Genetic Heterogeneity in Familial Hypercholesterolemia: Evidence for Two Different Mutations Affecting Functions of Low-Density Lipoprotein Receptor

[cholesterol metabolism/atherosclerosis/hyperlipidemia/hydroxymethylglutaryl-CoA reductase (NADPH)]

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ABSTRACT Studies in cultured fibroblasts from patients with the clinical syndrome of homozygous familial hypercholesterolemia have disclosed two different mutations affecting the functions of the low density lipoprotein receptor. One of these mutations, described previously, results in a functionless receptor that does not bind low density lipoproteins. In the cells of six patients who appear to be homozygous for this mutant allele, i.e., receptor-negative homozygotes, low density lipoproteins neither suppress hydroxymethylglutaryl-CoA reductase (NADPH) [mevalonate:NADP+ oxidoreductase (CoA-acylating) EC 1.1.1.34] activity nor stimulate cellular cholesterol esterification, even when examined in the presence of concentrations of lipoprotein 500 times higher than those required to produce maximal biologic effects in normal cells. The second type of mutation, described herein, results in a receptor that has a reduced but not absent function. Fibroblasts from three subjects who possess this mutation, i.e., receptor-defective homozygotes, show partial suppression of the same enzyme activity and a detectable increase in cholesterol esterification capacity in the presence of high levels of low density lipoproteins. It was calculated that their degree of function could be achieved if they possessed only about 10% of the normal binding of low density lipoprotein. This level of binding was too low to be reliably detected by the 125I-labeled low density lipoprotein binding assay. The finding of a second class of mutant cells in which a defect in low density lipoprotein binding is associated with simultaneous defects in both suppression of hydroxymethylglutaryl-CoA reductase activity and stimulation of cholesterol ester formation provides further evidence for the coordinate control of these two processes by the low density lipoprotein receptor.

Recent studies in cultured human fibroblasts have led to the identification of a cell surface receptor that binds serum low density lipoproteins (LDL) and thereby regulates cellular cholesterol metabolism (1-3). Binding of LDL to this receptor produces four known secondary metabolic events: i, the cholesterol of LDL is transferred into the cell (4); ii, the cellular mechanism for esterification of cholesterol is stimulated (5); iii, the activity of hydroxymethylglutaryl-CoA reductase (NADPH) [mevalonate:NADP+ oxidoreductase (CoA-acylating) EC 1.1.1.34], the rate-controlling enzyme in cholesterol biosynthesis, is suppressed (6, 7); and iv, the protein component of LDL is degraded to its constituent amino acids (2, 3). These actions of the LDL receptor can be studied either by direct measurement of 125I-labeled LDL binding to fibroblasts (1-3) or by assays of the effect of LDL on any one of the four secondary metabolic events (2-7).

We have previously reported that fibroblasts obtained from five patients who exhibit the typical clinical syndrome of homozygous familial hypercholesterolemia (FH) neither bind LDL at the receptor site nor manifest any of the functions of the LDL receptor, even when examined in the presence of LDL concentrations 500 times higher than those required to produce maximal biologic effects in normal fibroblasts (1-7). Thus, the receptor defect in these homozygotes' cells appeared to be due not to a reduced binding affinity for LDL but rather to an absence of functional receptor molecules (1-3). Cells from 10 typical FH heterozygotes, including six of the parents of the homozygous patients, were shown to contain about half the normal number of LDL receptor molecules on their surface (3, 8). However, since the binding affinity for LDL was normal, the only detectable functional LDL receptor in the heterozygotes' cells represents the product of the single normal allele (3, 8).

In this and in previous publications, we use the term FH homozygote in a phenotypic sense to denote a distinct clinical syndrome characterized by the following three features: i, marked hypercholesterolemia, i.e., serum cholesterol levels greater than 600 mg/dl, ii, the childhood onset of cutaneous xanthomas that are especially prominent in the interdigital webs, and iii, premature coronary vascular disease (9-11). As clearly shown by the genetic studies of Khachadurian, this phenotype results when an individual inherits two mutant FH alleles—one from his heterozygous mother and the other from his heterozygous father (9, 12). However, these two mutant alleles need not be identical. In genetic terms, the FH homozygous phenotype might arise from any one of the following genetic combinations: i, true homozygosity involving two identical mutant alleles at the same locus (as in SS or CC hemoglobinopathy); ii, a genetic compound state involving two nonidentical mutant alleles at the same locus (as in SC hemoglobinopathy); or iii, double heterozygosity involving two nonidentical genes each arising from a different locus (as in the case of two nonidentical subunits that comprise a single protein molecule).

