Binding, internalization, and degradation of low density lipoprotein by normal human fibroblasts and by fibroblasts from a case of homozygous familial hypercholesterolemia

(cell membrane receptors/cholesterol biosynthesis)

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ABSTRACT Skin fibroblasts from a patient with homozygous familial hypercholesterolemia (HFH) were compared with normal skin fibroblasts with regard to binding, internalization, and degradation of iodinated human low density lipoprotein (LDL). Like other cell lines from HFH patients, the mutant cells showed no suppression of sterol synthesis by LDL. Surface binding, measured at 0° to eliminate the appreciable internalization that was shown to occur at 37°, was on the average slightly less for HFH cells than normal cells at low LDL concentrations but comparable or even greater at high LDL concentrations (>80 μg of LDL protein per ml). A major defect observed was in the rate of internalization of LDL at 37°, which was only 1-10% of that in normal cells. LDL degradation was also markedly reduced but not to the same extent. Thus, a larger fraction of the LDL taken up appeared to be degraded by the mutant cells. The most striking defect observed, then, was not in surface binding of LDL but in rate of LDL internalization. While this might be secondary to a defect in specific binding sites of LDL, the magnitude of the observed differences in binding at low temperature seems too small to account for the huge differences in internalization (15- to 115-fold).

When normal human skin fibroblasts are exposed for 16-20 hr to a medium containing lipoprotein-deficient fetal calf serum there is a marked increase in cholesterol synthesis (1) associated with a parallel increase in the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (2). If low density lipoprotein (LDL) is added to the lipoprotein-deficient serum (LDS), however, and the cells are incubated for an additional 6 hr or more, the rate of cholesterol synthesis and the level of the reductase are once again suppressed (2). Brown, Goldstein, and their coworkers have shown that skin fibroblasts from patients with homozygous familial hypercholesterolemia (HFH) fail to show this LDL-induced suppression of the reductase although they do show suppression upon addition of cholesterol itself to the medium (2, 3). These investigators have carried out a series of elegant studies showing that this loss of LDL control of cholesterol synthesis is associated with a decrease in the uptake and degradation of the LDL apoprotein (4). They postulate that the primary defect in the mutant fibroblasts lies in the total or nearly total absence of specific, high-affinity cell-surface receptors for LDL. However, the evidence for specific high-affinity receptor sites is so far largely indirect, based primarily on analysis of LDL binding and degradation as a function of LDL concentrations. Moreover, binding was usually measured in cells exposed to labeled LDL at 37° for several hours, during which time an appreciable amount of the lipoprotein might be internalized rather than surface-bound.

We report here comparative studies of normal fibroblasts and of fibroblasts from a patient with the classical clinical features of HFH. Special attention was given to the determination of surface-bound LDL (as opposed to internalized LDL) by measuring LDL binding at 0° and by measuring that fraction of bound LDL released from the cells by brief digestion with trypsin. Bierman et al. (5) have presented evidence that the latter corresponds to surface-bound LDL and additional support for that interpretation is presented here.

METHODS

Mutant fibroblasts were grown from a skin biopsy of a 19-year-old female with HFH. The clinical features and in vivo LDL turnover of this patient have been reported (patient P4 in ref. 6; patient 2 in ref. 7). Control skin fibroblasts (line B.B.) were grown from a prepubial biopsy of a normal infant. Normal fibroblasts (line S.A.) from a 22-year-old man were also studied and the results were comparable.

Cells were maintained in F-12 medium or Dulbecco's modification of Eagle's minimal essential medium (DME) containing penicillin (50 units/ml), streptomycin (50 μg/ml), and 10% (vol/vol) fetal calf serum (FCS; Irvine Scientific Co.). At least 24 hr before study the medium was changed to fresh DME containing 10% FCS. Cells were studied between the 8th and 20th passages. Comparison of line P.A. at the 9th and 20th passages was made with respect to LDL uptake and inhibition of sterol synthesis and comparable results were obtained. Plastic petri dishes (60 mm) were seeded with 1 to 2 × 10⁶ cells and used 4-6 days later. Growth curves showed that the normal cell line (B.B.) reached a plateau at 1 to 2 × 10⁶ cells (approximately 600 μg of protein) in 6-8 days. The mutant cells grew somewhat more slowly and there were fewer cells per dish at confluency (6 to 8 × 10⁵ cells) and less protein (200-300 μg). Protein per cell was comparable for the two lines throughout the growth curve (normal, 0.39 ng per cell; mutant, 0.37). By inspection the mutant cells in culture showed a larger surface area at all stages of the growth cycle.

