryl ester in cultured human blood monocytes and intimal smooth muscle cells isolated from normal aorta. Native LDL from healthy subjects failed to induce intracellular lipid accumulation. We have demonstrated by laser correlative photometry and gel filtration chromatography that in vivo and in vitro modified lipoproteins form aggregates under cell culture conditions. The degree of modified lipoprotein aggregation directly correlated with the ability of these lipoproteins to elevate the cholesteryl ester content of cultured cells. Modified lipoprotein aggregates isolated by gel filtration induced a threefold to fivefold elevation in cellular cholesteryl ester content. Aggregates of ^{125}I-modified LDL were taken up and degraded fivefold to sevenfold more effectively as compared with nonaggregated lipoproteins. The uptake and degradation of ^{125}I-labeled aggregates were strongly inhibited by unlabeled aggregates, latex beads, and cytochalasin B but not by native or acetylated LDL. These data indicate that uptake of lipoprotein aggregates occurred by phagocytosis. Obtained results suggest that modified lipoprotein aggregation may be the key condition for lipid accumulation. (*Circulation Research* 1992;71:218–228)

Key Words • atherosclerosis • cell cultures • modified lipoproteins • lipoprotein aggregation • lipid accumulation

The accumulation of cholesteryl esters in vascular cells is one of the earliest manifestations of atherosclerosis. Despite the intensive investigative efforts made, the mechanisms underlying lipid deposition in the cells remain unclear. Low density lipoprotein (LDL) has been suggested to be a source of accumulated intracellular lipids.[1-3] However, native LDL isolated from the blood of healthy subjects fails to induce intracellular lipid accumulation in cultured cells.[4-6] At the same time, accumulation is observed in vitro when cells are incubated with LDL chemically modified by acetylation, methylation, glycosylation, oxidation, desialylation, or treatment with malondialdehyde, glutaraldehyde, or 4-hydroxynonenal.[3,5,7-13]

Recently we have reported that in vitro modified LDL forms aggregates under the conditions of cell culture.[14] A direct correlation between the degree of aggregation of oxidized, glycosylated, desialylated, or malondialdehyde-treated LDL and an increase in intracellular cholesteryl ester content was established. Furthermore, the removal of aggregates from modified LDL preparations by filtration resulted in a marked suppression of lipid accumulation in cultured cells. These findings allowed us to conclude that aggregation of in vitro modified LDL is a necessary step in intracellular lipid deposition.

At present, several types of modified LDL have been shown to occur in human blood. Curtiss and Witztum[15] have demonstrated the presence of nonenzymatically glycosylated LDL in the blood of hyperglycemic diabetic patients. We have recently shown that LDL isolated from the blood of atherosclerotic patients was able to induce intracellular lipid accumulation in cultured aortic cells[16,17] and differed from native LDL by a lower content of sialic acid; i.e., it appeared to be a desialylated lipoprotein.[11,12] Lipoprotein (a), which differs from LDL by the presence of an additional apoprotein is also considered to play an important role in the deposition of intracellular lipids.[18,19]

In the present study, we demonstrated that in vivo modified LDL caused lipid accumulation in cultured human intimal smooth muscle cells and monocytes. We tested the hypothesis that modified lipoprotein aggregates but not single particles caused intracellular lipid accumulation. To this end we showed that 1) in vivo modified LDLs were able to form aggregates, 2) these aggregates caused the accumulation of cholesteryl esters in cultured cells, and 3) the removal of aggregates from LDL preparation prevented the intracellular cholesteryl ester accumulation. We also attempted to examine the mechanism underlying the interaction between LDL aggregates and vascular cells.

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Materials and Methods

Materials

Fetal calf serum, Medium 199, fungizone, penicillin/streptomycin, and glutamine were obtained from GIBCO Europe, Paisley, UK. Kits for total and free cholesterol determinations were purchased from Boehringer Mannheim, Mannheim, FRG. Polycarbonate filters (pore diameters of 0.1, 0.22, and 0.45 μm) were from Nuclepore Corp., Pleasanton, Calif. Sodium iodide [^{125}I] was from Isotop Co., Moscow. Sepharose CL-2B was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Disposable tissue culture materials were purchased from Corning Glass Inc., Corning, N.Y. Microtiter plates were obtained from Costar Corp., Cambridge, Mass. All other reagents were from Sigma Chemical Co., St. Louis, Mo.

Preparation of Lipoproteins

LDL (1.019–1.050 g/cm^3) was isolated by sequential ultracentrifugation in a preparative ultracentrifuge after appropriate adjustment of density with solid NaBr[20] from the pooled blood of 12 healthy subjects, from 12 patients with coronary heart disease (CHD) with angiographically documented stenosis of coronary arteries, and from 12 non–insulin-dependent diabetic patients. The characteristics of CHD patients and healthy donors have been described previously.[16,17,21] Characteristics such as age and sex were similar in the groups of healthy donors and in patients with CHD and diabetes mellitus. The mean cholesterol levels were similar among the groups: 198±13, 195±10, and 184±5 mg/dl for CHD patients, diabetic patients, and healthy subjects, respectively. In the diabetic patients, the disease had been present for 3–12 (7.3±1.2) years, the fasting plasma glucose was 11.3±0.5 mmol/l, and the level of glycosylated hemoglobin (hemoglobin A_{Ic}) was 9.2±0.4%. None of the CHD patients or healthy donors had diabetes mellitus, and the fasting plasma glucose was 5.6±0.3 and 5.3±0.2 mmol/l, respectively.

