Sialic acid content of human low density lipoproteins affects their interaction with cell receptors and intracellular lipid accumulation

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Abstract Low density lipoproteins (LDL) isolated from the plasma of patients with angiographically demonstrable coronary heart disease (CHD) induced accumulation of triglycerides, free cholesterol, and cholesteryl esters in cultured macrophages, smooth muscle cells, and endothelial cells derived from uninvolved intima of human aorta, but not in skin fibroblasts or hepatoma cells. The sialic acid content of LDL from CHD patients was 40–75% lower than that from healthy donors. There was a negative correlation between LDL sialic acid content and the LDL-induced accumulation of total intracellular cholesterol. Neuraminidase treatment of LDL from normal healthy donors produced sialic acid-depleted LDL (Ds-LDL) which was able to stimulate intracellular lipid accumulation. Neuraminidase treatment of LDL from CHD patients further increased its capacity to induce intracellular lipid accumulation. Sialic acid-poor LDL isolated by affinity chromatography of LDL from CHD patients induced a 2- to 4-fold increase of free and esterified cholesterol in human intimal smooth muscle cells. Binding, uptake, and degradation of ^{125}I-labeled Ds-LDL by macrophages and endothelial cells were 1.5- to 2-fold higher than for native LDL. Binding and uptake of Ds-LDL was inhibited 64–93% by the addition of 20-fold excess acetylated LDL (Ac-LDL); in the inverse experiment, the level of inhibition was 35-54%. These data indicate that a sialic acid-poor form of LDL isolated from CHD patients can interact with both native and scavenger LDL receptors. A sialic acid-poor form of LDL may be a naturally occurring ligand that interacts with the scavenger receptor(s) on macrophages and endothelial cells.—**Orekhov, A. N., V. V. Tertov, I. A. Sobenin, V. N. Smirnov, D. P. Via, J. Guevara, Jr., A. M. Gotto, Jr., and J. D. Morrisett.** Sialic acid content of human low density lipoproteins affects their interaction with cell receptors and intracellular lipid accumulation. *J. Lipid Res.* 1992. **33:** 805–817.

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The reduction of LDL sialic acid content by neuraminidase treatment may confer potentially atherogenic properties on this lipoprotein; this change of LDL may mimic a modification that occurs in vivo (1, 2). Unlike native LDL, neuraminidase-treated LDL (Ds-LDL) induces lipid (preferably cholesteryl ester) accumulation in cultured human aortic cells, which represents a step toward their transformation into foam cells. Although it has been shown that LDL that has undergone in vitro chemical modification leads to foam cell formation (1, 3), a chemically modified form of LDL has not yet been isolated from the blood of CHD patients. We have found that a large fraction of LDL circulating in the blood of patients with coronary atherosclerosis has a reduced sialic acid content (1, 4). This sialic acid-poor form of LDL has been shown to stimulate intracellular lipid accumulation in cultured cells, while LDL obtained from normal healthy donors, which is more highly sialylated, had no effect on this phenomenon. Our results suggest that a sialic acid-poor form of LDL (SAP-LDL) may be a potentially atherogenic lipoprotein occurring in vivo.

The present study confirms our earlier observations that LDL isolated from the blood of a patient with coronary artery disease has reduced sialic acid content, and further demonstrates that a sialic acid-poor form of LDL has the capacity to induce intracellular lipid accumulation. The interaction of LDL preparations having different sialic acid content with cultured cells is described in this study.

Abbreviations: Ac-LDL, acetylated low density lipoproteins; BSA, bovine serum albumin; CHD, coronary heart disease; DMEM, Dulbecco's modified Eagle medium; Ds-LDL, neuraminidase-treated LDL resulting in depletion of sialic acid; FCS, fetal calf serum; LDL, low density lipoproteins isolated at density 1.020–1.063 g/ml; LPDS, lipoprotein-deficient serum; RCA, ricin agglutinin; SAP-LDL, sialic acid-poor LDL isolated from plasma of CHD patients; SAR-LDL, sialic acid-rich LDL isolated from plasma of CHD patients; TBARS, thiobarbituric acid-reactive substance.

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MATERIALS AND METHODS

LDL isolation and characterization

LDL was isolated from blood drawn in Moscow or Houston after subjects had fasted overnight for 18 h. The donor group included healthy male individuals between 30 and 50 years of age. These individuals had no history of ischemic heart disease and showed no signs of myocardial ischemia upon physical examination or during physical exercise (stress test). The group of coronary artery disease (CHD) patients was composed of males of 35–50 years of age with effort angina pectoris of the II–IV functional class as determined by the method of Campeau (5). The degree of stenosis of one to three coronary arteries assessed by selective coronary arteriography was 75% or higher. The total plasma cholesterol levels varied from 190 to 220 mg/dl for CHD patients and from 160 to 225 mg/dl for healthy subjects. None of the donors had diabetes mellitus or arterial hypertension. In Moscow, subjects ceased medications for 7 days prior to plasma donation. In Houston, subjects maintained their medication regimen and no differences were noted in the properties of the LDL isolated from these two groups. No differences were noted between the properties of SAP-LDL isolated in smaller quantities from normal patients and those isolated in much larger quantities from CHD patients.

LDL (d 1.019–1.063 g/ml) was isolated by ultracentrifugation in a NaBr density gradient according to Lindgren (6) as described earlier (7). Lipoprotein oxidation and proteolysis were inhibited by addition of 20 μM butylated hydroxytoluene and 0.5 mM phenylmethylsulfonyl fluoride to plasma at the time of collection. After recentrifugation, isolated LDL was dialyzed against phosphate-buffered saline (PBS), filtered through a 0.22-μm polycarbonate filter (Nucleopore Corp., Pleasanton, CA), and stored at 4°C for not longer than 7 days. For studies in Houston using LDL isolated in Moscow, LDL samples were transported by air to Houston by one of the Moscow-based investigators within 24 h of isolation. Transport was in PBS containing BHT and PMSF, as indicated above, and also included 5 mM EDTA, 0.01% sodium azide, and 1 mM ϵ-aminocaproic acid. Samples were maintained at 4°C during transport. Such samples were extensively dialyzed against PBS with 0.1 mM EDTA prior to use in cell experiments or further fractionation by affinity chromatography as detailed below. All LDL preparations were refiltered immediately before addition to the cultured cells. No functional differences were noted in cell culture experiments between LDL isolated and/or fractionated in Moscow and transported to Houston, and those isolated and fractionated in Houston.

Lipids were extracted from LDL preparations with chloroform–methanol 2:1 (v/v) according to Folch, Lees, and Sloane Stanley (8), and separated by thin-layer chromatography for quantification by scanning densitometry as described elsewhere (9). Sialic acid concentrations were determined according to Warren (10) in isolated LDL after hydrolysis in 0.1 N H_2SO_4 for 1 h. Sialic acid content was also determined according to Svennerholm (11) by color reaction with resorcinol. Results obtained by both methods were identical. About 70–75% of the total sialic acid was protein-linked; the rest was present in lipid glycoconjugates. Total protein was determined according to Lowry et al. (12). Thiobarbituric acid-reactive substances (TBARS) were determined according to Yagi (13). Native and modified LDL were iodinated with iodine monochloride (14) and ^{125}I (Isotex) to a specific activity of 200–400 cpm/ng for most experiments.

Isolation of sialic acid-poor LDL and preparation of Ds-LDL

Total LDL obtained from plasma of CHD patients was separated into two subfractions by affinity chromatography using ricin agglutinin (RCA_{120}) coupled to Sepharose as described earlier by Tertov et al. (15). Subfraction 1 (SAP-LDL) of a total LDL preparation interacts with RCA_{120} probably through terminal galactose, possesses low sialic acid content compared to subfraction 2 (SAR-LDL) which does not bind to RCA_{120} and possesses normal sialic acid content, comparable to that of native LDL from healthy donors. The sialic acid-poor fraction accounts for 28–60% of the LDL in CHD patients (avg 48 ± 7 in 10 donors) and 4–15% (avg 10 ± 3 in 10 donors) in normal individuals. Recovery from the columns (SAP-LDL + SAR-LDL) was always 95–98% of the total protein applied.