Human ¹²⁵I-LDL (density 1.019-1.063 g/ml) was prepared as previously described (8). Less than 1% of total ¹²⁵I in the final preparations was trichloroacetic acid-soluble; less than 4% was in lipid (9). Specific radioactivities ranged from 118 to 1240 cpm/ng of protein. No ¹²⁵I-LDL was used more than 4 weeks after preparation.
The integrity of the \(^{125}\text{I}-\text{LDL}\) was tested by diluting it up to 58-fold with unlabeled LDL and measuring total cell-associated radioactivity after 3 hr at 37° at a constant total LDL concentration (20 \(\mu\)g/ml). The amount of radioactivity retained by the cells decreased in strict proportion to the extent of dilution (i.e., calculated cell-associated LDL in ng of LDL per mg, at each final specific activity, was essentially the same).

LDL was prepared by adjusting the density of whole FCS to 1.24 and centrifuging 72 hr at 180,000 \(\times\) g. The floating fraction was separated by tube-slicing and the infranatant fraction was dialyzed for 72 hr against 0.15 M NaCl and finally against Dulbecco’s phosphate-buffered saline (PBS) (10).

Cells used for binding and uptake studies were washed three times with PBS and preincubated 15–16 hr in DME containing enough LDS to provide a total protein concentration of 2.5 mg/ml (approximately 7% LDS, vol/vol). On the morning of study this medium was replaced with fresh medium of the same composition. Cells to be studied at 0° were placed on a packing of crushed ice for 15 min before the addition of \(^{125}\text{I}-\text{LDL}\). During incubation the dishes on ice were held in a 4° cold room.

At the end of the experiment the medium was drawn off and an aliquot was directly assayed for total \(^{125}\text{I}\). Degradation was determined on a 1-ml aliquot of medium. Carrier serum (0.1 ml) and trichloroacetic acid (final concentration, 10%) were added and the mixture was heated at 100° for 5 min. After centrifugation at 3000 \(\times\) g for 10 min, an aliquot of the supernatant fraction was treated to remove free \(^{125}\text{I}\) (5). All degradation data refer to noniodide, acid-soluble radioactivity. To calculate net degradation, the values obtained in media incubated with cells at 0° or in media incubated without cells at 37° were subtracted from each experimental result.

After removal of the medium, cells were washed six times with PBS at room temperature (37° incubations) or at 0° (0° incubations). Two milliliters of 0.05% trypsin in Versene buffer was added to each plate and incubated at 37° for 4–6 min. The cells were collected and the plates were also scraped with addition of two 1-ml aliquots of DME containing 10% FCS to arrest trypsin degradation of the LDL. The cells were separated by centrifugation at 3000 \(\times\) g for 10 min at 4° and an aliquot of the supernatant fluid was assayed for \(^{125}\text{I}\) radioactivity (trypsin-releasable radioactivity).

The LDL radioactivity released by trypsinization is provisionally identified as LDL bound to the surface of the cell (bound LDL) and that not released as LDL taken into the cell interior (internalized LDL).

The routine method for washing the cells was directly compared with that of Goldstein and Brown (11). Cells were incubated 3 hr at 0° with \(^{125}\text{I}-\text{LDL}\) at 10, 20, and 40 \(\mu\)g/ml. Using our standard procedure, LDL binding was 44, 65, and 120 ng/mg for control cells and 48, 70, and 128 for mutant cells; using the Goldstein and Brown procedure (except that the dishes were resting on ice throughout), LDL binding was 39, 70, and 109 ng/mg for the normal cells and 35, 72, and 121 for the mutant cells, respectively. Shaking during the exposure of cells to \(^{125}\text{I}-\text{LDL}\) did not alter the results. Binding at 0° in the presence of human serum instead of FCS reduced binding by 10–15% but the effect was the same for normal and mutant cells.

