Low density lipoprotein degradation by mononuclear cells from normal and dyslipoproteinemic subjects

(lipoprotein receptors/familial hypercholesterolemia/abetalipoproteinemia)

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ABSTRACT Three major characteristics of cell surface low density lipoprotein (LDL) receptor activity in fibroblasts or lymphocytes are high-affinity LDL binding or degradation, specificity for LDL, and "inducibility"—that is, the ability to increase when cells are cultured in the absence of lipoproteins. Cells from patients with receptor-negative homozygous familial hypercholesterolemia (FH) have been reported to express none of these characteristics, and the patients are thought to have a genetic absence of LDL receptors. We found that, although induced receptor-negative lymphocytes degraded less LDL than did normal lymphocytes, the curves for LDL degradation versus LDL concentration were biphasic, with greater concentration-dependence at LDL concentrations $< 60 \mu\text{g/ml}$, indicating high-affinity LDL degradation. The percentage of specific LDL degradation by induced receptor-negative lymphocytes was two-thirds of normal with LDL at $10 \mu\text{g/ml}$ and increased to normal at $50 \mu\text{g/ml}$, an LDL concentration still within the range of high-affinity degradation. Receptor-negative lymphocytes could be induced by incubation in the absence of lipoproteins to degrade twice as much LDL as they did when freshly isolated. Freshly isolated cells from abetalipoproteinemic patients and one receptor-negative patient degraded as much LDL as did fresh normal cells. The findings indicate that receptor-negative lymphocytes have a mechanism for facilitated uptake of LDL that resembles that of normal lymphocytes, although it is not as efficient.

Familial hypercholesterolemia (FH) is a disease characterized by high plasma concentrations of β (low density) lipoproteins, whose major protein is apolipoprotein B. Its autosomal dominant pattern of inheritance (1, 2) offers the opportunity to study patients who are either heterozygous or homozygous for the defect that leads to the disease. Abetalipoproteinemia is a recessive disease in which all lipoproteins containing apolipoprotein B are absent. In order to characterize patients with these diseases biochemically, we used the *in vitro* assay of LDL degradation by lymphocytes first described by Ho *et al.* (3). These investigators, using cells incubated without lipoproteins ("induced" cells), demonstrated defective binding and degradation of LDL by both fibroblasts and lymphocytes from FH patients (3–7). This defect was particularly marked in receptor-negative homozygous FH patients and was attributed to a genetic absence of high-affinity LDL binding sites or receptors on their cells (6). We studied LDL degradation by normal, FH, and abetalipoproteinemic lymphocytes under a variety of experimental conditions. These included: (i) different times of incubation at constant LDL concentration, (ii) different LDL concentrations for the same time period, (iii) omission of incubation in lipoprotein-deficient serum (LPDS) for cells from both normal subjects and receptor-negative patients, and (iv) treatment of het-

erozygous FH patients with the lipid-lowering drug colestipol before lymphocyte isolation. We also examined the degree to which LDL degradation by induced lymphocytes was specific for LDL. Lymphocytes were used to study LDL receptor activity because lymphocyte LDL receptors are genetically identical to fibroblast LDL receptors (3).

METHODS

Subjects. Lymphocytes were obtained from 16 normal volunteers, 9 FH heterozygotes on no drugs, 3 FH heterozygotes on the bile acid-binding resin colestipol (10–20 g daily), 2 receptor-negative FH homozygotes (O.C. and D.R.), and 2 patients with abetalipoproteinemia (1). Before blood was drawn for the studies, informed consent in writing was obtained from the subjects or their parents. The homozygous FH patients were two of the six patients from the original group of receptor-negative FH homozygotes described by Goldstein *et al.* (8). The determination of FH in those subjects designated as heterozygotes was based on criteria used previously to distinguish between FH and familial combined hyperlipidemia (2). The three FH heterozygotes on colestipol had a 22% decrease in mean ($\pm$ SD) plasma total cholesterol from $325 \pm 27 \text{ mg/100 ml}$ before treatment to $255 \pm 21 \text{ mg/100 ml}$ during treatment, which compares favorably with the average 12–17% decrease in plasma total cholesterol reported in a previous study of the drug's efficacy (9).