LDL and fetuin were depleted of sialic acid by treatment with agarose-linked *Clostridium perfringens* neuraminidase (Sigma Chemical Co., St. Louis, MO) according to Camejo et al. (16). One hundred μl (0.9 U/ml) of neuraminidase was added to 1 ml LDL or fetuin solutions (2 mg/ml) and incubated at 37°C with constant stirring for 2 h. Control LDL was incubated with 100 μl agarose-linked bovine serum albumin. No loss of sialic acid from control LDL or fetuin was detectable. Protein degradation of ^{125}I-labeled LDL during incubation with neuraminidase was determined according to Goldstein, Brunschede, and Brown (17). After treatment of LDL with the agarose-bound neuraminidase, less than 0.01% radioactivity was found in the supernatant fraction after precipitation with trichloroacetic acid. Twenty μg of each sample, before and after treatment by neuraminidase-agarose, was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, according to the method of Laemmli (18) to detect the presence of proteolytic activity in the neuraminidase-agarose. Four percent polyacrylamide gels were stained with Coomassie Brilliant Blue dye R-250. There was no detectable proteolytic action of the neuraminidase-agarose on apoB-100 as judged from the ab-

sence of peptides with M_r <500 kDa. By electron microscopic analysis, LDL, Ds-LDL, and SAR-LDL and SAP-LDL were monodispersed. By agarose gel electrophoresis there was no significant shift in electrophoretic mobility between normal LDL (R_f 0.166), SAP-LDL (R_f 0.151) and Ds-LDL (R_f 0.143) as compared to Ac-LDL (R_f 0.454). There was no functional difference revealed by cell studies with Ds-LDL or SAP-LDL generated from LDL isolated in Houston or Moscow.

Cell culture studies

"Potential atherogenicity" of isolated LDL or plasma manifested in cell culture was evaluated as described elsewhere (7) and is expressed here as an increment of the total cholesterol content of cultured cells. Total cholesterol content of control cells (cultured without LDL) was used as the 100% value. An isolated LDL or plasma sample was considered "potentially atherogenic" if its addition to a primary culture of human intimal aortic cells (LDL concentration 100 μg/ml,) increased the intracellular cholesterol level by 2- to 3-fold (100–200% increment above the control level) within a 24-h incubation (7).

Subendothelial cells for culture were isolated from grossly normal intima of human thoracic aorta by dispersion with 0.15% collagenase as described in detail elsewhere (9). Isolated cells were suspended in the growth medium containing Medium 199, 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml fungizone (all reagents from Grand Island Biological Co., Grand Island, NY), and seeded into Linbro^R 24-well tissue culture plates (Flow Laboratories Ltd., U.K.) at a density of 2–4 × 10^4 cells per cm^2 of the growth area. 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 as defined by their ultrastructural and immunofluorescent features (9). Over 90% of cultured cells interacted with monoclonal antibody HHF35 (muscle actin specific monoclonal antibody). The medium was changed every day. Starting from the 7th day in primary culture, cells were incubated for 24 h in the medium containing LDL (100 μg protein/ml) and 10% lipoprotein-deficient serum of a healthy donor. On the 8th day, cells were rinsed twice with PBS containing 0.2% bovine serum albumin, followed by three rinses with PBS. Total cellular lipids were then determined as described below. Each value represents a mean of three determinations in three wells. Most experiments were performed using cultures obtained from three or four isolations of cells from aortas of different donors. No qualitative discrepancy was observed between the results obtained in different experiments.

Human aortic endothelial cells were isolated with Dispase^R treatment as described in detail by Antonov et al. (19). Mononuclear cells of human peripheral blood were isolated on a Ficoll-Paque gradient and cultured in growth medium as described earlier (20). Fibroblast cultures were started from skin biopsies of healthy individuals and incubated in DMEM, containing 10% FCS and were used in the studies between the 5th and 10th passage. Other cultured cells used in the experiments were as follows: P388D_1 murine macrophage cell line derived from DBA/2 mouse was cultured in RPMI 1640 (10% FCS) (21); bovine aortic endothelial cell line BFA/1c was cultured in Hams F-10/20% FCS (22); and human cell line HepG2 hepatocytes were cultured in DMEM (10% FCS) (23). Lipids were extracted from cells with n-hexane–isopropanol 3:2 (v/v) according to Hara and Radin (24). The total cholesterol content in the lipid extracts was determined using Boehringer Mannheim Monotest^R, Cholesterol CHOD-PAP Method (Boehringer Mannheim GmbH, Mannheim, Germany). Cellular phospholipids, triglycerides, free cholesterol, and cholesteryl esters were separated by thin-layer chromatography and measured by scanning densitometry as described earlier (9).

For the LDL uptake and degradation experiments, cells were incubated with 10 μg/ml radiolabeled lipoproteins for 5 h at 37°C. At the end of the incubation period, the medium was processed for determination of trichloroacetic acid-soluble radioactivity as previously described (21). Cell monolayers were washed and dissolved in 0.1 N NaOH to determine bound plus internalized (uptake) lipoprotein. For the LDL binding study, cells were incubated for 2 h at 4°C with 10 μg/ml ^{125}I-labeled, lipoprotein preparation. After washing, cells were dissolved in 0.1 M NaOH for determination of bound lipoprotein.

The significance of differences was evaluated by dispersion analysis methods using a BMDP statistical program package (25). Significance of the correlation coefficient difference from zero was estimated using the Fisher z-transformation (26).

RESULTS

LDL sialic acid content and cellular lipid accumulation potential

Table 1 illustrates the capacity of LDL isolated from plasma of a CHD patient to induce intracellular lipid accumulation in cultured cells. After 24 h of incubation with this LDL, levels of unesterified and/or esterified cholesterol were increased in intimal smooth muscle cells, aortic endothelial cells, blood monocytes, and P388D_1 macrophages. The triglyceride content increased only in smooth muscle cells. The phospholipid content remained unchanged in all cell types studied. The CHD patient's LDL induced no intracellular lipid accumulation in cultured fibroblasts or hepatoma cells. Native LDL obtained from plasma of healthy donors induced no changes in cellular lipid content in parallel experiments (data not shown).

TABLE 1. Effects of LDL from a CHD patient on intracellular accumulation of lipids

	Intracellular Lipid Content			
	PL	UC	TG	CE
	μg/mg cell protein			
Human intimal smooth muscle cells				
– LDL	101.7 ± 12.2	24.7 ± 1.5	13.7 ± 1.9	27.7 ± 1.5
+ LDL	112.0 ± 4.5	35.6 ± 1.9[a]	22.3 ± 1.2[a]	106.9 ± 5.3[a]
Human aortic endothelial cells				
– LDL	64.7 ± 4.8	8.0 ± 0.6	3.3 ± 0.3	6.3 ± 0.9
+ LDL	68.7 ± 3.4	13.3 ± 0.6[a]	3.7 ± 0.7	12.0 ± 1.2[a]
Bovine aortic endothelial cells				
– LDL	nd	4.3 ± 0.3	nd	0.8 ± 0.1
+ LDL	nd	4.5 ± 0.2	nd	2.1 ± 0.1[a]
Human blood monocytes				
– LDL	26.0 ± 3.2	12.6 ± 1.5	3.7 ± 0.7	4.6 ± 0.6
+ LDL	31.0 ± 3.6	25.6 ± 1.2[a]	4.0 ± 0.6	14.8 ± 2.3[a]
Murine P388D_1 macrophages				
– LDL	33.3 ± 1.2	8.7 ± 0.9	2.3 ± 0.3	1.5 ± 0.2
+ LDL	36.3 ± 3.8	11.0 ± 1.5	2.3 ± 0.2	5.3 ± 0.9[a]
Human skin fibroblasts				
– LDL	81.3 ± 2.3	15.3 ± 0.9	3.3 ± 0.9	1.8 ± 0.2
+ LDL	94.0 ± 6.7	16.6 ± 1.9	3.3 ± 0.7	2.0 ± 0.2
HepG2 hepatocytes				
– LDL	nd	6.9 ± 0.9	nd	0.9 ± 0.2
+ LDL	nd	6.3 ± 0.2	nd	1.1 ± 0.1