The cells were washed by suspending in 4 ml of PBS and recentrifuging at 3000 \(\times\) g for 20 min. The pellet was assayed for \(^{125}\text{I}\) radioactivity and then dissolved by incubation for 16 hr at 37° in 0.2 ml of 1 M KOH. The cell digest was diluted with water to a final volume of 1 ml and aliquots were removed for protein determination (12) and for determination of \(^{125}\text{I}\) radioactivity in the lipid fraction.

Sterol synthesis was measured in cells preincubated in DME containing 7% LDS for 18 hr and then incubated for 6 hr in 2 ml of fresh LDS medium containing various concentrations of LDL. Control cultures were maintained for the same period in 7% FCS. After the 6 hr incubation, 2 \(\mu\)Ci of \([\text{1-14C]}\text{acetate}\) (specific activity = 58 mCi/mmol) was added and incubations were continued for 1 hr. Incorporation into sterols remained linear for 3 hr under these conditions. Increasing total acetate concentration did not increase the calculated rate of incorporation (\(\mu\)mol of acetate/mg of protein per hr). The medium was removed and the cells were washed three to five times with cold PBS and harvested. The cell pellet was dissolved as described above, an aliquot was taken for protein assay, the remainder was saponified, and nonsaponifiable lipids were extracted with hexane. The hexane extract was washed with 0.1 M sodium acetate and aliquots were added to 10 ml of toluene scintillation fluid for determination of \(^{14}\text{C}\).

**RESULTS**

Sterol synthesis from \([\text{1-14C]}\text{acetate}\) in normal cells incubated for 24 hr in LDS was 6- to 10-fold greater than in cells maintained in whole 10% FCS. Incubation of these cells with LDL (20–120 \(\mu\)g/ml) for 6 hr suppressed acetate incorporation to levels 20–42% that seen in the cells incubated in LDS alone. In contrast, sterol synthesis in the mutant cells was not inhibited even at the highest concentrations of LDL tested (120 \(\mu\)g of LDL cholesterol per ml).

As shown in Fig 1, during incubation of normal fibroblasts at 37° with \(^{125}\text{I}-\text{LDL}\) binding was rapid initially and showed little further increase beyond 30 min. Internalization equaled or exceeded binding even in short incubations and by 3 hr was more than twice as great. Degradation showed a definite lag period (almost no measurable degradation at 30 min), after which it was almost linear from 1 to 3 hr. Essentially similar results were obtained at 10 \(\mu\)g of \(^{125}\text{I}-\text{LDL}\) protein per ml.

The results of a typical experiment comparing the two cell lines with respect to binding and internalization (3-hr
incubation) as a function of LDL concentration are shown in Fig. 2. At 0° (panel A) very little LDL was internalized by either cell line. Binding increased as a function of LDL concentration but departed from linearity in both cell lines. At all concentrations the normal cells bound somewhat more LDL than the mutant cells but the difference was less than 2-fold. The monolayers in this experiment were confluent.

At 37° (panel B) internalization was greatly increased in the normal cells, as expected. In striking contrast, the mutant cells showed almost no increase in internalization at 37°. This difference in internalization was reflected in the partition of cell-associated LDL between that released by trypsin and that not released. At 0° almost all was released by trypsin from both cell lines: normal cells, 87.3 ± 6.1% (SD; n = 18); mutant cells, 88.1 ± 2.7% (n = 14). At 37° only 29.0 ± 8.2% was released by trypsin from normal cells (n = 17) while 76.5 ± 7.5% was released from the mutant cells (n = 14), underscoring their limited capacity to internalize.

Table 1 summarizes data from six experiments comparing the two cell lines and at LDL concentrations ranging from 1 to 20 μg/ml. The last column shows that the amount of LDL internalized at 37° by the normal cells ranged from 13 to 115 times that internalized by the mutant cells under the same conditions. The difference in binding at 37° was much less striking. Finally, the difference in binding at 0° was small at best. Indeed, in some experiments there was no significant differences in binding at 0°; in others the normal cells bound about twice the LDL bound by the mutant cells and in only a few incubations did the ratio exceed 2:1.

