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Research Article

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Cholesteryl Ester Accumulation in Mouse Peritoneal Macrophages Induced by β-Migrating Very Low Density Lipoproteins from Patients with Atypical Dysbetalipoproteinemia

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ABSTRACT The $d < 1.006$ lipoproteins of patients in a kindred with atypical dysbetalipoproteinemia induced marked cholesteryl ester accumulation in mouse peritoneal macrophages. The affected family members had severe hypercholesterolemia and hypertriglyceridemia, xanthomatosis, premature vascular disease, the apo-E3/3 phenotype, and a predominance of cholesterol-rich β-very low density lipoproteins (β-VLDL) in the $d < 1.006$ fraction. When incubated with mouse peritoneal macrophages, the $d < 1.006$ lipoproteins or β-VLDL from the affected family members stimulated cholesteryl [^{14}C]oleate synthesis 15- to 30-fold above that caused by normal, control $d < 1.006$ lipoproteins (VLDL). The ability of the β-VLDL to stimulate macrophage cholesteryl ester accumulation was greatly reduced as a consequence of treatment with hypolipidemic agents, which specifically reduced the concentration of β-VLDL. Two important differences were noted in a comparison of the β-VLDL from these atypical dysbetalipoproteinemic subjects with that of classic E2/2 dysbetalipoproteinemics: (*a*) the β-VLDL from the atypical subjects were severalfold more active in stimulating cholesteryl ester accumulation in macrophages, and (*b*) both the intestinal and hepatic β-VLDL from the atypical subjects were active. The triglyceride-rich, α_2-migrating VLDL from the affected family members constituted <10% of the $d < 1.006$ fraction and were similar to normal VLDL in that they did not stimulate cholesteryl ester synthesis in the macrophages.

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Several lines of evidence indicate that the macrophage accumulation of cholesteryl esters was induced by a receptor-mediated uptake process and that the β-VLDL were bound by a specific β-VLDL receptor. First, the uptake and degradation of the lipoproteins and the induction of cholesteryl ester formation displayed qualities of high affinity, saturable kinetics. Second, the uptake and degradation process was inhibited when the lysyl residues of the β-VLDL apoproteins were modified by reductive methylation. Third, the β-VLDL from the affected subjects competed with diet-induced canine ^{125}I-β-VLDL for the same cell surface receptors, but did not compete with chemically modified low density lipoproteins. Finally, the receptor-mediated uptake of these β-VLDL resulted in lysosomal degradation of the lipoproteins, which could be prevented by incubating the cells with chloroquine. Normal, triglyceride-rich VLDL were also degraded when incubated with the macrophages, but they were not degraded by the same receptor-mediated process responsible for the degradation of the β-VLDL of the patients. The degradation of the VLDL was not abolished by reductive methylation of the lipoproteins or by treatment of the cells with chloroquine. These studies demonstrate that the β-VLDL from subjects with atypical dysbetalipoproteinemia are taken up by macrophages via the same receptor-mediated process responsible for the uptake of diet-induced β-VLDL. The accelerated vascular disease seen in these patients may be the result of high concentrations of β-VLDL capable of binding to and delivering large quantities of cholesterol to macrophages and converting them into cells resembling the foam cells of atherosclerotic lesions.

INTRODUCTION

Similarities between arterial wall foam cells and tissue macrophages suggest that macrophages may be the progenitors of certain foam cells that are involved in atherogenesis. This hypothesis is supported by experimental data obtained from studies of rabbits and monkeys. These animals, when fed cholesterol-enriched diets, developed arterial wall foam cells that demonstrate some of the characteristics of tissue macrophages (1–3). These characteristics include the presence of cell surface receptors for C'3, Fc, β-very low density lipoproteins (β-VLDL),[1] and chemically modified low density lipoproteins (LDL), as well as high acid lipase activity.

In initial studies, mouse peritoneal macrophages incubated with naturally occurring lipoproteins of human subjects failed to produce the massive accumulation of cholesterol that is typical of arterial wall foam cells. Lipoprotein entry into cells, as indicated by the accumulation of lipoprotein protein or cholesterol, was possible only when LDL were modified in vitro by acetoacetylation (4), acetylation (5), malondialdehyde treatment (6), or preincubation with rabbit aortic endothelial cells (7).

The first demonstration of naturally occurring lipoproteins causing cholesteryl ester accumulation in macrophages involved the incubation of mouse peritoneal macrophages with a subfraction of VLDL obtained from animals fed diets high in cholesterol and fat (8, 9). The cholesteryl ester-enriched, $d < 1.006$ lipoproteins of this subfraction of VLDL have β-electrophoretic mobility and are referred to as β-VLDL. The β-VLDL are taken up by a specific receptor on the macrophage cell surface and then internalized by endocytosis. Degradation of the internalized β-VLDL occurs within lysosomes; the cholesterol is reesterified by acyl-CoA:cholesterol acyltransferase in the cytoplasm, and cholesteryl esters accumulate within the macrophages. No other lipoproteins from normal or cholesterol-fed animals have been shown to stimulate cholesteryl ester accretion. Furthermore, the lack of competition between chemically modified LDL and β-VLDL in binding to macrophages suggests that there are different receptors for these two lipoproteins (9).