Cells were cultured for 24 h with media containing 10% LDS with or without 100 μg/ml LDL, isolated from a patient with coronary heart disease. The LDL used for these experiments had a sialic acid content of 10.5 nmol/mg protein. Cells were washed and lipids were extracted with hexane–isopropanol 3:2. Major classes of lipids were separated by thin-layer chromatography and quantified as described in Materials and Methods. Data represent a mean of three determinations ± SEM. Abbreviations: PL, phospholipid; UC, unesterified cholesterol; TG, triglyceride; CE, cholesteryl ester; nd, not determined.

[a]Significant difference from control, $P < 0.05$.

Fifty of 53 LDL preparations obtained from CHD patients' plasmas induced cholesterol accumulation in cultured intimal cells and macrophages. In contrast, only 1 out of 33 LDL preparations obtained from healthy donors' plasma induced appreciable accumulation of cholesterol in cultured cells. These results are fully consistent with our previous report on the behavior of CHD patients' plasma: 90% of CHD plasma samples induced cellular lipid accumulation, while <1% of samples from normal subjects' plasma produced this effect (20).

Fig. 1 shows the sialic acid content of LDL isolated from the plasma of healthy donors and CHD patients. On average, the sialic acid content of CHD patients' LDL was about 2-fold lower than that of healthy donors' LDL ($P < 0.05$). Three CHD patients had LDL with sialic acid levels comparable to that of LDL from normal healthy donors; these patients had LDL unable to induce cholesterol accumulation (Fig. 1). Of the remaining 50 CHD patients, a major proportion had LDL with ≤20 nmol sialic acid per mg LDL protein; these LDL caused cholesterol accumulation. Of the LDL obtained from 33 healthy donors, only one sample that had a sialic acid content comparable to that from CHD patients (Fig. 1) caused intracellular lipid accumulation. Hence, there appears to be a clear correlation between the sialic acid content of LDL and its capacity to induce the accumulation of cellular cholesterol. A negative correlation was established with a correlation coefficient of −0.88 (**Fig. 2**).

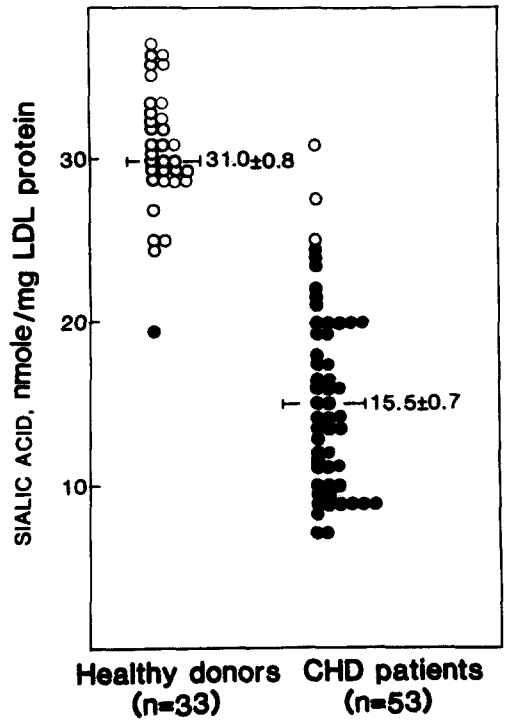

Fig. 1. The content of sialic acid in the LDL of patients with coronary atherosclerosis and in the LDL of healthy donors. Sialic acid was measured in LDL isolated from the blood plasma of healthy donors (left portion of panel) and CHD patients (right portion of panel). Samples that caused cholesterol accumulation are shown in closed circles and those that did not in open circles. Cholesterol accumulation in intimal smooth muscle cells was measured as detailed in Materials and Methods.

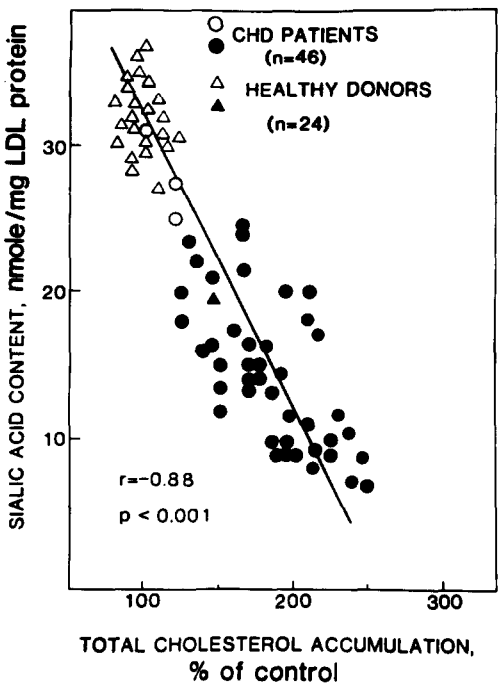

Fig. 2. Correlation between the sialic acid content of patients' LDL and LDL-induced cholesterol accumulation. Details are the same as in legend to Fig. 1. Samples causing cholesterol accumulation are shown in closed symbols and those that did not in open symbols. Cholesterol accumulation in intimal human smooth muscle cells was measured as detailed in Materials and Methods.

LDL subfractions of high and low sialic acid content were isolated from total LDL preparations (from plasma of CHD patients) by affinity chromatography over immobilized ricin agglutinin (RCA_{120}). RCA_{120} has a high affinity for the terminal D-galactose residues (27). Most of the D-galactose residues of SAR-LDL are probably masked by sialic acids, therefore this form of LDL passes through the column unretarded (15). In experiments with cultured smooth muscle cells, the SAR-LDL (subfraction 2, see Methods section) did not stimulate lipid accumulation. In contrast, concentrations of cholesteryl ester were increased 3.7-fold in cells incubated with the SAP-LDL (subfraction 1). SAP-LDL also stimulated accumulation of free cholesterol and triglycerides, but in lesser amounts (**Table 2**).

Individual LDL preparations isolated from the plasma of patients and healthy donors were treated with neuraminidase (Ds-LDL) to reduce their sialic acid content (**Table 3**). This treatment potentiated the capacity of these LDL preparations to induce intracellular lipid accumulation, converting LDL that initially did not exhibit this property to LDL that did (Table 3).

Cellular metabolism of LDL with differing sialic acid content

To determine the interactions of LDL with cells, resulting in the intracellular accumulation of cholesterol, we used preparations of LDL obtained from normal healthy donors, normal LDL that had been treated with neuraminidase (Ds-LDL), total LDL from CHD patients, and RCA_{120}-Sepharose-chromatographed subfractions of LDL containing different amounts of sialic acid. **Table 4** shows the sialic acid and TBARS content for these LDL preparations. LDL fractions which did or did not induce cellular lipid accumulation differed considerable in their sialic acid content, but were very similar in TBARS.