That such genetic heterogeneity may exist in the FH syndrome is indicated by our present findings in three additional FH homozygotes. These patients show clinical features similar to those of the originally described homozygotes, yet their fibroblasts differ biochemically in that their LDL receptor is capable of a low level of function. At high concentrations of LDL, these three mutant cell lines show low but detectable cholesterol ester formation and a partial suppression of hydroxymethylglutaryl CoA reductase activity. By means of a standardized assay that quantifies the effect of LDL on cellular cholesterol ester formation, this new class of
mutant cells, designated receptor-defective FH homozygotes, can be distinguished from the originally described receptor-negative FH homozygotes as well as from FH heterozygotes. These data suggest that at least two different mutations affecting the functions of the LDL receptor may each be associated with the FH homozygous phenotype.

**METHODS**

**Patients.** Table 1 summarizes the clinical data on patients whose fibroblasts were used in the present studies. The control group includes six healthy subjects and two hyperlipidemic patients with familial combined hyperlipidemia (CHL), a recently described inherited disorder that causes hypercholesterolemia and can be differentiated from FH on clinical and genetic grounds (13). The two subjects with CHL are siblings from a large family in which nine members have hyperlipidemia manifested as one of several different lipoprotein types, i.e., types IIA, IIB, and IV. E.F. has a serum cholesterol of 320 mg/dl, a serum triglyceride of 140 mg/dl, and a type IIA lipoprotein pattern. Her brother, C.B., has a serum cholesterol of 285 mg/dl, a serum triglyceride of 387 mg/dl, and a type IIB lipoprotein pattern. Two of the FH heterozygotes studied (P.C. and M.P.) are parents of a receptor-negative FH homozygote (J.P.). The other FH heterozygote (M.S.) is the mother of a receptor-negative FH homozygote (R.S.). All three of these heterozygotes manifest elevated serum cholesterol (350-450 mg/dl), normal serum triglyceride, and tendon xanthomas, but none of the clinical features unique to FH homozygotes. Of the six unrelated receptor-negative FH homozygotes, all but O.C. have been described previously (3-5). Each of the three unrelated receptor-defective FH homozygotes meets the clinical and genetic criteria for diagnosis of a FH homozygote, as outlined above. A more detailed report of the clinical and genetic findings in these receptor-defective FH homozygotes and in their heterozygous parents is being prepared in collaboration with their physicians, Dr. Robert Lees of Boston and Dr. John P. Kane of San Francisco.

**Cells.** Skin biopsies were obtained with informed consent, and all fibroblast cultures were established in our laboratory. Biopsy specimens were obtained from the deltoid region in all patients listed in Table 1 except for D.S. and D.W. (foreskin), A.P. (inguinal skin), and J.P. (abdominal skin). All cells were grown in monolayer and used between the 5th and 20th passage. Cell lines were maintained in a humidified incubator (5% CO₂) at 37°C in 75 cm² stock flasks (Falcon) containing 10 ml of growth medium consisting of Eagle's minimum essential medium (Gibco, Cat. no. F-11) supplemented with penicillin (100 units/ml); streptomycin (100 μg/ml); 20 mM Tricine-Cl (pH 7.4); 24 mM NaHCO₃; 1% (v/v) nonessential amino acids; and 10% (v/v) fetal calf serum (Flow Laboratories). All experiments were done in a similar format: confluent monolayers of cells from stock flasks were dissociated with 0.05% trypsin-0.05% EDTA solution and were seeded (day 0) at a concentration of 1 X 10⁶ cells per dish into 60 × 15 mm dishes (Falcon) containing 3 ml of growth medium with 10% fetal calf serum (FC serum). On day 3 the medium was replaced with 3 ml of fresh growth medium containing 10% FC serum. On either day 4 or day 6, when the cells were not yet confluent, each monolayer was washed with 2 ml of Dulbecco's phosphate-buffered saline, and 2 ml of fresh medium containing 5% (v/v) lipoprotein-deficient human serum (LPD serum) was added (final protein concentration, 2.5 mg/ml). All experiments were initiated either on day 5 or day 7 after the cells had been incubated for 24 hr in the presence of LPD serum.