Of importance regarding atherogenesis is the lack of autoregulation of the number of β-VLDL receptors on the surface of macrophages. Increasing the cholesteryl ester content of the cells does not result in complete repression of the number of receptors for β-VLDL, as occurs with the apo B,E receptors on fibroblasts and arterial smooth muscle cells after incubation with LDL (9). Exposure of tissue macrophages in the arterial wall to β-VLDL may lead to unregulated uptake of β-VLDL cholesterol and thus result in the creation of foam cells. Macrophages with the morphologic characteristics of foam cells have been shown to develop from mouse peritoneal macrophages incubated with canine β-VLDL (10).

The purpose of the present study was to investigate the interaction between macrophages and the β-VLDL of patients from a family with a newly described hyperlipidemic disorder. The affected members of this family were both hypercholesterolemic and hypertriglyceridemic; they also had high concentrations of β-VLDL and premature atherosclerosis (11). The subjects' lipoprotein abnormalities resembled those of patients with primary dysbetalipoproteinemia (type III hyperlipoproteinemia), although their lipoproteins did not have the classic apo E isoform pattern (i.e., E2/2) that is typical of primary dysbetalipoproteinemia (12, 13). Instead, the affected family members appeared to be homozygous for an apo E isoform with the electrophoretic mobility of E3, or to be heterozygous for two isoforms with electrofocusing properties similar to E3 (11). High concentrations of β-VLDL that have a marked propensity to stimulate macrophage cholesteryl ester accumulation will be shown to constitute a second feature that distinguishes patients with this disorder from those with more classic dysbetalipoproteinemia. The reactivity of the classic dysbetalipoproteinemic patients' β-VLDL is less pronounced (14).

METHODS

Human lipoproteins. All subjects (patients and controls) fasted for 12 h before a 500-ml plasmapheresis. The $d < 1.006$ fractions and LDL ($d = 1.02$–1.05) were isolated and washed as previously described (8). The β-VLDL of the patients were isolated from the $d < 1.006$ fractions by Geon-Pevikon block electrophoresis (15). Subfractionation of the β-VLDL by 4% agarose column chromatography was also performed as previously described (14). The β-VLDL were radioiodinated with iodine-125 (^{125}I) (9). More than 98% of the ^{125}I-radioactivity of the ^{125}I-labeled β-VLDL was precipitated by 10% trichloracetic acid, and <2% of the ^{125}I-radioactivity was extracted into chloroform/methanol. Protein concentrations were determined by the Lowry method (16). Polyacrylamide gel electrophoresis, using sodium dodecyl sulfate, was performed as described (17).

Canine lipoproteins. The hyperlipidemic canine lipoproteins were isolated from foxhounds fed a semisynthetic, coconut oil-cholesterol diet for 2–6 mo as described (8). The canine $d < 1.006$ fraction contained principally β-VLDL and was isolated by ultracentrifugation as described above for normal human VLDL.

Chemical modifications. Lysyl residues of the lipoproteins were modified by acetoacetylation with diketene or by reductive methylation as described (18).

Cultured mouse macrophages. Mouse peritoneal mac-

[1] *Abbreviations used in this paper:* AcAc, acetoacetylated; apo, apolipoprotein; β-VLDL, β-migrating very low density lipoproteins; DMEM, Dulbecco's modified Eagle's medium.

rophages were harvested from unstimulated mice using phosphate-buffered saline as described (5). Either 5.0×10^5, 1.5×10^6, or 3×10^6 cells were dispensed into 16-, 35-, or 60-mm plastic tissue culture dishes, respectively. After incubation for 2 h, the dishes were washed three times with Dulbecco's modified Eagle's medium (DMEM) without serum to remove nonadherent cells. The cultured macrophages were incubated for 18–24 h at 37°C in DMEM containing 20% fetal calf serum. The cells were washed once with DMEM and then used in the experiments.

Lipoprotein degradation and cholesteryl [^{14}C]oleate synthesis. The extent of proteolytic degradation of the iodinated lipoproteins was determined by previously published procedures (5, 19). The amount of cholesteryl [^{14}C]oleate formed during the incubation of cultured macrophages with [1-^{14}C]oleate complexed to albumin was determined by previously described procedures (20). Details of each experiment are presented in the respective figure captions or table legends.

Cellular cholesterol and cholesteryl ester mass. The mass of cellular cholesterol and cholesteryl esters was determined as described (21), except that the initial extraction solvent contained 5 μg stigmasterol and 2.0 μg stigmasteryl oleate to serve as internal standards instead of [^{3}H]cholesteryl oleate. The stigmasteryl oleate was synthesized essentially as described by Patel et al. (22) for the synthesis of cholesteryl esters. The samples were analyzed by gas-liquid chromatography (23).

Plasma and lipoprotein lipid determinations. The concentrations of unesterified cholesterol, total cholesterol, and triglycerides were measured by standard enzymatic procedures (Bio-Dynamics, Boehringer-Mannheim Corp., Indianapolis, IN).