Lipoproteins. LDL (density = $1.025\text{--}1.050 \text{ g/ml}$) were isolated from normal plasma by sequential flotation in the ultracentrifuge (10). LPDS (density $> 1.25 \text{ g/ml}$) was prepared by using solid KBr for density adjustment (11) before ultracentrifugation at $100,000 \times g$ for 18 hr. After extensive dialysis, the LDL and LPDS were characterized by paper electrophoresis, double immunodiffusion, and immunoelectrophoresis (10). Protein concentration was determined by the method of Lowry *et al.* (12).

LDL was iodinated with Na^{125}I by a previously described modification of the McFarlane technique (13). After extensive dialysis, 97–99% of the radioactivity was precipitable by 10% trichloroacetic acid. Final specific activities were between 100 and 400 cpm/ng of protein.

Collection and Preparation of Mononuclear Cells. Mononuclear cells were isolated from peripheral blood by the methods of Boyum (14) and Ho *et al.* (3). Cell viability ranged from 97% to 99%. If LDL degradation was to be measured in freshly isolated cells, the cells were incubated with LDL at this time, under the same conditions as for cells preincubated in LPDS.

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Abbreviations: FH, familial hypercholesterolemia; LDL, low density lipoproteins; LPDS, lipoprotein-deficient serum.

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Prior to incubation with LDL, the freshly isolated cells (about 85% lymphocytes and 15% monocytes) were "preincubated" in RPMI 1640 culture medium (Microbiological Associates) with glutamine and LPDS (3, 15) at an average protein concentration of 7 mg/ml to induce maximal LDL uptake and degradation. These cells are subsequently referred to as "induced" cells. The final cell concentration was between 1.8 and 2.0×10^6 cells per ml. Penicillin/streptomycin mixture was added to each flask to give a final antibiotic concentration of ≈ 300 units/ml and $300 \mu\text{g/ml}$, respectively. The culture flasks were kept in a humidified incubator with 5% CO_2 at 37°C for 66 ± 1 hr. This length of preincubation was sufficient to induce the maximum possible LDL binding (3).

After preincubation in LPDS, the contents of the flasks for each subject were pooled, leaving behind monocytes, which adhered to the sides of the flask (4). Differential counts of stained smears showed that the preincubated cell preparations contained less than 1% monocytes. Cell viabilities ranged from 97% to 99%. Cell counts averaged $1.5 \pm 0.4 \times 10^6$ cells per ml; in this range, LDL degradation is linearly proportional to cell concentration (4). Aliquots (2 ml) of pooled lymphocyte suspensions were incubated with LDL in a humidified incubator at 37°C with 5% CO_2 . Cells were incubated with $10 \mu\text{g}$ of ^{125}I -LDL (^{125}I -LDL) per ml and the desired quantities of unlabeled LDL. The times of incubation and amounts of LDL are indicated in the figure legends. Cell protein averaged $120 \mu\text{g}$ (range, 88 – $152 \mu\text{g}$) per flask.

LDL Metabolism. After centrifugation of each incubation mixture, the supernatant was treated with an equal volume of 10% trichloroacetic acid; the acid-soluble material was treated with KI and H_2O_2 (16). After chloroform extraction to remove free iodine, an aliquot of the aqueous phase was assayed in a gamma counter. Cell-free control samples, which had been incubated with LDL under the same conditions as the cells, were treated in the same way; the values obtained were used to correct the cell degradation results. The average variation from the mean for duplicate samples was 3% (range, 1–6%).

The cell pellets remaining after centrifugation of the incubation mixtures were washed, layered over fetal calf serum (7), and centrifuged at $8,000 \times g$ for 2.5 min. After removal of the supernatant, the pellets were assayed in the gamma counter. All results were corrected by subtracting the cell-associated cpm

at zero time from subsequent cpm and were used to calculate LDL accumulation.