Lipoprotein oxidation and proteolysis were prevented by the addition of 20 μM butylated hydroxytoluene and 1 mM phenylmethylsulfonyl fluoride to plasma. After recentrifugation, LDL preparations were dialyzed against phosphate-buffered saline (PBS), filtered through a 0.22-μm polycarbonate filter, and stored for 7 days. The preparations were refiltered immediately before each experiment. Glycosylation was performed by a 14-day incubation with 50 mM glucose.[22] LDL was desialylated by incubation with agarose-linked neuraminidase for 2 hours at 37°C.[11] Apolipoprotein B concentration was determined by an enzyme-linked immunosorbent assay as previously described.[23] The sialic acid content was determined by the method of Warren.[24] The extent of LDL glycosylation was evaluated as fructosyl lysine content by the method of Kruse-Jarres et al[25] using Boehringer Mannheim kits. The level of thiobarbituric acid (TBA)–reactive products in LDL preparations was determined according to Yagi.[26]

LDL was iodinated by the iodine monochloride method.[27] Over 98% of ^{125}I in labeled lipoprotein preparations was precipitated with 10% trichloroacetic acid.

Lipoprotein (a) was isolated from 1.050–1.120 g/ml lipoproteins of CHD patients by gel filtration on Sepharose 4B, according to Enholm et al.[28]

Determination of the Degree of Lipoprotein Aggregation and the Size of Lipoprotein Aggregates

The degree of lipoprotein aggregation was evaluated by the method based on the analysis of light transmission fluctuations in LDL suspension.[29] The relative dispersion of the optical density fluctuations caused by random changes in the number of particles in the optical channel reflects the deviations in their average size, i.e., the degree of aggregation. The optical density fluctuations were measured using a semiconductor laser with collimating optics (wavelength, 860 nm). The aggregate size was determined by methods of quasielastic laser scattering on an Autosizer 2 (Malvern Instrument, UK).

For the analysis of lipoprotein aggregation, native and modified lipoproteins were passed through a Sepharose CL-2B column (25×0.6 cm) at a flow rate of 0.15 ml/min. Fractions (0.30 ml) were collected, and total cholesterol content was determined in each fraction.

Examination of Lipoprotein–Lipoprotein Interactions

Ninety-six–well microtiter plates were precoated with freshly prepared native and modified lipoproteins (1 μg LDL protein per well) and incubated for 1 hour at 37°C. Then the wells were washed with 0.2% bovine serum albumin in PBS, and 0.01–100 μg/ml ^{125}I-LDL was added to each well. After a 1-hour incubation at 37°C, the wells were washed thoroughly with PBS, and radioactivity was measured.

Cell Culture

Subendothelial cells for culture were isolated from grossly normal intima by dispersion of human aortic tissue with 0.15% collagenase and suspended in the growth medium containing Medium 199, 10% fetal calf serum, 2 mM L-glutamine, and antibiotics. Cells were seeded into 24-well tissue culture plates at a density of 2–4×10^4 cells per 1 cm^2 of growth area.[30] The cells were cultured at 37°C in a humidified CO_2 incubator (95% air–5% CO_2). The primary cultures contained a mixed cell population made up primarily of typical and modified smooth muscle cells (>95%) as defined by the ultrastructural and immunofluorescent features. The medium was changed every day. Starting from the seventh day in primary culture, cells were incubated for 6 hours in medium containing native and modified LDLs (100 μg protein/ml) and 10% lipoprotein-deficient serum from a healthy donor (>1.250 g/ml) prepared by ultracentrifugation.[20] Human peripheral blood monocytes were isolated on Ficoll-paque gradient as described previously.[21] Monocytes were incubated for 6 hours in medium containing 50 μg/ml lipoprotein. Medium and lipoprotein preparations were filtered (pore size, 0.22 μm) immediately before their addition to the cell culture. After incubation, the cells were rinsed, and cellular lipids were determined as described below. Cellular protein content was determined by the method of Lowry.[31]

Determination of Lipids

Intracellular lipids were extracted with a mixture of *n*-hexane:isopropanol (3:2 vol/vol) as described elsewhere.[32] To determine the cholesteryl ester content, the amount of free and esterified cholesterol was measured

TABLE 1. Characteristics of Lipoprotein Preparations

Lipoprotein	Composition %					TBA-reactive substance level (nmol/mg apoB)	Sialic acid content (nmol/mg apoB)	Fructosyl lysine content (nmol/g apoB)
	Protein	PhL	Cho	TG	CE			
Healthy subjects								
LDL	20.5	23.1	12.5	5.5	38.3	1.1	30.8	23.9
Desialylated LDL	20.8	23.6	12.3	5.3	38.0	1.0	8.4	22.8
Glycosylated LDL	20.4	23.7	12.0	5.4	38.5	1.3	28.4	44.7
CHD patients								
LDL	22.0	22.8	12.2	6.0	37.0	1.0	10.2	21.7
Lipoprotein (a)	28.8	21.4	10.2	4.0	35.6	0.9	89.5	28.3
Diabetic patients								
LDL	21.5	21.6	12.2	6.2	38.5	1.2	15.8	31.9

PhL, phospholipids; Cho, free cholesterol; TG, triglycerides; CE, cholesteryl esters; TBA, thiobarbituric acid; apoB, apolipoprotein B; LDL, low density lipoprotein; CHD, coronary heart disease.

by the method of Siedel et al[33] using Boehringer Mannheim kits. Lipids were extracted from lipoproteins with a mixture of chloroform:methanol (2:1 vol/vol) according to Folch et al.[34] Phospholipids and neutral lipids were separated by thin-layer chromatography and measured by scanning densitometry.[30]

Examination of Lipoprotein Uptake and Degradation

Cells were incubated with labeled lipoprotein or labeled lipoprotein aggregates for 6 hours at 37°C. After incubation, an aliquot of the culture medium was taken to determine LDL degradation by the presence of the trichloroacetic acid–soluble (noniodide) ^{125}I.[3] To determine the ^{125}I uptake, cells were rinsed three times with PBS containing 0.2% albumin and seven times with PBS alone, after which they were dissolved in 0.1N NaOH to measure ^{125}I radioactivity.