RESULTS

The hyperlipidemic members of this kindred, ranging in age from 22 mo to 69 yr, had elevated concentrations of cholesteryl ester-enriched, $d < 1.006$ lipoproteins with β-electrophoretic mobility. The hyperlipidemia of the affected family members was expressed at an early age (evident at 22 mo of age), and the onset of xanthomas and atherosclerosis was also premature (11). Lipid values (Table I) obtained at the time of lipoprotein isolation were similar to those previously reported (11). A paucity of sample from patient IV-1, 22-mo-old son of III-2, precluded measurement of the cholesterol concentrations of the major lipoprotein fractions, but the cholesterol-to-triglyceride ratio of the $d < 1.006$ fraction was 0.51, indicative of cholesterol enrichment similar to that seen in the $d < 1.006$ fraction of classic dysbetalipoproteinemic patients (24). Treatment of patient I-1 (see pedigree, Fig. 1 of reference 11) with 1.0 g clofibrate, twice daily, and patient II-1 with 0.7 g nicotinic acid, three times daily, markedly reduced the cholesterol content of the VLDL ($d < 1.006$) fraction (Table I), but the β-VLDL did not disappear entirely.

In an attempt to evaluate the role of the $d < 1.006$ lipoproteins of affected family members in the pathogenesis of tissue cholesterol accumulation associated with hyperlipidemia, the ability of these lipoproteins to induce cholesteryl ester accumulation in mouse peritoneal macrophages was investigated. In initial studies, the $d < 1.006$ lipoproteins of the affected subjects were isolated by ultracentrifugation, but were not subjected to further subfractionation. These lipoproteins were then incubated with mouse peritoneal macrophages in the presence of [^{14}C]oleate, and the incorporation of [^{14}C]oleate into the cellular cholesteryl esters was determined. The assay of [^{14}C]oleate incorporation into cellular cholesteryl esters (see Fig. 1 and Table II) is an isotopic measure of cellular cholesteryl ester synthesis and accumulation. As shown in Fig. 1, $d < 1.006$ fractions from patients III-2, III-3, and III-4 markedly stimulated [^{14}C]oleate incorporation into cellular cholesteryl esters; this stimulation was dose dependent, a characteristic of high affinity, receptor-mediated binding and uptake. The $d < 1.006$ lipoproteins obtained from a normal subject (control VLDL) failed to stimulate [^{14}C]oleate incorporation. A comparison of all six affected family members from four generations (ages ranging from 22 mo for IV-1 to 66 yr for I-1) and 14 normal controls is shown in Table II. When these patients were untreated, their $d < 1.006$ lipoproteins stimulated [^{14}C]oleate incorporation into cholesteryl esters to an extent that was 15- to 30-fold above that which was induced by the $d < 1.006$ lipoproteins obtained from 14 normolipidemic individuals. Treatment of two patients (I-1 and II-1) with hypolipidemic agents, however, profoundly diminished the rate of cholesteryl ester synthesis induced by these lipoprotein

TABLE I

Plasma Lipid and Lipoprotein Cholesterol Concentrations of Patients with Atypical Dysbetalipoproteinemia

Patient*	Plasma		Lipoprotein cholesterol‡		
	Cholesterol	Triglycerides	VLDL	LDL	HDL
	mg/dl		mg/dl		
I-1	218	190	130	60	28
I-1§	215	185	67	127	21
II-1	630	623	375	222	33
II-1‖	237	219	62	148	27
III-2	433	730	252	161	20
III-3	196	241	86	85	15
III-4	301	282	109	173	19
IV-1	312	289	ND	ND	ND

ND, not determined.

* As described in reference 11.

‡ VLDL, $d < 1.006$ lipoproteins; LDL, $d = 1.006$–1.063; high density lipoproteins (HDL), $d = 1.063$–1.21.

§ Treated with 1.0 g clofibrate, twice daily.

‖ Treated with 0.7 g nicotinic acid, thrice daily.

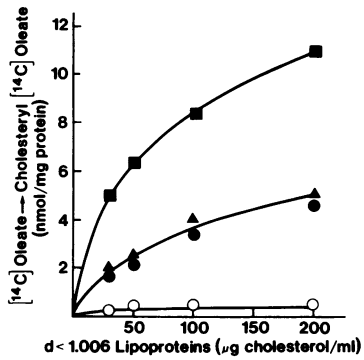

FIGURE 1 Cholesteryl ester formation in macrophages incubated with varying concentrations of $d < 1.006$ lipoproteins of affected family members and a normolipidemic control. Each monolayer received 1 ml of DMEM containing 0.2 mM [^{14}C]oleate and varying concentrations of $d < 1.006$ lipoproteins. The lipoproteins were obtained from patients III-2 (■), III-3 (▲), and III-4 (●), and a normolipidemic subject (○). After incubation for 16 h at 37°C, the cellular content of cholesteryl [^{14}C]oleate was determined.

fractions (Table II), despite the persistence of cholesterol-enriched β-VLDL in the plasma of these patients.