RESULTS

When LPDS-treated lymphocytes from normal and FH subjects were incubated at 37°C with $10 \mu\text{g}$ of ^{125}I -LDL protein per ml for up to 4 hr, LDL accumulation (Fig. 1A) reached a plateau by 2 hr, while LDL degradation (Fig. 1B) was linear up to 4 hr, by polynomial regression analysis. At each time point, cells from heterozygous FH subjects on no drug degraded about 40% as much LDL and cells from FH heterozygotes who were taking colestipol degraded about 65% as much LDL as did normal cells (Fig. 1B). Thus, cells from the FH subjects on colestipol degraded substantially more LDL than did cells from similar FH subjects on no drug. In the presence of $10 \mu\text{g}$ of LDL per ml, cells from the two receptor-negative homozygotes degraded the least LDL. After 4 hr, receptor-negative cells from subject D.R. had degraded about 9% as much LDL and cells from receptor-negative donor O.C. had degraded 2.5% as much LDL as did normal cells.

The mean concentration curves for degradation by induced normal and heterozygous FH lymphocytes were biphasic, with greater concentration dependence at LDL concentrations $< 60 \mu\text{g/ml}$ and less concentration-dependence at higher concentrations (Fig. 2). Brown and Goldstein and co-workers, who obtained similar results with both fibroblasts and lymphocytes (3–5), have designated the more concentration-sensitive part of the curve "high-affinity" degradation and the less concentration-sensitive part "low-affinity" degradation (5). The differences in LDL degradation between cells from heterozygotes on or not on colestipol were more marked at higher LDL concentrations (Fig. 2) than when LDL was at $10 \mu\text{g/ml}$ (Fig. 1B). At 60 – $250 \mu\text{g}$ of LDL per ml, degradation by cells from colestipol-treated heterozygotes was essentially the same as normal (although the cells had been in culture for 3 days before LDL degradation was assayed), whereas degradation by cells from untreated heterozygotes ranged from about 50% of normal at $60 \mu\text{g/ml}$ to 65% of normal at $250 \mu\text{g/ml}$. At $1,000 \mu\text{g}$ of LDL per ml, degradation by cells from untreated heterozygotes was about 60% of normal (results not shown).

Like the time curves, the concentration curves for LDL degradation by induced receptor-negative lymphocytes were much

FIG. 1. Accumulation and degradation of LDL by normal and FH lymphocytes as a function of time. Aliquots (2 ml) of induced lymphocytes from normal (●), heterozygous FH patients on colestipol (□), heterozygous FH patients on no drugs (■), and two receptor-negative homozygous FH patients were incubated at 37°C for the indicated times with $10 \mu\text{g}$ of ^{125}I -LDL per ml (100 – 400 cpm/ng of LDL protein). LDL accumulation (A) and degradation (B) were measured as described. In A each point is the mean value $\pm$ SEM for three or more values (corresponding values in B are cited in parentheses) obtained for lymphocytes from four (14) normals, one (three) heterozygous FH patient(s) on colestipol, four (nine) heterozygous FH patients on no drug, homozygous FH patient O.C. (▲), or homozygous FH patient D.R. (▼).

FIG. 2. Effects of LDL concentration on its degradation by normal and heterozygous FH lymphocytes. Aliquots (2 ml) of induced lymphocytes were incubated for 4 hr at 37°C with 10 μ g of 125 I-LDL per ml (100–400 cpm/ng of LDL protein) and increasing concentrations of unlabeled LDL. Degradation was measured as described. Each point is the mean value ($\pm$ SEM) obtained for lymphocytes from 12 normals (●), three heterozygous FH patients on colestipol (□), or eight heterozygous FH patients on no drugs (■).

lower than normal, but they did have shapes that were similar to those of normal cells, with steeper slopes below 60 μ g of LDL per ml, followed by more gradual slopes at higher LDL concentrations (Fig. 3). Regression analysis of the receptor-negative curves indicated that each was fitted by a two-component or quadratic equation better than by a linear equation; higher-order equations produced no better fit.