Statistical Analysis

The significance of differences of group mean values was evaluated by multiple *t* test of one-way analysis of variance using a BMDP statistical program package.[35] The Bonferroni method was used to compare the experimental groups with the control group.[36]

Results

Characterization of Lipoprotein Preparations

Table 1 shows the characteristics of lipoprotein preparations examined in the present study. The protein and lipid compositions of LDLs obtained from healthy subjects and CHD or diabetic patients were essentially similar. There was no significant difference in the content of TBA-reactive substance between LDL preparations obtained from healthy subjects and from CHD and diabetic patients. The sialic acid content in the LDL of CHD patients was threefold lower compared with that in the LDL of healthy subjects. A high fructosyl lysine content in the LDL of diabetic patients that was due to enhanced glycosylation of LDL was also found (see Table 1).

In healthy subjects desialylation of LDL by treatment with agarose-linked neuraminidase lowered the sialic acid content to 29% of the initial level and had no effect on other parameters. Fructosyl lysine content was twofold higher, and TBA-reactive substance content was not changed in glycosylated in vitro LDL as compared with native LDL (Table 1).

As for lipoprotein (a), the protein/lipid ratio and the ratios between the major lipid classes were similar to those in native LDL. The sialic acid content was sevenfold higher in lipoprotein (a) than in donors' LDL, and the TBA-reactive substance content did not differ significantly from that of LDL.

Effect of Lipoproteins on Intracellular Lipid Accumulation

Figure 1 illustrates the effects of lipoproteins on the cholesteryl ester content in smooth muscle cells cul-

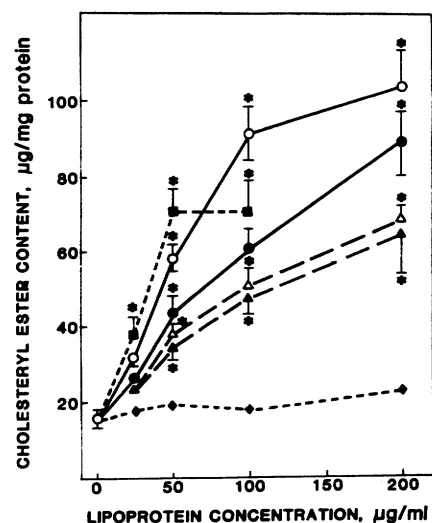

FIGURE 1. *Graph showing the effects of native and modified lipoproteins on the cholesteryl ester content in cultured smooth muscle cells from human aortic intima. Cells were incubated for 6 hours in Medium 199 containing 5% lipoprotein-deficient serum from healthy subjects and the indicated concentrations of low density lipoprotein from the following sources: healthy subjects (◆), glycosylated low density lipoprotein (▲), diabetic patients (○), lipoprotein (a) (■), patients with coronary heart disease (●), and desialylated low density lipoprotein (△). Control cells were cultured in Medium 199 containing 10% lipoprotein-deficient serum. Data are mean±SEM of four determinations.* *p<0.05 *compared with the control value.*

Table 2. Effect of Native and Modified Lipoproteins on Cholesteryl Ester Accumulation in Human Monocytes

	Cholesteryl ester content (μg/mg protein)
Control	6±1
Native LDL of healthy subjects	9±1
LDL of CHD patients	22±2*
LDL of diabetic patients	28±2*
Lipoprotein (a)	12±1*
Desialylated LDL	32±2*
Glycosylated LDL	14±1*

LDL, low density lipoprotein; CHD, coronary heart disease. Values are mean±SEM of four determinations.

Monocytes were incubated for 6 hours in Medium 199 containing 10% lipoprotein-deficient serum and freshly filtered (pore diameter, 0.22 μm) lipoprotein preparations at a concentration of 50 μg protein/ml. Control cells were incubated in Medium 199 containing 10% lipoprotein-deficient serum.

*$p<0.05$ vs. control.

tured from grossly normal human aorta. After 6 hours of incubation, the native LDL of healthy subjects induced no significant change in the intracellular cholesteryl ester content throughout the concentration range examined. By contrast, starting from a concentration of 25 μg/ml, the LDL of CHD patients significantly raised the cholesteryl ester content level in cultured cells. The intracellular cholesteryl ester content also increased after the cells had been incubated with the LDL of diabetic patients. Neuraminidase-treated as well as glycosylated LDL caused significant cholesteryl ester accumulation in cultured smooth muscle cells (Figure 1).

Lipoprotein (a) at a concentration of 25–100 μg/ml induced a twofold to threefold increase in esterified, but not free, cholesterol content of cultured smooth muscle cells (Figure 1). The 6-hour incubation of intimal cells with LDL from CHD or diabetic patients or with in vitro modified LDL produced no significant increase in the intracellular content of free cholesterol (data not shown).

We also examined the effects of LDL and lipoprotein (a) on cholesteryl ester content of human peripheral blood monocytes. LDL from CHD and diabetic patients and lipoprotein (a) at a concentration of 50 μg/ml promoted a twofold to threefold rise in intracellular cholesteryl esters in cultured monocytes (Table 2). Similar effects were observed when the cells had been incubated with neuraminidase-treated (desialylated) or in vitro glycosylated lipoproteins (Table 2).

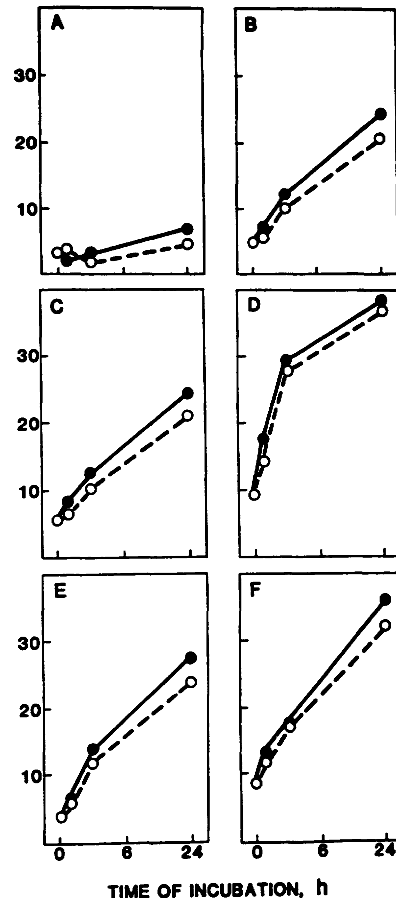

Figure 2. *Graphs showing the kinetics of changes in the average size (ordinate, in arbitrary units) of lipoprotein particles during incubation under the conditions of cell culture as determined by laser correlative photometry. Freshly filtered (pore size, 0.22 μm) preparations of low density lipoprotein were obtained from the following sources: healthy subjects* (panel A), *patients with coronary heart disease* (panel B), *desialylated low density lipoprotein* (panel C), *diabetic patients* (panel D), *glycosylated low density lipoprotein* (panel E), *and lipoprotein (a)* (panel F). *These preparations were incubated in Medium 199 containing 5% low density lipoprotein with (●) or without (○) smooth muscle cells from grossly normal human aortic intima for the indicated time period (in hours).*