To validate this assay as an accurate indicator of cellular cholesteryl ester accumulation, an additional experiment was performed in which the $d < 1.006$ fraction from three of the affected subjects was incubated with mouse peritoneal macrophages, and the increases in cellular cholesteryl ester accumulation were directly determined by gas-liquid chromatography (Table III). The $d < 1.006$ lipoproteins of affected family members induced increases in cellular cholesteryl ester accumulation four- to sevenfold above those induced by the $d < 1.006$ fraction of control subjects. Thus, an increase in the cholesteryl ester mass in mouse peritoneal macrophages paralleled incorporation of [^{14}C]oleate into cellular cholesteryl esters in the same series of experiments. However, the extent of the relative increase as measured by [^{14}C]oleate incorporation was greater than the extent of the relative increase in cholesteryl ester mass measured directly by gas-liquid chromatography.

In the studies described above, unfractionated $d < 1.006$ lipoproteins were used. In previous studies of $d < 1.006$ lipoproteins from cholesterol-fed animals, it was shown that cholesterol-enriched, β-migrating subfractions of the $d < 1.006$ lipoproteins (β-VLDL) were responsible for inducing the greatest increases in macrophage cholesteryl ester accumulation (8, 9). To determine whether the β-VLDL of these family members constituted the subfraction active in stimulating macrophage cholesteryl ester accumulation, the $d < 1.006$ lipoproteins of patient II-1 were subfractionated by Geon-Pevikon preparative electrophoresis into α_2- and β-migrating subfractions (Table IV). In two other patients, III-2 and III-4, preparative electrophoresis was not required to isolate β-VLDL, because virtually all of the lipoproteins in their $d < 1.006$ fraction were β-VLDL, as judged by paper electrophoresis. The β-VLDL of patient II-1 were rich in cholesterol, and the cholesterol-to-protein ratio of the β-VLDL was greater than that of the α_2-VLDL (Table

TABLE II
*Effect of Hyperlipidemic VLDL on Macrophage Cholesteryl Ester Synthesis**

Subject	Multiple increase in cholesteryl ester synthesis above no lipoprotein control
Family A, patient I-1‡	23
II-1	28
III-2	31
III-3	22
III-4	21
IV-1	53
I-1 (Clofibrate Rx)	1.3
II-1 (Nicotinic A, Rx)	1.9
Normal $d < 1.006$ g/ml (n = 14)	1.4 (0–2.2)

* Each monolayer received 0.3 ml DMEM containing the indicated lipoprotein at a concentration of 300 μg of lipoprotein cholesterol/ml. After a 16-h incubation at 37°C, the cellular content of cholesteryl [^{14}C]oleate was determined. Control values were obtained by incubating macrophages in medium lacking additional lipoproteins. In the control dishes, cholesteryl [^{14}C]oleate formation ranged from 0.4 to 1.97 nmol/mg of cell protein.

‡ All subjects were initially studied while they were receiving no medication. In a second experiment, $d < 1.006$ g/ml lipoproteins were obtained from patient I-1 and II-1 after treatment for 8 wk with the indicated drugs.

TABLE III
Content of Cholesterol in Macrophages after Incubation with Human $d < 1.006$ Lipoprotein Fractions

$d < 1.006$ lipoproteins	Cellular cholesterol content	
	Free	Ester
	μg sterol/mg protein	
None	33	2.3
Normal	24	1.0
III-2	40	6.4
III-3	36	4.5
III-4	40	7.2

Each dish contained the indicated lipoprotein at a concentration of 300 μg of cholesterol/ml. After incubation at 37°C for 24 h, the cellular content of free and esterified cholesterol was determined. Values represent the mean of triplicate dishes for each $d < 1.006$ lipoprotein sample.

TABLE IV
Compositions of Subfractions of VLDL of Patient II-1

Lipid	Lipoprotein subfractions			
	α_2-VLDL	β-VLDL	β-VLDL-I	β-VLDL-II
	% composition by wt			
Total cholesterol	16	33	28	41
Triglycerides	58	45	50	38
Phospholipid	18	14	17	11
Protein	8	8	5	10
Cholesterol/protein	2.0	4.1	5.6	4.1

IV). The ability to stimulate cholesteryl ester synthesis clearly resided wth the β-VLDL, whereas the α_2-VLDL were no more effective in stimulating cellular cholesteryl ester synthesis than the $d < 1.006$ fractions from control subjects (Table V).

The β-VLDL of affected family members could be further subfractionated into two subclasses (β-VLDL-I and β-VLDL-II) by 4% agarose gel permeation chromatography (Table IV). Data are shown for patient II-1, but similar results were obtained with analogous subfractions from patients III-2 and III-4. The β-VLDL-I eluted in the void volume and contained lipoprotein particles with a mean diameter of 76 nm (±28 SD, $n = 100$), whereas the next fraction to elute, β-VLDL-II, contained smaller particles with a mean diameter of 42 nm (±13 SD, $n = 100$). The diameters

TABLE V
*Stimulation of Macrophage Cholesteryl [^{14}C]Oleate Synthesis by Subfractions of VLDL**

Experiment	Lipoprotein subfractions			
	α_2-VLDL	β-VLDL	β-VLDL-I	β-VLDL-II
	increase in [^{14}C]oleate incorporation above no lipoprotein control‡			
1 (Pt. II-1)	0.8	24	—	—
2 (Pt. II-1)	1.9	12	12.8	20.8
3 (Pt. III-2)§	—	28.7	13.7	27
4 (Pt. III-4)§	—	25.2	26.2	23.7
Normals ($n = 14$)	0–2.2	—	—	—

* The final lipoprotein cholesterol concentration in experiments 1 and 2 was 300 μg/ml of incubation medium; in experiments 3 and 4, it was 100 μg/ml.