Experiments were performed to assess which lipoprotein receptor(s) might be responsible for the binding and metabolism of in vitro-generated, partially desialylated LDL. The involvement of the normal LDL receptor was examined using human skin fibroblasts, aortic endothelial cells, hepatoma HepG2 cells, and the macrophage cell line $P388D_1$. The involvement of the acetylated LDL (scavenger) receptor was examined using the $P388D_1$ line, while the role of the galactose receptor was examined using the HepG2 cell line.

TABLE 2. Effect of sialic acid content of LDL isolated from a CHD patient on lipid content of smooth muscle cells cultured from uninvolved human aortic intima

Lipoprotein Preparation	Intracellular Lipid Content			
	PL	UC	TG	CE
	μg/mg cell protein			
Control	95.3 ± 5.8	21.7 ± 2.3	11.0 ± 0.6	14.5 ± 0.3
Total LDL	105.0 ± 9.2	38.7 ± 4.8[a]	16.0 ± 1.2[a]	36.3 ± 3.4[a]
Subfraction 1: sialic acid-poor LDL	88.0 ± 7.5	41.3 ± 4.8[a]	18.0 ± 0.6[a]	54.0 ± 5.7[a]
Subfraction 2: sialic acid-rich LDL	87.6 ± 9.6	18.7 ± 0.9	10.0 ± 1.5	16.3 ± 1.2

Smooth muscle cells were cultured for 7 days in medium 199, containing 10% fetal calf serum. On the 7th day, cells were washed and fresh medium containing 10% LPDS and 100 μg/ml LDL preparations was added. Control cells were incubated in the absence of lipoproteins. The sialic acid content of the total LDL was 14.7 nmol/mg protein. The sialic acid-rich fraction contained 24.5 nmol sialic acid/mg protein while the sialic acid-poor fraction contained 6.9 nmol/mg protein. After 24 h incubation, cells were washed and lipids were extracted with hexane–isopropanol 3:2. Major classes of lipids were separated by thin-layer chromatography and determined as described in Materials and Methods. Data represent a mean of three determinations ± SEM. Abbreviations: PL, phospholipids; UC, free cholesterol; TG, triglycerides; CE, cholesteryl esters.

[a]Significant difference from control, $P < 0.05$.

TABLE 3. Effects of neuraminidase treatment of LDL on its sialic acid content and capacity to accumulate total cholesterol in cultured intimal smooth muscle cells

Subject	Cholesterol Accumulation		Sialic Acid Content	
	Before Treatment	After Treatment	Before Treatment	After Treatment
	% of control		*nmol/mg LDL protein*	
Healthy donors				
1	104 ± 8	209 ± 7[ab]	27.8	16.1
2	111 ± 8	190 ± 13	32.0	17.4
CHD patients				
1	169 ± 15[a]	232 ± 9[ab]	15.5	9.0
2	157 ± 6[a]	314 ± 37[ab]	14.6	7.5

LDL isolated from two healthy subjects and two coronary atherosclerosis patients were treated with neuraminidase as described in Materials and Methods. Cells were cultured for 7 days in medium 199, containing 10% fetal calf serum. On the 7th day, cells were washed and fresh medium containing 10% of LPDS and 100 μg/ml of native and neuraminidase-treated LDL preparations was added. Control cells were incubated in the absence of lipoproteins. After 24 h incubation, cells were washed and lipids were extracted with hexane–isopropanol 3:2. Total cholesterol content was determined by an enzymatic method as described in Materials and Methods. Data represent a mean of three determinations ± SEM.

[a]Significant difference from control, $P < 0.05$.

[b]Significant difference from cells incubated with untreated LDL, $P < 0.05$.

Results of binding of native and modified LDL to fibroblasts and macrophages at 4°C are shown in **Table 5**. The level of specific binding of native LDL and LDL treated with neuraminidase to fibroblasts was similar. A 20-fold excess of unlabeled native LDL decreased the binding of Ds-LDL by 60%. Unlabeled Ds-LDL reduced the binding of native LDL to a comparable extent (52%). A similar pattern of competition was observed with fibroblast experiments in which both uptake (binding plus internalization) and degradation were examined (**Table 6**).

Very different results were obtained when the binding of LDL fractions to the macrophage cell line P388D_1 was examined. This cell line possesses both native LDL and acetylated LDL (scavenger) receptors. As seen in Table 5 under control measurements, approximately 2-fold more Ds-LDL than native LDL was specifically bound to the P388D_1 cells. ^{125}I-labeled Ds-LDL was very effectively competed by excess Ac-LDL (93% reduction), but not by native LDL (14% reduction). Unlabeled Ds-LDL reduced by 54% the binding of ^{125}I-labeled Ac-LDL. A similar pattern of results was seen in uptake and degradation experiments (Table 6). Addition of Ds-LDL inhibited the binding, uptake, and degradation of native LDL in these macrophages (Tables 5 and 6). These results suggest that in vitro sialic acid-depleted LDL interacts with the receptors for native LDL and Ac-LDL on P388D_1 cells. The major interaction appears to be with the latter receptor although additional sites for Ds-LDL on the macrophage not shared with Ac-LDL cannot be excluded, in that Ac-LDL was a more effective competitor for Ds-LDL than Ds-LDL was for Ac-LDL. Similar uptake and degradation results were obtained with bovine endothelial cells that also have both the native LDL and Ac-LDL receptor. The experiments in Tables 5 and 6 were conducted at a single concentration of competitor. To rule out the possibility of spurious results from a single point competition, experiments were conducted in fibroblasts and macrophages at 4°C (**Fig. 3** and **Fig. 4**) and at 37°C (**Fig. 5** and

TABLE 4. Content of sialic acid and TBARS in LDL preparations

Lipoprotein Preparation	Sialic Acid Content	TBARS Level
	nmol/mg protein	
Healthy subjects		
Total LDL	24.7 ± 2.0	1.5 ± 0.2
Sialic acid-depleted LDL (neuraminidase-treated)	13.6 ± 1.4	1.6 ± 0.2
CHD patient		
Total LDL	13.2 ± 1.0[a]	1.7 ± 0.2
Subfraction 1: sialic acid-rich LDL	21.0 ± 0.3[b]	1.9 ± 0.1
Subfraction 2: sialic acid-poor LDL	6.4 ± 0.6[ab]	1.5 ± 0.2

Sialic acid content and level of thiobarbituric acid-reactive substances were determined as detailed in Materials and Methods. Data represent mean of three determinations ± SEM.

[a]Significant difference from healthy subject LDL preparation, $P < 0.05$.

[b]Significant difference from total LDL preparation, $P < 0.05$.

TABLE 5. Binding of native and modified ^{125}I-labeled LDL by human skin fibroblasts and P388D$_1$ macrophages at 4°C

Competitor	Binding		
	^{125}I-Labeled Native LDL	^{125}I-Labeled Ds-LDL	^{125}I-Labeled Ac-LDL
	ng/mg protein/2 h		
Fibroblasts			
None	6.3 ± 0.6		
Native LDL	1.1 ± 0.1[a]		
Ds-LDL	2.5 ± 0.7[a]		
Ac-LDL	6.0 ± 1.8		
None		8.1 ± 0.6	
Ds-LDL		1.4 ± 0.1	
Native LDL		2.9 ± 0.3[a]	
Ac-LDL		7.1 ± 1.2	
P388D$_1$ macrophages			
Control	11.4 ± 0.9		
Native LDL	2.0 ± 0.6[a]		
Ds-LDL	1.7 ± 0.6[a]		
Ac-LDL	10.4 ± 1.4		
None		29.6 ± 1.1	
Ds-LDL		5.8 ± 0.2[a]	
Native LDL		21.3 ± 1.2	
Ac-LDL		1.7 ± 0.6[a]	
None			757 ± 110
Ac-LDL			108 ± 09[a]
Native LDL			562 ± 102
Ds-LDL			302 ± 52[a]

Cell cultures were incubated for 24 h in media containing 10% lipoprotein-deficient serum. Cells were cooled to 4°C and then maintained for 2 h with 10 μg/ml ^{125}I-labeled lipoproteins in the presence or absence of a 20-fold excess of unlabeled native or modified lipoproteins. The native LDL had a sialic acid content of 26.8 nmol/mg protein while the Ds-LDL had a sialic acid content of 8.9 nmol/mg protein. At the end of the incubation period, cultures were washed and cells were dissolved in 1.0 N NaOH. Values represent a mean of three determinations ± SEM.