Lipoprotein Aggregation Under the Conditions of Cell Culture

The alteration of lipoprotein particle average size under the conditions of cell culture was estimated by analyzing light transmission fluctuations in LDL suspensions. Figure 2 illustrates the kinetics of average particle size increase for lipoproteins incubated at 37°C in Medium 199 containing 5% lipoprotein-deficient serum in the absence of cultured cells. With native LDL isolated from the blood of healthy subjects, there was no appreciable change in the average size of particles up to 24 hours of incubation (Figure 2A). In contrast, with LDL from CHD or diabetic patients and with lipoprotein (a), the significant increase of the average particle size occurred within 6 hours of incubation, reflecting LDL aggregation (Figures 2B, 2C, and 2F); similar effects were observed for desialylated (Figure 2D) or glycosylated LDL (Figure 2E). We failed to detect any further increase of LDL aggregation when incubated at 37°C in the presence of intimal smooth muscle cells (Figure 2) or human monocytes (data not shown). A direct and strong correlation ($r=0.88$, $n=28$, $p<0.01$) was established between the degree of lipoprotein aggregation and cholesteryl ester accumulation in cultured intimal cells.

The formation of LDL aggregates during incubation was also demonstrated by gel filtration on Sepharose CL-2B. Figure 3 shows the elution profiles of LDL from healthy donors and from CHD and diabetic patients as well as modified LDL, either freshly filtered or incubated

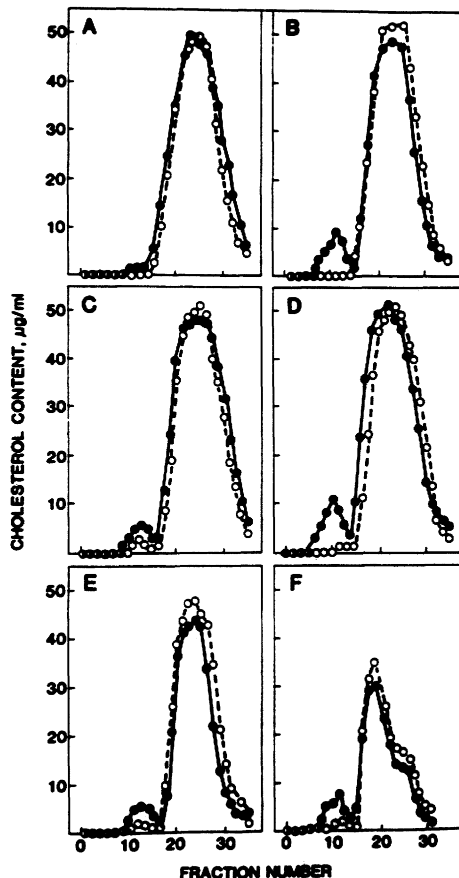

FIGURE 3. *Graphs showing the formation of low density lipoprotein aggregates of native and modified lipoproteins that were freshly filtered (pore size, 0.22 µm)* (○) *or preincubated for 6 hours at 37°C* (●). *Preparations of low density lipoprotein were obtained from the following sources: healthy subjects* (panel A), *patients with coronary heart disease* (panel B), *desialylated low density lipoprotein* (panel C), *diabetic patients* (panel D), *glycosylated low density lipoprotein* (panel E), *and lipoprotein (a)* (panel F). *These preparations were applied to a Sepharose CL-2B column (25×0.6 cm) at a flow rate of 0.15 ml/min. The total cholesterol content was determined in 0.30-ml fractions.*

for 6 hours with intimal cells. A subfraction with a higher molecular weight appeared after incubation of modified LDL and LDL from CHD and diabetic patients but not after incubation of LDL from healthy subjects. The total cholesterol content in this subfraction accounted for 3–7% of cholesterol applied to the column, suggesting that up to 7% of lipoprotein might aggregate within 6 hours of incubation. The diameters of glycosylated LDL, desialylated LDL, and lipoprotein (a) aggregates were 70–200, 100–500, and 200–400 nm, respectively, as determined by laser spectroscopy. The average diameter of LDL particles from healthy subjects was 27 nm, ranging from 24 to 29 nm. The average diameter of nonaggregated lipoprotein (a) particles was 35 nm.

LDL Binding to Native and Modified Lipoproteins

To determine whether native LDL can form a part of an LDL aggregate, we have examined the interaction between ^{125}I-labeled and modified lipoproteins. As seen