The finding that the concentration curves obtained with lymphocytes from receptor-negative homozygous FH patients were biphasic like normal curves raised the question of whether receptor-negative cells were also inducible. Concentration curves produced by freshly isolated cells were compared with the curves from induced cells (Fig. 3). The results indicated that receptor-negative cells were indeed inducible. Induced cells from subject D.R. (Fig. 3C) degraded about twice as much LDL as did her freshly isolated cells at each LDL concentration studied. Induced cells from subject O.C. (Fig. 3B) degraded the same amount of LDL as did her fresh cells at 10 μ g of LDL per ml, but at 50 μ g/ml (still within the high-affinity range), her induced cells also degraded twice as much LDL as did her fresh cells. The magnitude of the increase in degradation, which was caused by induction of the receptor-negative cells, was smaller than the increase for normal cells (Fig. 3A), particularly with LDL at 10 μ g/ml, where the increase for normal cells was almost 20-fold. At higher LDL concentrations, the increase between fresh and induced normal cells was only about 5-fold.

Another important finding shown in Fig. 3 was that the difference in LDL degradation between normal and receptor-negative cells was much less for fresh cells than for induced cells. In fact, there was no difference for receptor-negative donor D.R.

(Fig. 3C), whose fresh cells degraded as much LDL as did fresh normal cells (Fig. 3A) at each LDL concentration studied. Fresh receptor-negative cells from subject O.C. (Fig. 3B) degraded as much LDL as did normal cells with LDL at 10 μ g/ml but fell to 30% of normal at 50 μ g/ml.

High-affinity LDL degradation has also been defined as that degradation which occurs through LDL uptake by cell surface receptors that are "specific" for LDL, whereas low-affinity degradation has been postulated to occur without the intervention of specific receptors, perhaps through bulk fluid endocytosis (17). Specific degradation is that degradation of radio-labeled ligand which is eliminated by an excess of unlabeled ligand (3, 5). If cells have few or no specific LDL receptors that can be competitively inhibited, as has been postulated for homozygous FH cells (6), then they should display little or no specific degradation. We examined (Table 1) the specificity of LDL degradation at two LDL concentrations within the high-affinity range, 10 and 50 μ g/ml. When lymphocytes were incubated with labeled LDL at 10 μ g/ml in the absence and presence of unlabeled LDL at 240 μ g/ml, specific degradation by receptor-negative cells was about 60% of their total degradation compared with about 90% for normal cells. There was no difference in the percentage of specific degradation between normal lymphocytes, either group of heterozygous FH cells, or abetalipoproteinemic cells. When cells were incubated with 125 I-LDL at 50 μ g/ml in the absence and presence of unlabeled LDL at 950 μ g/ml, degradation was about 90% specific for all cell groups.

Evidence that LDL receptors respond to apolipoproteins E (18) and B came from studies of cells from two subjects with abetalipoproteinemia (Fig. 4). If the receptors recognized only apolipoprotein B, they should have been operating at their maximal capacity in freshly isolated abetalipoproteinemic mononuclear cells because the patients' plasmas contained no apolipoprotein B. However, we found that incubation in LPDS enhanced LDL degradation by the induced lymphocytes compared with freshly isolated cells by about 5-fold with LDL at 10 μ g/ml (Fig. 4A), about 4-fold at 60 μ g/ml, and about 3-fold at 125 and 250 μ g/ml (Fig. 4B). Similar results have been reported by Reichl and co-workers (19) and by Ho *et al.* (20). Blum

Table 1. Percentage of specific LDL degradation by induced lymphocytes*

Cell type	Specific degradation, % $\pm$ SD	
	125 I-LDL, 10 μ g/ml	125 I-LDL, 50 μ g/ml
Normal	92 $\pm$ 3 (12)	90 $\pm$ 2 (6)
Hetero FH on colestipol	87 $\pm$ 2 (3)	
Hetero FH on no drugs	86 $\pm$ 7 (6)	90 $\pm$ 2 (3)
Homo FH		
O.C.	57 (1)	95 (1)
D.R.	63 (1)	
Abetalipoproteinemic	92 (2)	

Hetero FH, heterozygous FH subject; homo FH, homozygous FH subject.