[a]Significant difference from control, $P < 0.05$.

TABLE 6. Cellular uptake and degradation of native and modified ^{125}I-labeled lipoproteins

Competitor	Uptake			Degradation		
	^{125}I-Labeled Native LDL	^{125}I-Labeled Ds-LDL	^{125}I-Labeled Ac-LDL	^{125}I-Labeled Native LDL	^{125}I-Labeled Ds-LDL	^{125}I-Labeled Ac-LDL
	ng/mg protein/5 h			*ng/mg cell protein/5 h*		
Fibroblasts						
None	84.4 ± 6.2	96.7 ± 2.7		305.5 ± 24.8	380.2 ± 28.5	
Native LDL	7.4 ± 0.9[a]	8.7 ± 1.8[a]		53.5 ± 1.3[a]	57.6 ± 20.5[a]	
Ds-LDL	4.5 ± 1.2[a]	10.9 ± 1.2[a]		68.7 ± 8.8[a]	101.2 ± 15.2	
Ac-LDL	84.2 ± 4.3			302.4 ± 15.4		
P388D$_1$ macrophages						
None	60.6 ± 5.9	99.9 ± 4.2	4270 ± 152	540.9 ± 29.6	802.0 ± 75.2	21808 ± 432
Native LDL	36.0 ± 3.3[a]	60.0 ± 2.3[a]	3618 ± 245	86.2 ± 8.3[a]	539.6 ± 10.5[a]	21956 ± 807
Ds-LDL	46.1 ± 2.8[a]	26.1 ± 1.3[a]	2765 ± 317[a]	166.9 ± 14.7[a]	512.2 ± 11.4[a]	13825 ± 1583[a]
Ac-LDL	66.2 ± 2.6	35.8 ± 6.4[a]	2022 ± 253[a]	595.5 ± 40.3	263.4 ± 30.9[a]	3824 ± 366[a]
Endothelial cells						
None	23.2 ± 1.3	24.8 ± 1.3	847.3 ± 46.5	112.3 ± 9.4	156.2 ± 7.3	424.3 ± 58.6
Native LDL	11.3 ± 0.5[a]	24.2 ± 4.1	825.2 ± 22.6	56.7 ± 12.1[a]	134.0 ± 2.7	393.4 ± 5.8
Ds-LDL	15.2 ± 0.4[a]	15.9 ± 1.2[a]	623.4 ± 35.4[a]	103.0 ± 2.9	121.0 ± 7.4[a]	315.5 ± 20.5[a]
Ac-LDL	22.6 ± 3.8	11.2 ± 0.8[a]	132.2 ± 14.0[a]	117.8 ± 4.7	38.2 ± 9.7	47.0 ± 12.1[a]

Cells were preincubated for 24 h in DMEM containing 10% LPDS; the cells were incubated for 5 h with the same medium containing 10 μg/ml of ^{125}I-labeled lipoprotein with or without 20-fold excess of unlabeled native and modified lipoprotein. The native LDL used had a sialic acid content of 26.8 nmol/mg protein while the Ds-LDL had a sialic acid content of 8.9 nmol/mg protein. The medium was analyzed for the determination of degraded LDL as described in Materials and Methods. Cells were washed and dissolved in 1 N NaOH for the determination of protein. Data represent a mean of three determinations ± SEM.

[a]Significant difference from cells incubated in the absence of unlabeled LDL, $P < 0.05$.

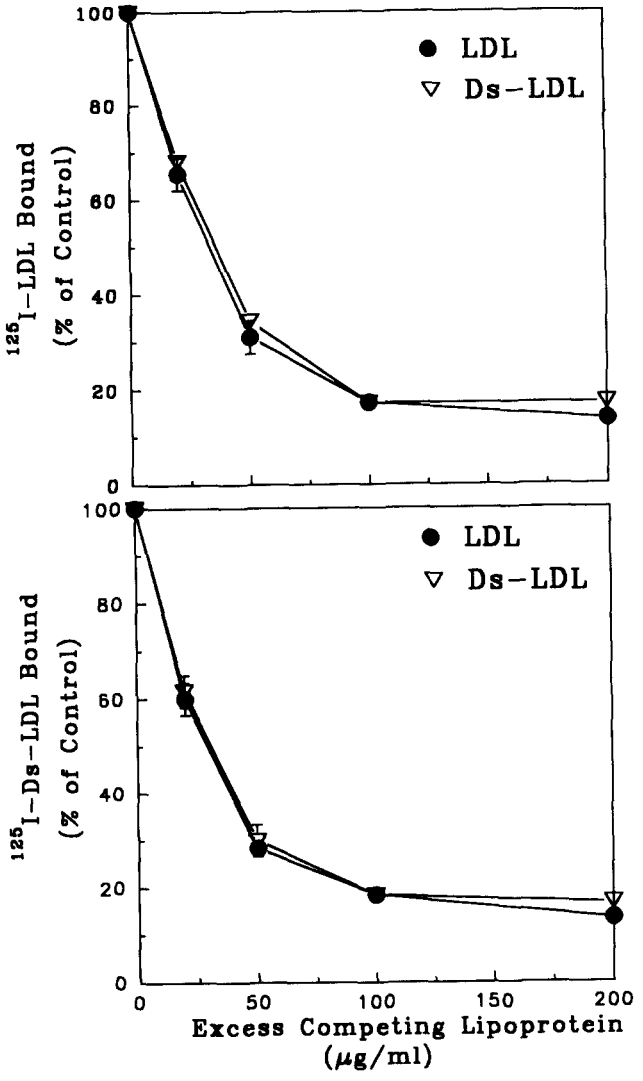

Fig. 3. Competition for binding of LDL and Ds-LDL to fibroblasts at 4°C over a broad range of competitor. Fibroblasts were incubated for 2 h at 4°C with ^{125}I-labeled LDL (upper panel) or ^{125}I-labeled Ds-LDL (lower panel) in the presence of the indicated concentrations of excess unlabeled lipoprotein. The 100% value in the absence of competitor was 5.8 ng/mg protein for LDL and 6.0 ng/mg for Ds-LDL. The sialic acid content of the LDL was 27.2 nmol/mg protein and that of the Ds-LDL was 8.9 ng/mg protein. Data represent triplicate determinations.

Fig. 6) over a broad range of competitors. The results confirm the patterns of cross-competitions seen in Tables 5 and 6 and rule out anomolous interactions dependent on the level of competitor.

We also explored the potential interaction of sialic acid depleted-LDL (Ds-LDL) with the galactose receptor of hepatocytes from the HepG2 cell line. Degradation of Ds-LDL was reduced 42% by 50 mM galactose (**Table 7**). Fetuin, a protein known to have a high content of sialic acid, was also used as a competitor. At a high concentration (200 μg/ml) of fetuin depleted of sialic acid by neuraminidase (91% sialic acid-free), uptake and degradation of Ds-LDL was reduced by 26 and 27%, respectively. These data suggest only a low level interaction of Ds-LDL with the galactose receptor.