If trypsin-releasable LDL corresponds to surface-bound LDL, and if the binding sites are not importantly altered by temperature, the value should be similar at 0° and at 37°. This is approximately the case for the normal cells, the overall ratio for binding at 37° to that at 0° being 1.12 ± 0.14 (SEM) in 19 experiments covering a range of LDL concentrations from 5 to 50 μg/ml. However, the corresponding ratio for the mutant cells was consistently less than unity, averaging 0.52 ± 0.06 (n = 15). The greater difference between the cell lines with respect to LDL binding at 37° versus that at 0° (Table 1) reflects primarily this differential effect of temperature on binding to the mutant cells.

Binding at 0° was studied over a wider range of LDL concentrations (Fig. 3). At LDL concentrations above 100 μg/ml, 0° binding by the mutant cells was comparable to or greater than that by the control cells (panel A). At low LDL concentrations (panel B) binding to the normal cells was greater than to the mutant cells but the curves appear to cross at about 60 μg of LDL per ml. The experiment shown in Fig. 3 was done using cells in logarithmic growth phase and at low cell density (100–170 μg of cell protein). It was repeated using nongrowing cells in confluent culture. The shapes of the curves were generally similar, a crossover occurring at about 60 μg of LDL per ml, but the absolute LDL bound per mg of cell protein was considerably less with the confluent cultures—½ to ¼ as much. While the plates for both cell lines were totally confluent, the average cell protein per plate was much higher for the normal cells (637 μg) than for the mutant cells (259 μg).

At 0° there was no measurable degradation of ¹²⁵I-LDL. Degradation of LDL during 3 hr incubations at 37° was much slower in the mutant cells, as shown in Table 2, ranging from 20 to 47% of that in the normal cells. In two 6-hr

![Figure 2](image-url)

**Fig. 2.** Comparison of binding (trypsin-releasable ¹²⁵I; open symbols) and internalization (residual cell-associated ¹²⁵I; closed symbols) by normal fibroblasts (circles) and mutant fibroblasts (triangles) incubated 3 hr either at 0° (panel A) or at 37° (panel B). Note: The ordinate scale in panel B covers five times the range of that in panel A. The monolayers were confluent: cell protein per dish for the normal cells, 729 ± 20 μg; for the mutant cells, 211 ± 8 μg (mean ± SEM).

Table 1. Summary of data comparing normal and mutant cell lines with regard to LDL bound and with regard to LDL internalized*

<table>
<thead>
<tr>
<th>Exp. no.</th>
<th>Bound at 0°</th>
<th>Bound at 37°</th>
<th>Internalized at 37°</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.02 ± 0.24 (3)</td>
<td>1.60 ± 0.45 (5)</td>
<td>14.5 ± 4.1 (6)</td>
</tr>
<tr>
<td>2</td>
<td>1.40 ± 0.30 (4)</td>
<td>3.85 ± 0.95 (4)</td>
<td>44.8 ± 9.6 (4)</td>
</tr>
<tr>
<td>3</td>
<td>0.91 ± 0.16 (4)</td>
<td>2.14 ± 0.16 (4)</td>
<td>13.2 ± 0.61 (4)</td>
</tr>
<tr>
<td>4</td>
<td>2.17 ± 0.45 (5)</td>
<td>4.31 ± 0.63 (6)</td>
<td>44.3 ± 1.7 (6)</td>
</tr>
<tr>
<td>5</td>
<td>3.86 (2)</td>
<td>8.31 ± 2.1 (3)</td>
<td>60.7 ± 7.0 (3)</td>
</tr>
<tr>
<td>6</td>
<td>—</td>
<td>4.69 ± 1.4</td>
<td>115.5 ± 9.3 (4)</td>
</tr>
</tbody>
</table>

Ratios are given as mean ± SEM. Number of observations is given in parentheses.

* Pooled data from six experiments covering a range of LDL concentrations from 1 to 20 μg/ml. The data in line 2 are from the experiment presented in detail in Fig. 2. The monolayers were preconfluent except in Exp. 2 where the cells were confluent (normal cells 729 ± 20 μg (mean ± SEM) and mutant cells 211 ± 8 μg of protein per dish). Three different ¹²⁵I-LDL preparations were used: preparation A in Exps. 1 and 2; preparation B in Exp. 3; preparation C in Exps. 4-6. Incubation times were 1 hr in Exps. 1 and 4; 6 hr in Exp. 6; 3 hr in the others.
experiments the difference was still greater, the mutant cells degrading only 5–12% as much LDL.