After preincubation in LPDS and subsequent incubation for 4 hr with LDL, the percentage of specific degradation for each cell type was obtained by subtracting from 100% the percentage of nonspecific degradation, which was calculated by dividing the ng of 125 I-LDL degraded in the presence of an excess of unlabeled LDL (nonspecific degradation) by the ng of 125 I-LDL degraded in the absence of unlabeled lipoprotein (total degradation). Total LDL degradation at various LDL concentrations is shown graphically in Figs. 2–4. For an 125 I-LDL concentration of 10 μ g/ml, the concentration of unlabeled LDL was 240 μ g/ml, whereas at 50 μ g/ml of 125 I-LDL, the concentration of unlabeled LDL was 950 μ g/ml.

*The number of patients is shown in parentheses.

FIG. 3. A comparison of LDL degradation by fresh mononuclear cells and induced lymphocytes from normal subjects and receptor-negative homozygous FH patients. The effects of LDL concentration on LDL degradation by freshly isolated mononuclear cells (---) and LPDS-induced lymphocytes (—) were examined. The results for normal cells (A) were mean values ($\pm$ SEM for three or more values) for fresh ($\circ$) and induced ($\bullet$) cells from two subjects whose individual values were the means of duplicate samples. Results for receptor-negative patient O.C.'s (B) induced ($\blacktriangle$) cells are mean values ($\pm$ SEM for three or more values) from two experiments, whereas results from her fresh ($\triangle$) cells are from a single experiment with duplicate samples. The same conditions were true for receptor-negative patient D.R.'s (C) induced ($\blacktriangledown$) and fresh ($\triangledown$) cells. Aliquots (2 ml) of cells were incubated for 4 hr at 37°C with 10 μ g of 125 I-LDL per ml (200–400 cpm/ng of LDL protein) and increasing concentrations of unlabeled LDL. Fresh cells were studied immediately after isolation; induced cells from the same blood sample were incubated with LPDS, as usual, before study. LDL concentrations, incubation volumes, and approximate cell number were kept the same for both fresh and induced cells so that direct comparisons of LDL degradation could be made between the two conditions.

et al. (21) have shown that the plasma of abetalipoproteinemic subjects contains normal amounts of apolipoprotein E; this could suppress high-affinity LDL receptors *in vivo* and lead to the lower LDL degradation observed in freshly isolated abetalipoproteinemic cells compared with induced cells. The induced abetalipoproteinemic cells responded to induction to the same extent as heterozygous FH cells. Comparison of Fig. 4 with Figs. 1B and 2 shows that induced abetalipoproteinemic lymphocytes degraded only about 45% as much LDL as did induced normal cells. However, fresh abetalipoproteinemic cells

(Fig. 4B) degraded as much LDL as did fresh normal cells (Fig. 3A).

DISCUSSION

LDL receptors are characterized by high affinity and specificity for LDL (6) and by inducibility (3)—that is, the ability to increase activity when lipoproteins are removed from the medium. The presence of high-affinity LDL binding or degradation is determined by the shape of the binding or degradation curve with increasing LDL concentration (5). If the concen-

FIG. 4. A comparison of LDL degradation by fresh mononuclear cells and induced lymphocytes from two abetalipoproteinemic patients. The effects of time (A) and LDL concentration (B) on LDL degradation by abetalipoproteinemic freshly isolated mononuclear cells (---) and LPDS-induced lymphocytes (—) were examined. For the time experiments, 2-ml aliquots of cells were incubated for the indicated times at 37°C with 10 μ g of 125 I-LDL per ml (280 cpm/ng of LDL protein). For the concentration experiments, the cells were incubated for 4 hr at 37°C with 10 μ g of 125 I-LDL per ml and increasing concentrations of unlabeled LDL. Results are mean values ($\pm$ SEM) obtained from two patients. Fresh cells were studied immediately after isolation; induced cells from the same blood sample were incubated with LPDS before study. LDL concentrations, incubation volumes, and approximate cell number were kept the same for both fresh and induced cells so that direct comparisons of LDL degradation could be made between the two conditions.

tration curve is biphasic, both high- and low-affinity binding sites are said to be functioning. We have found that lymphocytes from two receptor-negative patients had biphasic concentration curves; the finding thus fulfilled one of the criteria for the presence of high-affinity LDL receptors.