‡ Cholesteryl [^{14}C]oleate formation in the absence of lipoprotein control dishes ranged from 0.4 to 1.97 nmol/mg cell protein.

§ In experiments 3 and 4, preparative electrophoresis was not required before gel permeation chromatography, as virtually all of the $d < 1.006$ fraction was β-VLDL. After incubation at 37°C for 16 h, the cellular content of cholesteryl [^{14}C]oleate was measured.

of these fractions resembled those described for subfractions of the β-VLDL of classic dysbetalipoproteinemic patients (14), and there were similarities in the major apoprotein constituents as well. Enrichment of apo B_{48} in the β-VLDL-I fraction and of apo B_{100} in the β-VLDL-II subfraction was observed (determination by 4% polyacrylamide gel electrophoresis). 11% polyacrylamide gel electrophoresis demonstrated enrichment of apo-B and apo-E in both β-VLDL-I and β-VLDL-II. Apolipoproteins B and E were also the predominant apoproteins in the α_2-VLDL, but there was a greater proportion of C apoproteins in α_2-VLDL compared with the β-VLDL and the β-VLDL subfractions. Comparison of the chemical compositions (Table IV) of these subfractions revealed that the β-VLDL-II were enriched in cholesterol compared with β-VLDL-I. Both β-VLDL subfractions from all three patients were highly active in stimulating cholesteryl ester formation in macrophages (Table V): when compared with cells lacking added lipoproteins, [^{14}C]oleate accumulation in macrophages was increased 13- to 27-fold by β-VLDL-I and β-VLDL-II. The extent of the stimulation observed with the β-VLDL-II was much greater than that previously observed for this fraction obtained from classic dysbetalipoproteinemics (14); fraction II from these latter patients exhibited only about one-third of the activity demonstrated by fraction I (11).

It was of interest to determine whether the cellular uptake of the β-VLDL from these patients was mediated by the same receptor as that described for β-VLDL obtained from cholesterol-fed animals (8, 9) and classic primary dysbetalipoproteinemic subjects (14), or whether the uptake was mediated by the receptor for chemically modified LDL, i.e., acetylated or acetoacetylated (AcAc) LDL. As shown in Fig. 2, the nonradiolabeled β-VLDL from both a cholesterol-fed dog and patient III-2 (11) were identical in their ability to displace canine ^{125}I-β-VLDL from binding to the macrophage β-VLDL receptor, whereas human LDL were much less effective in competing with the ^{125}I-β-VLDL. This suggested that the binding and uptake of the β-VLDL from the affected subjects occurred via the same receptor responsible for canine β-VLDL uptake by macrophages. It was also demonstrated that the β-VLDL from affected family members could not competitively inhibit the binding and degradation of AcAc LDL by mouse peritoneal macrophages (data not shown). Moreover, the addition of fucoidin, previously shown to inhibit the binding of chemically modified LDL to the modified LDL binding site, inhibited both the binding and degradation of ^{125}I-AcAc LDL (5, 9), but did not inhibit the receptor-mediated binding of the patients' β-VLDL

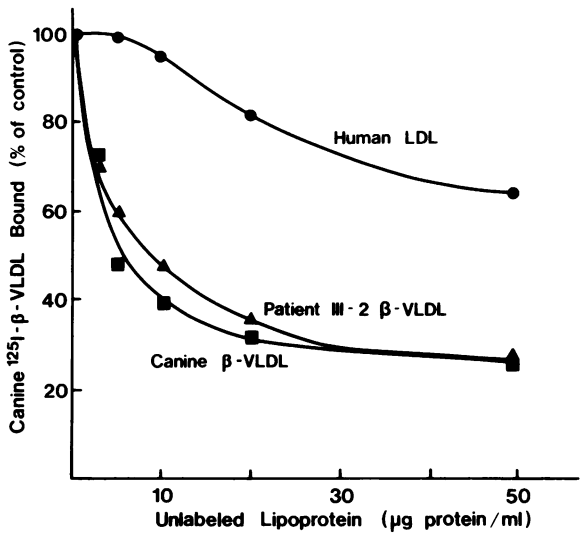

FIGURE 2 Effect of canine β-VLDL (■), patient III-2 β-VLDL (▲), and normal human LDL (●) on the binding of canine ^{125}I-β-VLDL by macrophages at 4°C. Each 35-mm dish of macrophages received 1.0 ml of DMEM containing 10% lipoprotein-deficient serum, canine hypercholesterolemic ^{125}I-β-VLDL (0.76 μg protein/ml; 3 μg cholesterol/ml), and the indicated concentrations of unlabeled lipoproteins. After incubation for 2 h at 4°C, the amount of ^{125}I-β-VLDL that bound to the cells was determined. The cells incubated with only canine ^{125}I-β-VLDL (100% value) bound 20 μg of radioiodinated apolipoprotein/mg macrophage protein.