If we assume that degradation presupposes internalization, the sum of the LDL internalized and that degraded during the incubation will approximate the integrated total of LDL internalized over the 3- or 6-hr interval. Relating the LDL degraded to this total should provide an index of the fraction of LDL internalized that undergoes degradation (Table 2, last column). On the average about 50% of the total LDL internalized by the normal cells was degraded by the end of the incubation but almost 90% was degraded in the case of the mutant cells. The results suggest that the mutant cells have no difficulty in degrading LDL once it has been internalized.

**DISCUSSION**

The mutant cells studied here show a marked defect in feedback inhibition of cholesterol synthesis by LDL, a marked defect in degradation of the apoprotein moiety of LDL, and a marked decrease in the total cell-associated 125I-LDL after incubation at 37°C. These findings agree with those of Brown, Goldstein, and coworkers in fibroblasts of other FH patients (4). When, however, cell-associated radioactivity was analyzed as two fractions—that released by trypsinization (assumed to be surface-bound) and that not released by trypsinization (assumed to be internalized)—it became evident that the outstanding difference between the normal and mutant lines lay in the inability of the latter to internalize LDL. The surface-bound LDL, measured at 0°C, was in many experiments not significantly different between the two cell lines. At LDL concentrations up to 50 μg/ml, the highest binding ratio observed at 0°C (normal/mutant) was 4.2 and the mean for all experiments was 1.7. Thus, even at low concentrations of LDL, where the differences should be maximal if the mutant line lacks high-affinity receptors, the differences were small. At very high LDL concentrations, binding by the mutant cells actually exceeded that by the control cells. Even in those experiments where a difference in bound LDL was observed (either at 0°C or at 37°C) this difference was small relative to the enormous difference in LDL internalized at 37°C (Table 1). The latter difference was as much as 50- to 100-fold. It appears that the major de-
fect in this particular cell line is related more to the process of internalization than it is to the surface binding of LDL.

Goldstein and Brown (11) measured "binding" at 37°C (including both surface-bound and internalized LDL) and observed a two-component curve for "binding" versus LDL concentration in normal fibroblasts but only a single-component curve (straight line) in HFH fibroblasts. The slope of the latter paralleled that of the second component of the former, compatible with the hypothesis presented, namely, that HFH cells lack high-affinity binding sites but retain a normal complement of low-affinity sites like those on normal cells. Further, there was excellent correlation between "binding" and several of the consequences of LDL-cell interaction: inhibition of cholesterol synthesis, degradation of LDL, and stimulation of cholesterol ester formation (4, 11). In the present study the "binding" curve for normal cells was readily resolved into just two components, the curve for the HFH cells was not strictly linear, and, as already mentioned, the absolute binding was not consistently lower in the HFH cells. Brown and Goldstein have reported some comparative data on binding at 4°C (13). If the data are not corrected for "nonspecific" binding, they show binding to normal cells not quite double that to the HFH cell lines. Thus, any marked abnormality in the number of binding sites at 37°C compared to the number at 4°C. Finally, the process of LDL internalization may not be fully analogous to endocytosis of other proteins. The lipid moiety of the lipoproteins could play a unique and significant role in their interaction with the cell membrane. Thus, there could be a defect evident only in uptake of lipoproteins or only in uptake of certain classes of lipoproteins.

The P.A. line studied here, like the HFH cells studied by Goldstein and Brown (3), showed no "feedback" control of sterol synthesis even at high levels of LDL. Yet at high concentrations of LDL in the medium, the mutant cells do degrade significant amounts of LDL. Although this is always less than the amount degraded by the normal cells at the same high concentration, it can be comparable to that degraded by normal cells at low LDL concentrations, yet there is not accompanying inhibition of sterol synthesis. Either one must postulate that interaction with specific receptors on the membrane is a necessary component of the control mechanism as suggested by Brown, Goldstein et al. (4, 15) or, alternatively, that the fate of LDL entering the mutant cells is different in some fashion, as suggested by the present result showing an apparently greater fractional degradation (Table 2).

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