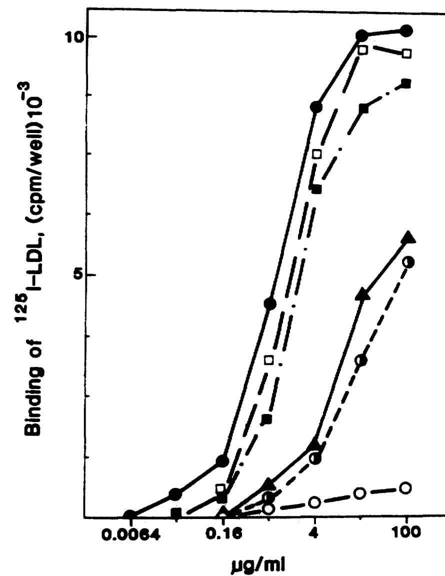

FIGURE 4. *Graph showing the interaction of native ^{125}I-labeled low density lipoprotein from healthy subjects (^{125}I-LDL) with native and modified lipoproteins. Freshly filtered (pore size, 0.22 µm) preparations of low density lipoprotein were obtained from the following sources: healthy subjects* (○), *patients with coronary heart disease* (□), *diabetic patients* (■), *desialylated low density lipoprotein* (●), *glycosylated low density lipoprotein* (◑), *and lipoprotein (a)* (▲). *These preparations were adsorbed on microtiter plates for 2 hours at 37°C. The wells were blocked by the addition of 2% bovine serum albumin, incubated for 1 hour with 0.01–100 µg/ml ^{125}I-LDL, and washed seven times with phosphate-buffered saline, and radioactivity was measured.*

from Figure 4, less than 0.01% of ^{125}I-LDL was bound to native lipoproteins. On the other hand, a fivefold to 10-fold more efficient binding was observed between ^{125}I-LDL and the LDL of patients or in vitro modified LDL. It can be concluded from these findings that native LDL can bind to modified LDL or lipoprotein (a) and thus participate in aggregate formation.

Effect of Aggregate Removal From LDL Preparations

To elucidate a contribution of lipoprotein aggregates in the accumulation of lipids by cultured cells, we decided to remove aggregated lipoproteins from LDL preparations. For this purpose, LDL preparations that were filtered and incubated for 6 hours at 37°C were passed again through polycarbonate filters with various pore diameters. Then LDL was added to cultured smooth muscle cells and incubated an additional 6 hours under the same conditions. Subsequently, LDL aggregation and intracellular cholesteryl ester content were analyzed. After the second filtration, the average diameters of aggregates in filtered preparations of lipoprotein (a), in vitro modified LDL, and patients' LDL were significantly reduced as compared with the diameters in the initial preparations (Table 3). At the same time, a lower cholesteryl ester accumulation was observed when cells were incubated with refiltered preparations. It should be pointed out that, after being passed through 0.1-µm filters, LDL preparations lost their ability to induce lipid accumulation in smooth muscle cells.

TABLE 3. Effect of Lipoprotein Filtration on the Accumulation of Cholesteryl Esters in Smooth Muscle Cells of Unaffected Human Aortic Intima and Human Blood Monocytes

	Pore size (μ m)	Average size (arbitrary units)	CE content (μg/mg protein)	
			SMCs	Monocytes
Control	...	...	41±3	8±1
Native LDL of healthy subjects	...	6.0	43±2	8±1
	0.45	6.2	40±2	9±1
	0.22	6.0	40±2	8±1
	0.10	5.9	42±2	8±1
LDL of CHD patients	...	27.7	123±2*	17±2*
	0.45	19.7	105±10*	13±2*
	0.22	12.6	75±5*	9±1
	0.10	7.9	52±6	9±1
LDL of diabetic patients	...	37.4	131±12*	26±2*
	0.45	32.4	128±6*	21±2*
	0.22	14.1	65±4*	16±1*
	0.10	7.8	46±4	10±2
Desialylated LDL	...	35.4	108±8*	21±2*
	0.45	18.1	73±6*	19±2*
	0.22	12.4	62±3*	15±1*
	0.10	7.8	41±2	12±1*
Glycosylated LDL	...	22.7	87±7*	12±1*
	0.45	14.5	56±4*	9±1
	0.22	9.5	49±3	7±1
	0.10	6.7	39±4	8±1
Lipoprotein (a)	...	25.4	92±6*	14±1*
	0.45	19.6	73±6*	12±1*
	0.22	14.5	58±2*	8±1
	0.10	8.1	38±4	8±1

CE, cholesteryl ester; SMCs, smooth muscle cells; LDL, low density lipoprotein; CHD, coronary heart disease. Values are mean±SEM of four determinations.

Preparations of freshly modified LDL were filtered through 0.22-μm filters and preincubated for 6 hours in Medium 199 containing 5% lipoprotein-deficient serum (0.5 mg apolipoprotein B/ml). After preincubation, lipoprotein preparations were carefully filtered through 0.1-μm, 0.22-μm, and 0.45-μm filters and added to cells at a concentration of 0.1 mg/ml. After 6 hours of incubation, cells were rinsed, and their CE content was measured.

*$p<0.01$ vs. control.

Experiments with a continuous filtration of the culture medium containing modified LDL were also performed. A schematic diagram of the apparatus for continuous filtration is shown in Figure 5. Medium 199 containing 5% lipoprotein-deficient serum and LDL preparations were filtered (pore size, 0.1 μm) for the whole incubation period at a flow rate of 0.5 ml/min. Continuous filtration of the culture medium abolished cholesteryl ester accumulation in cultured intimal smooth muscle cells, whereas the cholesteryl ester content of cells incubated with lipoprotein (a), modified LDL, and patients' LDL in the absence of continuous filtration increased approximately twofold (Figure 6).

Gel filtration was also used to remove LDL aggregates. Preparations of lipoprotein (a), in vitro modified LDL, and patients' LDL caused intracellular lipid deposition in intimal smooth muscle cells initially, but when passed through a Sepharose CL-2B column, they lost their ability to exert such an effect (Table 4).