Finally, we examined the uptake and degradation of the affinity-purified (ricin-Sepharose) SAP-LDL subfraction by fibroblasts and P388D$_1$ cells. The pattern of uptake and degradation of SAP-LDL isolated from plasma and that of Ds-LDL prepared by neuraminidase treatment

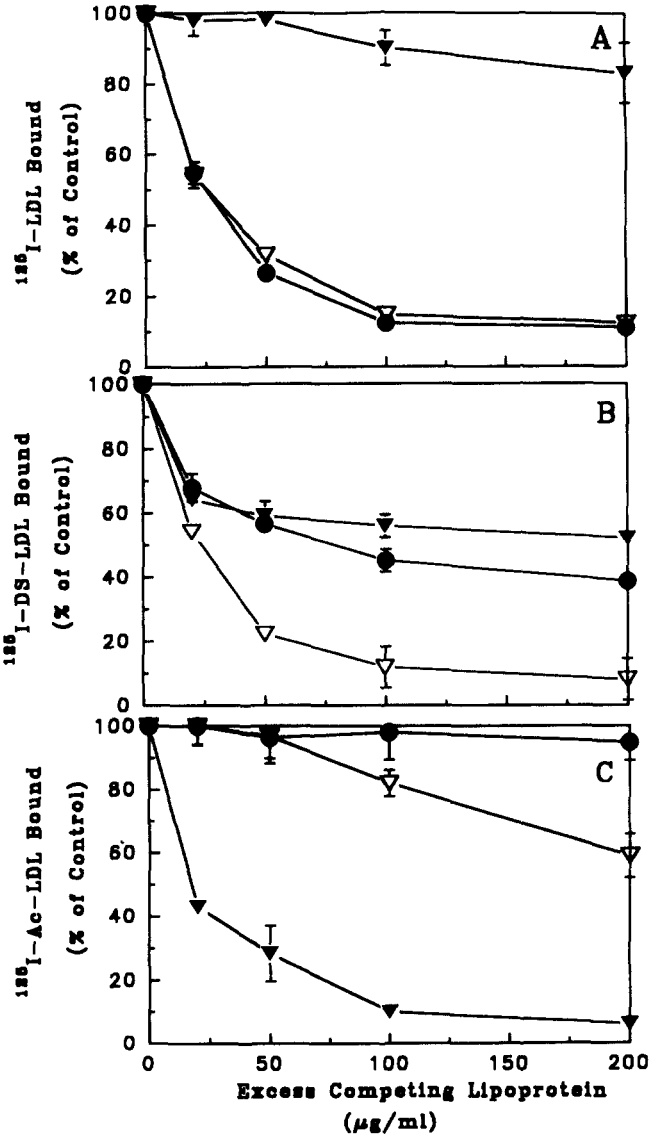

Fig. 4. Competition for binding of lipoproteins at 4°C to P388D$_1$ over a broad range of competitor. P388D$_1$ cultures were incubated at 4°C with 10 μg/ml of ^{125}I-labeled LDL (panel A), 10 μg/ml ^{125}I-labeled Ds-LDL (panel B), or 10 μg/ml ^{125}I-labeled Ac-LDL (panel C) in the presence of excess LDL (●), excess Ds-LDL (▽), and excess Ac-LDL (▼). After 2 h, cells were washed and dissolved in NaOH to determine bound lipoprotein as detailed in Materials and Methods. The 100% binding in the absence of competitor was 16.2 ng/mg protein for LDL, 30.7 ng/mg protein for Ds-LDL, and 251.4 ng/mg protein for Ac-LDL. The sialic acid content of the LDL was 27.2 nmol/mg protein, for Ds-LDL 8.9 nmol/mg protein, and for Ac-LDL 27.2 nmol/mg protein. The data represent the average of triplicate determinations.

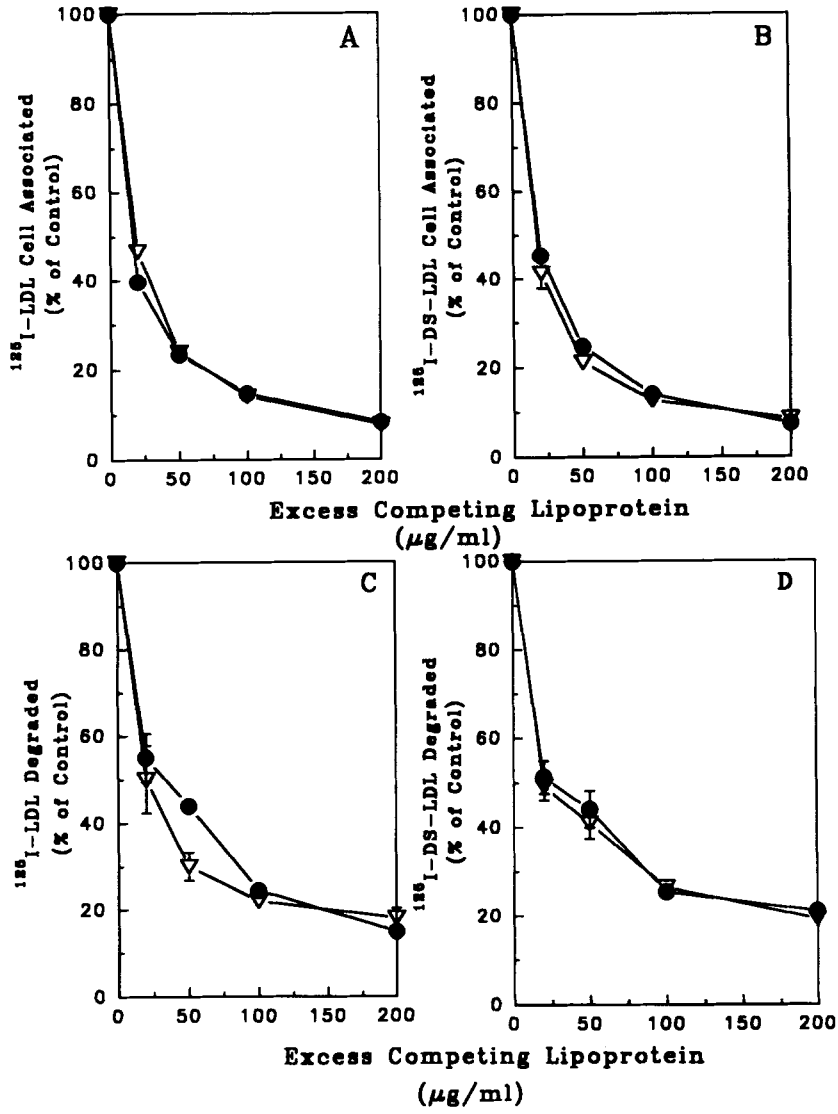

Fig. 5. Competition for LDL and Ds-LDL metabolism in human skin fibroblasts over a range of competitors. Human skin fibroblasts were incubated for 5 h at 37°C with 10 μg/ml of ^{125}I-labeled LDL (panels A, C) or ^{125}I-labeled Ds-LDL (panels B, D) in the presence and absence of excess LDL (●) and Ds-LDL (▽). At the end of the incubation period, media was removed for determination of lipoprotein degradation (panels C, D) and cell-associated radioactivity (panels A, B). The 100% cell-associated radioactivity point in the absence of competitor was 184 ng/mg cell protein for LDL and 198 ng/mg cell protein for Ds-LDL, while the 100% degradation point was 216 ng/mg cell protein for LDL and 193 cpm/ng cell protein for the Ds-LDL. The sialic acid content of the LDL was 27.2 nmol/mg protein and the Ds-LDL was 8.9 nmol/mg protein. Data are the average of triplicate points.

were similar (Tables 6 and **Table 8**). Thus, LDL derived from the plasma of CHD patients appears to interact with the receptor for native LDL, and with the scavenger receptor for Ac-LDL.