(data not shown). Thus, it appears that the uptake of β-VLDL from these patients was mediated by a specific macrophage receptor for β-VLDL. The VLDL from control subjects did not appear to interact with this receptor and did not stimulate cholesteryl ester accumulation in macrophages.

Despite the inability of the control VLDL to stimulate cholesteryl ester accumulation (Fig. 3 *A*, *C*), a significant amount of this α_2-VLDL was degraded by the macrophages (Fig. 3 *B*, *D*). As shown in Fig. 3 *D*, when the α_2-VLDL of control subjects and the β-VLDL of affected subjects were added to dishes based on equal concentrations of cholesterol, there was equivalent degradation of the lipoproteins. To investigate further any possible role the β-VLDL receptor might play in the uptake and degradation of normal VLDL, the lysyl residues of the apolipoproteins of a patient's β-VLDL and those of control VLDL were modified by reductive methylation. This modification has previously been shown to inhibit binding, uptake, and degradation of canine β-VLDL by macrophages, indicating that the lysyl residues of the apolipoproteins are involved in the lipoprotein uptake mediated by the β-VLDL receptor (25). If receptor binding were a prerequisite for normal VLDL degradation, reductive methylation would inhibit binding and prevent any subsequent degradation of VLDL. As shown in Fig. 4, reductive methylation of β-VLDL from an affected subject reduced [^{14}C]oleate incorporation in macrophage cholesteryl esters nearly to control levels. Furthermore, as shown in Fig. 5, the reductive methylation of β-VLDL from patient III-2 reduced degradation of β-VLDL by 83%. However, the same treatment had much less effect on the degradation of control VLDL.

Another indication of a difference in the mechanism by which normal VLDL and β-VLDL are degraded was demonstrated by chloroquine treatment of macrophages (Fig. 5). Chloroquine treatment of cells has been shown to inhibit the lysosomal degradation of lipoproteins that have entered the cell by means of receptor-mediated endocytosis (9, 26). When mouse peritoneal macrophages were treated with chloroquine, the degradation of β-VLDL decreased by 72%. The effect of chloroquine in preventing the degradation of control VLDL was considerably less, implying that degradation did not depend on receptor-mediated endocytosis. The precise mechanism by which normal VLDL are degraded remains to be determined.

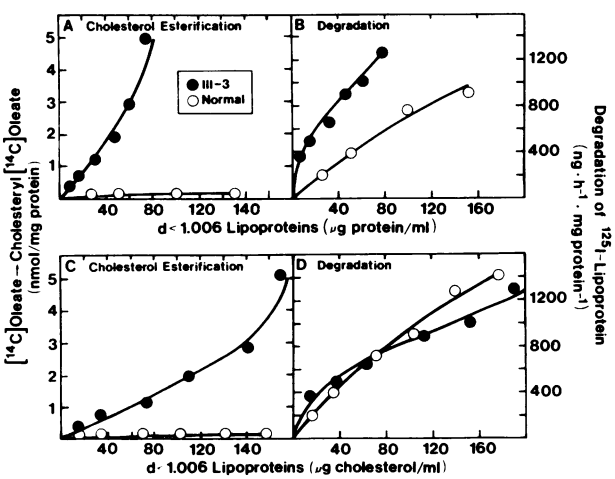

FIGURE 3 Comparison of the ability of the β-VLDL of patient III-3 (●) and normal, control VLDL (○) to stimulate cellular cholesterol esterification (*A*, *C*) or to be degraded (*B*, *D*) by macrophages. Each monolayer (*A*, *C*) received 1 ml DMEM containing 0.2 mM [^{14}C]oleate, 10% lipoprotein-deficient serum, and the indicated concentrations of ^{125}I-β-VLDL of patient III-3 or control ^{125}I-VLDL. Plates were prepared in triplicate for each concentration. After incubation for 16 h at 37°C, the cellular content of cholesteryl [^{14}C]oleate was determined. To compare degradation (*B*, *D*), the medium from the same plates was removed, and the proteolytic degradation products were determined as described in references 5 and 19. The half-bracket at the top of each column indicates one standard deviation.

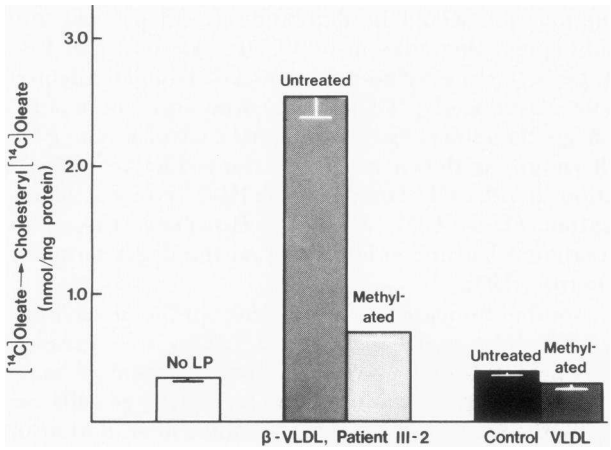

FIGURE 4 Effect of reductive methylation of $d < 1.006$ lipoproteins upon the ability of β-VLDL from patient III-2 and control VLDL to stimulate cholesteryl ester accumulation in macrophages. Each monolayer received 1 ml of DMEM containing 0.2 mM [^{14}C]oleate and the lipoprotein at a concentration of 150 μg of lipoprotein cholesterol. After incubation for 5 h at 37°C, the cellular content of cholesteryl [^{14}C]oleate was determined. No LP, dishes without added lipoproteins.