Effect of Aggregates of Modified LDL on Intracellular Cholesteryl Ester Accumulation

The subfractions of aggregated LDL isolated from total LDL preparations by gel filtration were incubated with smooth muscle cells to determine their effect on intracellular lipid accumulation. As follows from the data presented in Table 4, CHD or diabetic patients' LDL aggregates as well as lipoprotein (a) aggregates

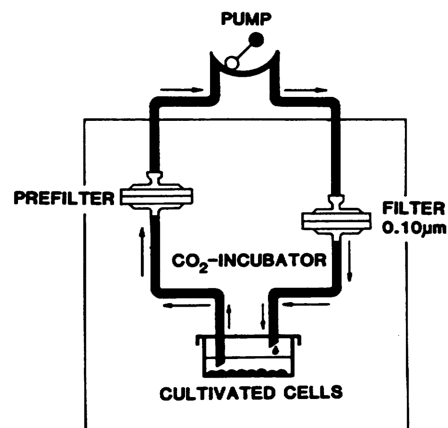

FIGURE 5. *Schematic diagram of the system for filtration of the culture medium.*

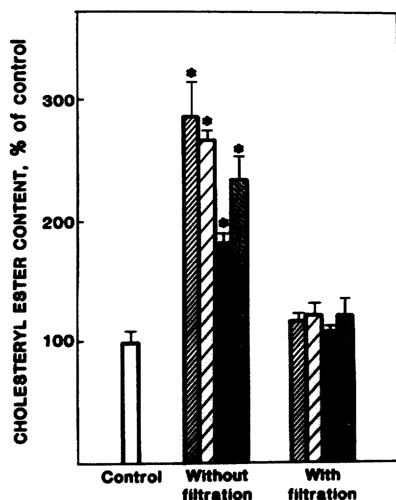

FIGURE 6. *Bar graph showing the effect of constant filtration of the culture medium on cholesteryl ester content in human aortic intimal smooth muscle cells. Smooth muscle cells from grossly normal human aortic intima were cultured for 6 hours in Medium 199 containing 10% lipoprotein-deficient serum and 100 μg/ml low density lipoprotein preparations from the following sources: patients with coronary heart disease (widely hatched bars), diabetic patients (densely hatched bars), desialylated low density lipoprotein (stippled bars), and glycosylated low density lipoprotein (solid bars). In some cultures the medium was filtered through a 0.1-μm filter throughout the whole period of incubation. Control cells were cultured in low density lipoprotein–free medium (open bar). Each value is the mean±SEM of three determinations.* *p<0.05 *compared with the control value.*

induced a fourfold to fivefold rise in intracellular cholesteryl ester content. Aggregates of in vitro desialylated and glycosylated LDL produced the same effect (Table 4). Similar results were obtained for cultured human peripheral blood monocytes (Table 5).

TABLE 5. **Effect of Aggregated Lipoprotein on the Cholesteryl Ester Content in Cultured Human Peripheral Blood Monocytes**

Sources of aggregates	Cholesteryl ester accumulation (% above control)
LDL of CHD patients	307±18*
LDL of diabetic patients	389±45*
Lipoprotein (a)	285±14*
Desialylated LDL	405±40*
Glycosylated LDL	288±23*

LDL, low density lipoprotein. Values are mean±SEM of four determinations.

Human peripheral blood monocytes were incubated for 6 hours in Medium 199 containing 10% lipoprotein-deficient serum and 50 μg/ml aggregated lipoproteins. Aggregated lipoproteins were obtained as indicated in the legend to Table 4. Control cells were incubated in the absence of lipoprotein aggregates.

*$p<0.05$ vs. the control value.

Uptake and Degradation of LDL Aggregates

The uptake of aggregates of desialylated or glycosylated ^{125}I-LDL by smooth muscle cells was fivefold to sevenfold higher than the uptake of native LDL or aggregate-free LDL preparations (Table 6). Interestingly, the uptake of modified LDL after the removal of aggregates was nearly the same as that of native LDL.

Degradation of aggregates of modified ^{125}I-LDL by cultured smooth muscle cells was approximately fourfold to fivefold greater than that of native LDL or aggregate-free LDL preparations (Table 6). Degradation of aggregate-free preparations of modified LDL was similar to that of native lipoproteins.

Similar results were obtained with cultured monocytes (Table 6). The uptake of aggregates of desialylated and glycosylated ^{125}I-LDL by intimal cells was threefold to fourfold greater than that of native LDL. The degradation rate for aggregated LDL by monocytes was twofold to fourfold greater than that for native LDL (Table 6).

To determine whether the receptors for native and modified LDL were involved in the interaction between cells and LDL aggregates, we examined the effect of excess of native unlabeled LDL on uptake and degradation of ^{125}I-LDL aggregates. A 20-fold excess of native or acetylated unlabeled LDL had no effect on uptake and degradation of ^{125}I-LDL aggregates (Table 7). On the other hand, the addition of a 20-fold excess of aggregated unlabeled LDLs resulted in a notable de-

TABLE 4. **Effects of Aggregated and Nonaggregated Low Density Lipoproteins on the Cholesteryl Ester Content in Cultured Smooth Muscle Cells**

	Cholesteryl ester accumulation (% above control)		
	Total lipoprotein	Nonaggregated lipoprotein	Aggregated lipoprotein
Native LDL of healthy subjects	7±12	4±7	...
LDL of CHD patients	103±7*	17±11	311±19*
LDL of diabetic patients	187±20*	5±8	323±14*
Lipoprotein (a)	84±6*	15±6	345±28*
Desialylated LDL	215±23*	19±12	305±17*
Glycosylated LDL	82±6*	3±6	308±10*

LDL, low density lipoprotein; CHD, coronary heart disease. Values are mean±SEM of four determinations.

Freshly filtered lipoprotein preparations were preincubated for 6 hours at 37°C. Aggregated and nonaggregated modified lipoproteins were separated by gel filtration on a Sepharose CL-2B column (1.6×90 cm) at a flow rate of 1.5 ml/min. Fractions of aggregated and nonaggregated lipoproteins were pooled and examined (concentration, 100 μg/ml) for their ability to induce cholesteryl ester accumulation in cultured smooth muscle cells from grossly normal human aortic intima.

*$p<0.05$ vs. control value.