DISCUSSION

The capacity of a lipoprotein particle such as LDL to cause intracellular cholesterol accumulation may be directly related to its sialic acid content. In our studies, isolated LDL with low sialic acid content induced greater intracellular cholesterol accumulation than LDL with higher sialic acid content. These two LDL populations do not differ significantly in TBARS, hence their difference in cellular metabolism cannot be attributed to differing degrees of lipid oxidation. These findings strongly suggest that the capacity of LDL to stimulate cholesterol accumulation in cultured cells, and perhaps in vivo, is directly related to its degree of sialylation.

Not all forms of LDL circulating in blood of CHD patients cause cellular cholesterol accumulation; only the sialic acid-depleted form of LDL produced this effect.

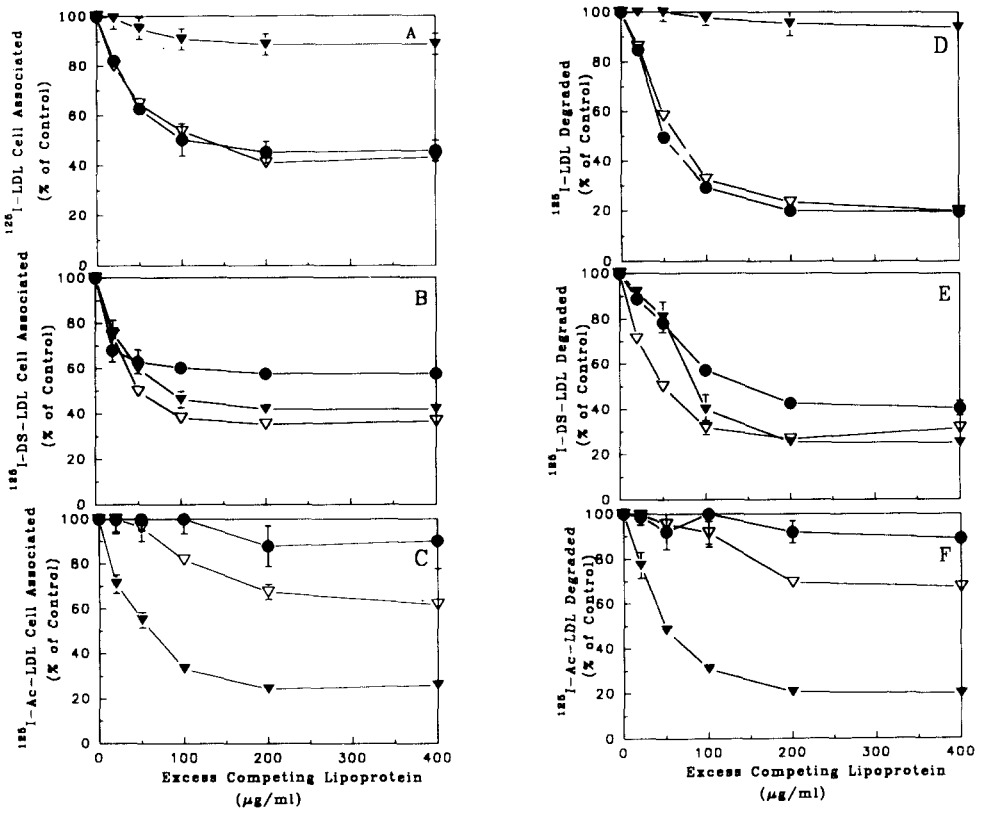

Fig. 6. Competition for metabolism of lipoproteins in P388D$_1$ at 37°C by normal LDL, Ds-LDL, and Ac-LDL. P388D$_1$ macrophages were incubated with ^{125}I-labeled LDL (panels A, D), ^{125}I-labeled Ds-LDL (panels B, E), or ^{125}I-labeled Ac-LDL (panels C, F) in the absence and presence of a 20-fold excess of LDL (●), Ds-LDL (▽), and Ac-LDL (▼). Cell-associated lipoprotein (panels A, B, C) and lipoprotein degradation (panels D, E, F) were determined as detailed in Materials and Methods. The cell-associated radioactivity in the absence of any competitor was 46.3 ng/mg protein for LDL, 76.3 ng/mg protein for Ds-LDL, and 1114 ng/mg protein for Ac-LDL. The cell degradation in the absence of competitor was 515 ng/mg protein for LDL, 908 ng/mg protein for Ds-LDL, and 5087 ng/mg protein for Ac-LDL. Data represent the average of triplicate determinations. The sialic acid content of the LDL was 27.2 nmol/mg protein, 8.9 nmol/mg protein for Ds-LDL, and 27.1 nmol/mg protein for Ac-LDL.

Thus, in the blood of over 95% of the CHD patients examined, we found a modified, sialic acid-poor form of LDL that determines the potential of a patient's plasma to cause lipid accumulation in arterial cells. Additional evidence indicating the presence of sialic acid-depleted LDL in the blood of CHD patients has recently been obtained. In these patients, we have found anti-LDL autoantibodies with much greater affinity for sialic acid-depleted LDL than for native LDL or chemically modified LDL (28). This observation strongly suggests that desialylation is a significant LDL modification that occurs in vivo. A potentially atherogenic subfraction of LDL has been isolated from the blood of healthy donors by Avagaro et al. (29). Another type of modified lipoprotein, glycosylated-LDL, has been discovered in the blood of patients with diabetes mellitus (30). In addition, there is accumulating evidence that oxidation may also be an in vivo modification that renders LDL more atherogenic (31). Taken together, these findings suggest that there are several in vivo modifications that render lipoproteins potentially atherogenic. We suggest that desialylation may be one of them.

The presence of large amounts of sialic acid-poor LDL in the plasma of CHD patients raises questions as to the mechanism for its continued presence. The rapid clearance of modified LDL has been clearly demonstrated by Mahley and colleagues (32) and Van Berkel, Nagelkerke, and Kruijt (33) by the reinjection of extensively modified LDL. The sialic acid-poor LDL described here is not extensively modified with regard to charge since its electrophoretic mobility in agarose gels is very similar to that of normal LDL. If the rate of clearance from circulation or degree of interaction with the scavenger receptor is related to the degree of charge modification, then one might expect slow clearance. Moreover, one would expect any charge modification to be in a net positive direction upon removal of sialic acids, suggesting that some property other than charge, perhaps apolipoprotein con-

TABLE 7. Effect of galactose and sialic acid-poor fetuin on uptake and degradation of sialic acid-depleted ^{125}I-labeled LDL by HepG2 cells

Addition	Uptake	Degradation
	ng/mg protein/5 h	
Exp. 1		
None	99.4 ± 6.5	216.2 ± 12.8
Ds-LDL, 200 μg/ml	7.5 ± 1.0[a]	38.4 ± 2.9[a]
Galactose, 50 mM	79.5 ± 7.2	126.3 ± 15.6[a]
Exp. 2		
None	64.6 ± 4.9	172.2 ± 13.4
Ds-LDL, 200 μg/ml	2.1 ± 0.4[a]	30.7 ± 6.6[a]
Fetuin		
50 μg/ml	68.1 ± 4.0	210.5 ± 17.8
100 μg/ml	63.7 ± 6.4	166.7 ± 5.5
200 μg/ml	61.2 ± 5.1	185.3 ± 20.3
Ds-fetuin		
50 μg/ml	66.7 ± 5.7	165.6 ± 10.4
100 μg/ml	52.8 ± 8.2	183.2 ± 17.2
200 μg/ml	47.9 ± 3.2	126.3 ± 10.5[a]

HepG2 hepatocytes were preincubated in DMEM, containing 10% of LPDS, and then were incubated for 5 h with the fresh LPDS containing LDL (10 μg protein/ml) that had been depleted of sialic acid by neuraminidase treatment and then radio-iodinated, and the indicated concentrations of galactose, native fetuin, and Ds-fetuin. The native LDL had a sialic acid content of 26.8 nmol/mg protein while the Ds-LDL had a sialic acid content of 8.9 nmol/mg protein. The sialic acid content of fetuin was 287 nmol/mg protein, and that of Ds-fetuin 25.8 nmol/mg protein. The galactose content of Ds-LDL was 25.4 nmol/mg protein and for Ds-fetuin 279 nmol/mg protein. Thus the galactose concentration in the medium at the highest level of asialofetuin represents a 22-fold molar excess over that in the Ds-LDL and the concentration of pure galactose used represents a 20-fold molar excess over the Ds-LDL. Uptake and degradation of lipoproteins as well as protein cellular content were determined. Data represent a mean of three determinations ± SEM.