DISCUSSION

Evidence has been presented to show that the entire β-VLDL subfraction of the $d < 1.006$ lipoproteins of a family with an atypical form of dysbetalipoproteinemia (E3/3 phenotype instead of E2/2 as in classic dysbetalipoproteinemia) was very potent in stimulating cholesteryl ester accumulation in mouse peritoneal macrophages. This accretion of cholesteryl esters depended on the binding and uptake of β-VLDL by a specific β-VLDL receptor on the macrophages. When α_2-VLDL from normolipidemic subjects were incubated with macrophages, there was virtually no stimulation of cellular cholesteryl ester synthesis, despite some interaction between the lipoproteins and the macrophages that permitted a significant amount of α_2-VLDL degradation.

The β-VLDL from the patients with the atypical (E3/3 homozygous) dysbetalipoproteinemia differed from those previously described for classic (E2/2 homozygous) dysbetalipoproteinemics (14). The β-VLDL from the atypical subjects were severalfold more active in stimulating cholesteryl ester accumulation in macrophages than the β-VLDL of the more classic subjects (14). Furthermore, unlike the β-VLDL of classic dysbetalipoproteinemics (14), both subfractions of the β-VLDL isolated by 4% agarose chromatography were active in stimulating cholesteryl ester formation in macrophages. Previously, it was shown that fraction I, apparently representing intestinal β-VLDL, was more active than fraction II, representing β-VLDL of probable hepatic origin (14). It is also unique for β-VLDL and the hyperlipidemia to occur at such an early age (the youngest affected patient in this kindred was 22 mo of age at diagnosis) and for there to be vertical transmission through four generations.

The nature of the determinant responsible for the uptake of β-VLDL by macrophages remains in question. However, the present studies implicate a protein in mediating the uptake, since reductive methylation of lysyl residues abolishes the delivery of cholesterol to macrophages. The specific apoprotein involved has not been identified, but further characterization of the β-VLDL from typical and atypical dysbetalipoproteinemic subjects will undoubtedly be helpful.

The role of receptor-mediated endocytosis in the uptake of the β-VLDL from the affected subjects was suggested by the following: (*a*) the stimulation of cholesteryl ester synthesis in the macrophages by β-VLDL, which was dose dependent (a characteristic of

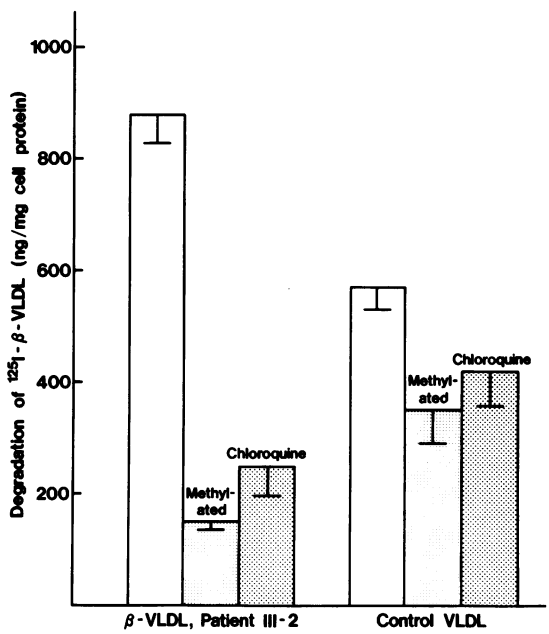

FIGURE 5 Effect of reductive methylation of the lipoproteins or chloroquine treatment of the cells on the degradation of β-VLDL or normal VLDL. Monolayers in triplicate received 1 ml DMEM containing 10% lipoprotein-deficient serum and 10 μg ^{125}I-lipoprotein protein or ^{125}I-methylated lipoprotein protein. Appropriate dishes were preincubated with 16 μM chloroquine 16 h before the beginning of the experiment, and the drug was also added during the 5-h experiment. After incubation for 5 h at 37°C, the medium was removed and the proteolytic degradative products were determined as described in references 5 and 19. The half-bracket at the top of each column indicates one standard deviation.

high affinity, receptor-mediated uptake, Fig. 1); (*b*) the modification of lysyl residues by reductive methylation, which prevented the stimulation of cholesteryl ester synthesis by β-VLDL (Fig. 4) and the degradation of ^{125}I-β-VLDL (Fig. 5); and (*c*) chloroquine treatment of the macrophages, which inhibited the degradation of β-VLDL (Fig. 5). It has been shown previously that reductive methylation prevents high affinity binding, uptake, and degradation of LDL and apo-E-enriched HDL_c by the apo B,E receptor of fibroblasts (18), and that the same modification of hyperlipidemic rabbit β-VLDL inhibits cholesteryl ester accumulation in macrophages (25). The present studies confirm these observations and extend them to β-VLDL obtained from human subjects.