Table 6. Uptake and Degradation of Aggregated and Nonaggregated Low Density Lipoproteins by Human Aortic Smooth Muscle Cells

	Uptake after 6 hours (nmol/mg protein)		Degradation after 6 hours (nmol/mg protein)	
	SMCs	Monocytes	SMCs	Monocytes
Healthy subjects				
Total ^{125}I-LDL	302+25	210+11	217+34	225+17
CHD patients				
Total ^{125}I-LDL	627+32*	407+39*	486+41*	486+27*
Nonaggregated ^{125}I-LDL	356+41	276+29	205+27	198+15
^{125}I aggregates	2,137+284*	1,245+137*	1,083+172*	735+74*
Desialylated LDL				
Total ^{125}I-LDL	715+63*	493+25*	457+26*	475+45*
Nonaggregated ^{125}I-LDL	417+52*	226+32	286+27	293+36
^{125}I aggregates	2,249+189*	1,347+106*	1,238+79*	863+92*
Glycosylated LDL				
Total ^{125}I-LDL	485+14*	356+13*	302+18*	452+33*
Nonaggregated ^{125}I-LDL	298+32	178+22*	242+27	235+21*
^{125}I aggregates	1,743+152*	853+106*	1,045+92*	672+15*

SMCs, smooth muscle cells; LDL, low density lipoprotein; CHD, coronary heart disease. Values are mean±SEM of four determinations.

Aggregated and nonaggregated ^{125}I-LDLs were separated by gel filtration on a Sepharose CL-2B column. Cells were cultured in the presence of 5 μg/ml isolated ^{125}I-LDL for 6 hours.

*$p<0.05$ vs. corresponding value for healthy subjects.

crease in the uptake of ^{125}I-LDL aggregates by intimal cells and monocytes.

To determine whether the uptake of LDL aggregates was mediated by phagocytosis, we examined the influence of the phagocytosis inhibitor cytochalasin B on the metabolism of ^{125}I-LDL aggregates by cultured cells. This agent inhibited uptake and degradation of ^{125}I-LDL aggregates by both smooth muscle cells and monocytes (Table 7), without any significant change in the uptake and degradation rate for the native LDL of healthy subjects.

Marked suppression (twofold to threefold) of uptake and degradation of ^{125}I-LDL aggregates by latex beads also confirmed the crucial role of phagocytosis in these processes in the case of cultured smooth muscle cells and monocytes (Table 7).

The aggregate–cell interaction is probably independent of the type of LDL modification. This is confirmed by the fact that the uptake of modified LDL aggregates can be suppressed by an excess of aggregated LDL modified in any other manner (Figure 7).

Discussion

In the present study we have demonstrated that the glycosylated LDL of diabetic patients, desialylated LDL

Table 7. Effects of Lipoproteins and Agents on the Uptake and Degradation of ^{125}I-Labeled Low Density Lipoprotein From Healthy Subjects and Aggregated ^{125}I-Labeled Low Density Lipoprotein From Patients With Coronary Heart Disease

	Uptake (% of control)		Degradation (% of control)	
Additions	SMCs	Monocytes	SMCs	Monocytes
^{125}I-LDL of healthy subjects				
Cytochalasin B	78±6	82±5	76±8	80±4
LDL of healthy subjects	49±5*	56±8*	54±3*	61±5*
Acetylated LDL	82±4	85±7	79±8	86±4
Aggregates of LDL of CHD patients	78±7	86±5	82±5	97±6
Aggregates of ^{125}I-LDL of CHD patients				
Cytochalasin B	28±2*	19±1*	23±1*	22±2*
Latex beads	39±4*	36±5*	24±3*	25±2*
LDL of healthy subjects	79±7	85±4	86±5	83±8
Acetylated LDL	93±6	88±8	92±5	89±7
Aggregates of LDL of CHD patients	32±5*	25±3*	24±2*	19±2*

SMCs, smooth muscle cells; LDL, low density lipoprotein; CHD, coronary heart disease. Values are mean±SEM of four determinations.

SMC from human aortic intima and human peripheral blood monocytes were cultured in Medium 199 containing 10% lipoprotein-deficient serum and either 5 μg/ml ^{125}I-LDL from healthy subjects or aggregated ^{125}I-LDL from CHD patients. Control cells were cultured without any additions.

*$p<0.05$ vs. the control value.

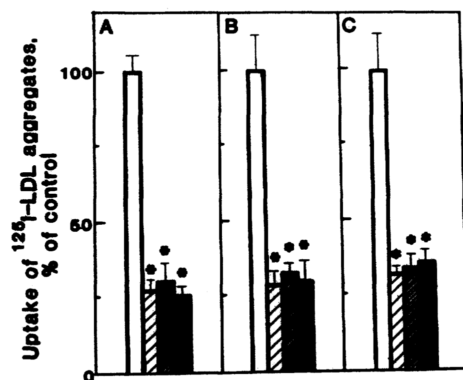

FIGURE 7. *Bar graphs showing the effect of unlabeled low density lipoprotein aggregates on the uptake of aggregated ^{125}I-labeled low density lipoprotein (^{125}I-LDL). Human aortic smooth muscle cells were incubated for 6 hours with 5 μg/ml aggregated ^{125}I-LDL from patients with coronary heart disease* (panel A), *from desialylated low density lipoprotein* (panel B), *or from glycosylated low density lipoprotein* (panel C) *in the absence (control, open bars) or in the presence of 100 μg/ml of unlabeled aggregated low density lipoprotein from the following sources: patients with coronary heart disease (widely hatched bars), desialylated low density lipoprotein (densely hatched bars), or glycosylated low density lipoprotein (solid bars). Each value is the mean±SEM of four determinations.* $^{*}p<0.05$ *compared with the control value.*

of CHD patients, and lipoprotein (a) induce the accumulation of cholesteryl esters in cultured human smooth muscle aortic cells and peripheral blood monocytes. It follows that all known modified forms of LDL isolated from human blood caused lipid accumulation regardless of the type of modification, suggesting that similar mechanisms may govern lipid accumulation that is promoted by different modifications of LDL in vivo. In a previous work we showed that in vitro modified LDL can aggregate and that aggregation of LDL plays a crucial role in intracellular lipid accumulation.[11] We suggested that aggregation of in vivo modified LDL also is an important step in the deposition of intracellular lipid.