Another criterion met by the receptor-negative lymphocytes that we studied was that of specificity for LDL. With LDL in the medium at 10 $\mu\text{g}/\text{ml}$, the percentage of specific degradation by these cells was two-thirds of normal, a figure far higher than could be expected if the receptor-negative cells degraded LDL by a nonspecific low-affinity process only. Furthermore, because specific, high-affinity LDL degradation predominates not only at 10 $\mu\text{g}/\text{ml}$ but also over a concentration range up to at least 50 $\mu\text{g}/\text{ml}$ (4), we examined specificity at the latter concentration. At 50 $\mu\text{g}/\text{ml}$, the percentage of specific LDL degradation by receptor-negative cells was normal. Additional evidence that receptor-negative lymphocytes might have LDL receptors came from our finding that, like normal cells, the cells from the two receptor-negative FH homozygotes whom we studied could be induced to degrade more LDL after incubation without lipoproteins.

Also of interest were the findings that freshly isolated normal, receptor-negative, and abetalipoproteinemic cells degraded similar amounts of LDL and that these cells had biphasic concentration curves, just as induced cells did. The latter suggests that fresh mononuclear cells also have a mechanism for facilitated uptake of LDL.

Although we found similar patterns of LDL degradation by normal, FH, and abetalipoproteinemic cells, LDL degradation did not reach normal levels in any of the induced cells from subjects not treated with colestipol. One possible explanation is that the binding sites on abnormal cells have lower affinities for LDL because the cells' genes specify receptors that differ structurally from the normal receptor. Recent work with monoclonal antibodies against LDL receptors gives some support to such a hypothesis (22–24). Alternatively, however, the decrease in LDL degradation by induced FH and abetalipoproteinemic cells may reflect varying degrees of LDL receptor suppression. That the suppression could originate *in vivo* and still be expressed *in vitro* is suggested by the data provided by heterozygous FH cells from colestipol-treated donors. Although these cells had been thoroughly washed during isolation and had neither autologous plasma nor colestipol in the medium for the three-day preincubation period, they degraded more LDL than did lymphocytes from patients who were not on colestipol; thus, the effects of the drug were detected *in vitro* long after the cells had been removed from plasma, and at a point in time more than three half-lives beyond the reported 20-hr half-life of the LDL receptor (17). Similarly, whatever factors suppressed LDL receptors on FH or abetalipoproteinemic cells *in vivo* might continue to act even after long incubation *in vitro*. Although lipid-lowering drug effects on LDL metabolism have been reported previously *in vivo* (25, 26) or in freshly isolated cells (27, 28), the enhancement of LDL degradation by colestipol that we observed was unexpected in cells so long after their isolation.

What might suppress LDL receptors *in vivo*? In abetalipoproteinemia, apolipoprotein E is a possibility. It is known to be a more potent suppressor of LDL receptors than apolipoprotein B (18) and is present in approximately normal quantities in abetalipoproteinemic plasma (21). In FH, circulating LDL might suppress the receptors, just as it does in normal cells. Kita *et al.* (29) suggested that the absence of LDL receptors in the Watanabe heritable hyperlipidemic rabbit may result from metabolic suppression by abnormally high plasma LDL levels rather than from a genetic defect. Similarly, LDL receptors in FH patients may be suppressed by the high plasma LDL concentra-

tions found in that disease. The high plasma LDL levels could be the result of overproduction of LDL. The latter has been reported by several laboratories (30–33).

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