To determine whether the β-VLDL from the affected subjects were taken up by the same receptor that has been shown to bind canine β-VLDL, further experiments were carried out. The displacement of canine ^{125}I-β-VLDL from macrophages by both nonradioiodinated canine β-VLDL and the β-VLDL of patient III-2 suggested that both lipoproteins were bound by the same receptor. Evidence that the uptake was not mediated by the receptor for chemically-modified LDL was obtained when the β-VLDL of the affected subjects failed to inhibit the uptake of ^{125}I-AcAc LDL by macrophages.

Of considerable interest was the amount of degradation of normal α_2-VLDL (Fig. 3) that occurred despite the lack of cellular cholesteryl ester accumulation stimulated by these lipoproteins. The relatively slight effect of reductive methylation on α_2-VLDL degradation, compared with the effect of reductive methylation on β-VLDL degradation (Fig. 5), suggests that the majority of the degradation of normal α_2-VLDL is not mediated by the β-VLDL receptor. This inference was supported by data obtained from experiments involving chloroquine treatment of macrophages. The inhibition of lysosomal functioning by chloroquine treatment had little effect on macrophage degradation of α_2-VLDL, whereas β-VLDL degradation was markedly decreased. This indicates that the proteolysis of α_2-VLDL may not be intralysosomal. Proteases may exist on the external cell surface of macrophages (27), and it may be at these sites where most of the α_2-VLDL degradation occurs. Alternatively, it has been proposed that apolipoprotein degradation by macrophages without concomitant cellular accumulation of cholesterol from the associated lipoprotein may be due to differences in the intracellular handling of cholesterol. Lipoproteins that gain entry to a cell via a specific lipoprotein receptor are degraded in lysosomes and stimulate cholesteryl ester synthesis, whereas nonreceptor-mediated uptake may allow apoprotein degradation but not a net increase in cellular cholesterol (28).

The increase in cellular cholesteryl esters induced by the affected patients' VLDL was documented by two separate methods, which provided varied estimates of the extent of cholesteryl ester accumulation. The incorporation of [^{14}C]oleate into cellular cholesteryl esters was 15- to 30-fold greater than that induced by control VLDL, whereas direct mass measurements indicated that cellular cholesteryl ester mass increased only four- to sevenfold above that induced by control VLDL. It is likely that the radioisotopic technique provides an overestimate of the increase in cellular cholesteryl esters compared with mass measurements for the following reason: Before the incubations, the macrophages contained no [^{14}C]oleate in their cholesteryl esters but did contain a significant amount of cholesteryl ester mass (2.3 μg sterol/μg cell protein, Table III). The lower (zero) background level of [^{14}C]oleate cholesteryl esters thus favors a greater relative increase in cellular [^{14}C]oleate cholesteryl esters compared with cells incubated with control VLDL, whereas the existence of a finite amount of cholesteryl ester mass at the start of the incubations favors a lower relative increase in cellular cholesteryl ester mass. Moreover, the continual hydrolysis and reesterification of cholesteryl esters via the macrophage cholesteryl ester cycle would promote reesterification of previously existing unlabeled cellular cholesteryl esters with [^{14}C]oleate without producing a net increase in cholesteryl ester mass (21). A similar disparity between the relative increases in macrophage cholesteryl ester mass and cholesteryl [^{14}C]oleate content was previously observed in studies of the accumulation of cholesteryl esters induced by incubating these cells with AcAc LDL (5).

The accumulation of cholesteryl esters in macrophages incubated with β-VLDL suggests a possible mechanism for the development of accelerated atherogenesis (10, 29). The β-VLDL of cholesterol-fed animals (10, 29) and the β-VLDL of patients with classic dysbetalipoproteinemia (14) stimulate cholesteryl ester accumulation in these cells in vitro and are associated with accelerated atherosclerosis. Recently, it has been shown that lipid-laden foam cells grown from aortic explants of atherosclerotic rabbits possess a receptor for β-VLDL, as well as having other characteristics of macrophages (3). This evidence further supports the intriguing hypothesis that β-VLDL and macrophages play a role in atherogenesis.

For reasons discussed above, the β-VLDL of the patients with atypical dysbetalipoproteinemia appear to be more active in causing cholesteryl ester accumulation in macrophages than the β-VLDL described

in subjects with classic dysbetalipoproteinemia (14). The presence of these particles in the plasma at an early age may account for the relatively early onset of clinically evident atherosclerosis and xanthomas in these patients. It is significant that in the two patients treated with hypolipidemic agents, the plasma concentrations of β-VLDL decreased, and furthermore, those β-VLDL still present in the plasma lacked the ability to stimulate cholesteryl ester accumulation in macrophages (Tables I and II). In addition, the treatment of these patients resulted in the resolution of their xanthomas (11).

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