[a]Significant difference from cells incubated in the absence of additional components, $P < 0.05$.

formational changes, dictates the interaction with the scavenger receptor. It is also clear from Tables 5–8 that cross-competition between Ac-LDL and SAP-LDL is not fully reciprocal. This may suggest that SAP-LDL has lower affinity than Ac-LDL for the scavenger receptor or that additional receptor(s) for the SAP-LDL are present. Either case might lead to slower clearance. In addition, since we know nothing of the mechanism(s) for the generation of SAP-LDL in plasma, we cannot exclude the possibility that the rate of generation of the material exceeds its rate of clearance.

To elucidate the mechanism(s) whereby LDL with low sialic acid content lead to lipid accumulation in cultured cells, we have explored how sialic acid-depleted LDL (formed in vitro by neuraminidase treatment) and sialic acid-poor LDL (formed in vivo by CHD patients) interact with cultured cells possessing various types of receptors. The involvement of the native LDL receptor, the scavenger receptor, and the galactose receptor has been assessed. The galactose receptor of hepatocytes was investigated to evaluate its possible interaction with LDL galactose residues exposed by desialylation. This interaction appeared to be weak. These results may be related to the observation that a high surface density of galactose on LDL would be necessary for this lipoprotein to bind to the galactose receptor (34). Measurement of glycosylation of asparagine residues in the apoB-100 molecule (35) suggests a low surface density of carbohydrate chains on the LDL particle and, hence a low density of terminal galactose residues.

TABLE 8. Uptake and degradation of sialic acid-poor LDL (SAP-LDL) isolated from a CHD patient by human skin fibroblasts and P388D$_1$ macrophages

	Uptake			Degradation		
Competitor	^{125}I-Labeled Native LDL	^{125}I-Labeled SAP-LDL	^{125}I-Labeled Ac-LDL	^{125}I-Labeled Native LDL	^{125}I-Labeled SAP-LDL	^{125}I-Labeled Ac-LDL
	ng/mg protein/5 h			*ng/mg protein/5 h*		
Fibroblasts						
None	66.1 ± 7.6	61.5 ± 3.3		136.4 ± 9.8	127.4 ± 8.0	
Native LDL	4.6 ± 0.5[a]	14.7 ± 2.8[a]		20.0 ± 2.0[a]	60.0 ± 14.1[a]	
SAP-LDL	45.8 ± 0.7[a]	30.9 ± 3.3[a]		86.5 ± 6.7[a]	47.6 ± 12.4[a]	
Ac-LDL		50.7 ± 2.1			149.7 ± 23.3	
P388D$_1$ macrophages						
None	41 ± 1	623 ± 39	1107 ± 50	189 ± 15	707 ± 83	4477 ± 350
Native LDL	7 ± 2[a]	485 ± 13[a]	1080 ± 60	64 ± 2[a]	468 ± 17[a]	3829 ± 155
SAP-LDL	30 ± 2[a]	346 ± 10[a]	516 ± 28[a]	138 ± 5[a]	293 ± 17[a]	2198 ± 115[a]
Ac-LDL	49 ± 5	260 ± 25[a]	295 ± 12[a]	158 ± 11	113 ± 27[a]	1940 ± 194[a]

Cell cultures were preincubated for 24 h in media containing 10% LPDS, then incubated for 5 h with the fresh media containing 10 μg/ml of iodinated lipoprotein with or without 20-fold excess of unlabeled native and modified lipoprotein. SAP-LDL were separated from total LDL by affinity chromatography on ricin agglutinin-agarose and labeled as described in Materials and Methods. The native LDL from the CHD patient had a sialic acid content of 13.2 nmol/mg protein and the sialic acid-poor fraction had a sialic acid content of 6.4 nmol/mg protein. Data represent a mean of three determinations ± SEM.

[a]Significant difference from cells incubated in the absence of unlabeled LDL, $P < 0.05$.

We have previously reported that Ds-LDL at 100 μg/ml begins to aggregate after 4 h at 37°C (4). However, it is unlikely that aggregation plays a role in the current finding that this type of particle binds to the scavenger receptor (4). Aggregation was observed to occur in those previous studies at high levels of lipoprotein (100 μg/ml). This aggregation is concentration-dependent and is not detectable at the level used for binding experiments, 10 μg/ml, by either light scattering or column chromatography of radiolabeled lipoprotein. In addition, aggregation does not occur during the 2-h incubation at 4°C even at levels of up to 100 μg/ml. Moreover, soluble aggregates of LDL, such as those produced by vortexing (36), still bind to the LDL receptor and are not competed by Ac-LDL. In contrast, both Ds-LDL and SAP-LDL are effectively competed by Ac-LDL in our studies. In addition, phagocytosis of aggregates cannot account for the uptake of Ds-LDL. If this were the case, one would not expect to observe competition of Ds-LDL by Ac-LDL or by Ds-LDL for Ac-LDL as the interaction of Ac-LDL with both macrophages and endothelial cells is receptor-mediated.

It is also unlikely that the slightly more positively charged Ds-LDL or SAP-LDL directly interact with Ac-LDL in solution to reduce the availability of either ligand for binding to the cell surface, creating the illusion of competition for Ac-LDL binding. In additional experiments, we mixed Ac-LDL and the sialic acid-depleted ligands and examined the mixtures by agarose gel electrophoresis after 5 h at 37°C. We found no shift of protein mass or of iodine label from the position of each ligand by itself, strongly suggesting that complexes between Ac-LDL and the other ligands do not form. Goldstein and colleagues (37) also noted that orosomucoid, depleted of sialic acid to increase its net positive charge, was not effective in blocking Ac-LDL binding even at concentrations as high as 5 mg/ml. In addition, when we pretreated cells at 4°C with a 20-fold excess of competitors followed by incubation with labeled ligands, the same pattern of competition was noted as when labeled ligands and unlabeled competitors were added together in solution. This clearly supports the idea that competition is occurring at the cell surface and is not the result of an artificial interaction between the lipoproteins in solution.

The uptake and degradation of Ds-LDL or SAP-LDL by fibroblasts were almost the same as that of native LDL. Ds-LDL or SAP-LDL was an effective inhibitor of native LDL uptake and degradation by fibroblasts and other cell types, indicating that it can bind to the native LDL receptor. In contrast, binding, uptake, and degradation of Ds-LDL or SAP-LDL by macrophages and endothelial cells possessing the scavenger receptor were greater than that of native LDL. The results suggest that Ds-LDL/SAP-LDL and Ac-LDL are competitive ligands. Since SAP-LDL/Ds-LDL can interact with the scavenger receptor, this form of lipoprotein might be regarded as one of this receptor's natural ligands. However, SAP-LDL/Ds-LDL was usually less effective than Ac-LDL in competing for Ac-LDL binding and/or degradation. It is not clear whether this reflects a difference in affinity for the Ac-LDL receptor or the presence of other types of binding sites for LDL with low sialic acid content on the macrophage/endothelial cell surface. Further studies will be needed to distinguish between these possibilities.

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