In this study, we provide evidence that LDL and lipoprotein (a) isolated from the blood of patients with CHD or diabetes mellitus can aggregate. By contrast, native LDL of healthy subjects does not aggregate under the conditions of cell culture used in this study. Thus, lipoprotein modification appears to be a necessary step in the formation of LDL aggregates. The mechanism of lipoprotein aggregation as yet remains unclear. The changes in the lipid or protein moieties leading to aggregation are unknown. These changes include an extremely wide range of chemical modifications. For example, in earlier work we have also demonstrated the aggregation of oxidized and malondialdehyde-treated LDLs.[11] On the other hand, we have found that modified lipoproteins bind to native LDLs, consistent with the findings of Ye et al[37] that LDLs bind to lipoprotein (a) and other apolipoprotein B–containing lipoproteins. Considered together, these data suggest that aggregates may consist of both modified and native LDLs.

The diameter of LDL aggregates varied from 50 to 500 nm. Assuming that the diameter of an LDL particle (25–27 nm) is not changed during aggregation and that the aggregate has a spherical form, one can estimate the number of particles forming the aggregate. The calculated number varies from 5 to 4,000 LDL particles per aggregate. The major proportion of aggregates would contain about 500 particles; thus, the uptake of one aggregate would be equal to deposition of several hundreds or thousands of LDL particles, which may account for the high rate of intracellular lipid accumulation when cells are incubated with aggregated LDL.

We have shown that removal of aggregates from the cultural medium abolished cholesteryl ester accumulation in cells. On the other hand, the accumulation was apparently promoted by aggregated LDL, because isolated LDL aggregates induced significant cholesteryl ester deposition regardless of the type of LDL modification. It follows from these observations that lipoprotein aggregation is an essential step in the process of intracellular lipid accumulation.

The uptake of aggregated LDL was severalfold more efficient than that of nonaggregated particles. It should be mentioned that the uptake of aggregated ^{125}I-labeled LDL was not significantly suppressed by an excess of native or acetylated LDL, suggesting that uptake of aggregates is not mediated by B,E-receptors, or scavenger receptors. Our data concerning the lack of effect of acetylated LDL on the uptake of spontaneously formed LDL aggregates agree with the results of Khoo et al[38] obtained in experiments with aggregates produced by vortexing an LDL suspension. On the other hand, they have also shown that a 20-fold excess of aggregated LDLs strongly suppresses the degradation of native LDL, which may indicate that the aggregate binding and uptake are mediated by B-receptors. But the calculation of aggregate number in the incubation medium (assuming an average diameter of aggregate to be ≈ 1 μm) shows the amount of aggregates to be approximately two orders lower than the number of native LDL particles. One can suggest that the lower rate of degradation of native ^{125}I-LDL is due to the aggregate-produced inhibition of LDL metabolism within the cell rather than to occupation of the major proportion of B,E-receptors by aggregates.

As other evidence of aggregate uptake via B,E-receptor, Khoo et al[38] adduced the data that heparin is able to inhibit ^{125}I-LDL aggregate degradation and that rate of methylated LDL degradation is lower as compared with native LDL aggregates. Heparin is a well-known inhibitor of LDL–receptor interaction. However, we have found that heparin induces the reduction of preformed LDL aggregate size and prevents the formation of new aggregates (authors' unpublished data). This fact can explain the lower rate of ^{125}I-LDL aggregate degradation in the presence of heparin. The number and diameter of aggregates formed by the vortexing of methylated LDL are lesser than those in the case of native LDL. It has been shown that smaller size and lower concentration of particles result in decreased uptake by macrophages.[39] Thus, the lower degradation of methylated LDL aggregates cannot be accepted as evidence for the aggregate uptake via the B,E-receptor. The possibility that a part of LDL aggregates is bound and internalized via the apolipoprotein

B,E-receptor cannot be excluded; however, we propose that the major proportion of aggregates does not enter the cell via this receptor. Definitive data on the role of apolipoprotein B,E-receptors and scavenger receptors in LDL aggregate uptake may be obtained in the experiments using anti-receptor antibodies.

Excess of unlabeled aggregates or latex beads decreased the uptake of ^{125}I-labeled lipoprotein aggregates. Also, cytochalasin B, an inhibitor of macrophage phagocytosis, decreased uptake and degradation of aggregates. These findings strongly suggest phagocytosis to be the mechanism responsible for the uptake of spontaneously formed LDL aggregates.

Fogelman et al[8] were the first to demonstrate that LDL cross-linked by glutaraldehyde induced cholesteryl ester accumulation in human monocytes/macrophages. Recently, Hoff et al[40] have reported that 4-hydroxynonenal produced upon lipid peroxidation is capable of lipoprotein cross-linking. Lipoprotein aggregates thus formed elevate the cholesteryl ester level in cultured macrophages; i.e., aggregates of covalently bound LDL exhibit atherogenicity. Khoo et al[38] have reported that aggregates prepared by vortexing LDL suspension promote lipid accumulation in macrophages but not in smooth muscle cells. We have found that in vitro modified LDLs spontaneously aggregate under the conditions of cell culture and that this aggregation is a necessary step in the process of cholesteryl ester accumulation within cultured human aortic smooth muscle cells. It was shown that in vivo modified LDLs also aggregate under these conditions.

At the present time, besides LDL aggregates, associates of lipoproteins and various connective tissue components, such as proteoglycans,[41,42] collagenase-resistent debris,[43] heparin-fibronectin–denaturated collagen,[44] and elastin[45] have been described. All of these associates are able to induce lipid accumulation in macrophages and smooth muscle cells. Associates between LDL and the artificial compounds dextran sulfate[46] or latex beads[43] also induce intracellular lipid accumulation. LDL–antibody complexes elicit the same effect.[45,47] Taken together, these data indicate that lipid accumulation in the cell occurs after the formation of essentially large LDL-containing particles. We assume that the formation of lipoprotein–lipoprotein complexes (aggregates) or lipoprotein–matrix component complexes (associates) with subsequent uptake of these complexes by phagocytosis is the major mechanism underlying massive accumulation of cholesterol esters in human aortic cells.

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