**Lipoproteins.** Human LDL (density 1.019-1.063 g/ml) and LPD serum (density > 1.215 g/ml) were obtained from the plasma of healthy subjects and prepared by differential ultracentrifugation (7). The concentration of the LDL preparations is expressed in terms of its protein content, which averaged 60% of the cholesterol concentration.

**Assays.** Hydroxymethylglutaryl-CoA reductase activity was measured in extracts of detergent-solubilized cells (7).

The rate of [1-¹⁴C]oleate incorporation into cholesteryl [¹⁴C]esters by intact cell monolayers was measured as follows (5): 1.09 mg of [¹⁴C]oleic acid in hexane (New England Nuclear Corp., 53 mCi/mmol) was evaporated to dryness and resuspended in 1.25 ml of a solution containing 7 mM non-radioactive sodium oleate, 12% bovine albumin (Armour, Fraction V), and 0.15 M sodium chloride prepared as described by Van Harken et al. (15). The final specific activity of the [1-¹⁴C]oleate-albumin solution was 18,700 cpm/nmol. Aliquots (20 μl) of this solution were added to 2 ml of culture medium to give a final oleate concentration of 0.1 mM. Under these conditions, the concentration of [1-¹⁴C]oleate was saturating for cholesteryl [¹⁴C]ester formation (5), and its incorporation into cholesteryl [¹⁴C]esters was linear with time for at least 4 hr (5). One or 2 hr after the addition of [1-¹⁴C]oleate to the culture medium, cell monolayers were harvested and their content of cholesteryl [¹⁴C]esters was determined by thin-layer chromatography (5).

Binding of ¹²⁵I-labeled LDL to intact cell monolayers was determined as described (2) and the ¹²⁵I-labeled LDL (specific activity, 69 cpm/ng of protein) was prepared by a modification (15) of the method of MacFarlane (16).

All values are expressed in terms of the cellular protein content as measured by the method of Lowry et al. (17).

**Table 1. Summary of clinical data**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Clinical diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>D.S.</td>
<td>M</td>
<td>Newborn</td>
<td>Normal control</td>
</tr>
<tr>
<td>D.W.</td>
<td>M</td>
<td>2</td>
<td>Normal control</td>
</tr>
<tr>
<td>A.P.</td>
<td>M</td>
<td>10</td>
<td>Normal control</td>
</tr>
<tr>
<td>D.B.</td>
<td>M</td>
<td>25</td>
<td>Normal control</td>
</tr>
<tr>
<td>F.J.</td>
<td>F</td>
<td>30</td>
<td>Normal control</td>
</tr>
<tr>
<td>W.E.</td>
<td>M</td>
<td>35</td>
<td>Normal control</td>
</tr>
<tr>
<td>E.F.*</td>
<td>F</td>
<td>53</td>
<td>CHL, Type IIa lipoprotein pattern</td>
</tr>
<tr>
<td>C.B.*</td>
<td>M</td>
<td>32</td>
<td>CHL, Type IIb lipoprotein pattern</td>
</tr>
<tr>
<td>P.C.†</td>
<td>F</td>
<td>38</td>
<td>FH heterozygote</td>
</tr>
<tr>
<td>M.P.†</td>
<td>M</td>
<td>40</td>
<td>FH heterozygote</td>
</tr>
<tr>
<td>M.S.‡</td>
<td>F</td>
<td>58</td>
<td>FH heterozygote</td>
</tr>
<tr>
<td>M.C.</td>
<td>F</td>
<td>6</td>
<td>FH homozygote, receptor-negative</td>
</tr>
<tr>
<td>D.R.</td>
<td>F</td>
<td>6</td>
<td>FH homozygote, receptor-negative</td>
</tr>
<tr>
<td>L.L.</td>
<td>M</td>
<td>10</td>
<td>FH homozygote, receptor-negative</td>
</tr>
<tr>
<td>J.P.</td>
<td>F</td>
<td>12</td>
<td>FH homozygote, receptor-negative</td>
</tr>
<tr>
<td>O.C.</td>
<td>F</td>
<td>18</td>
<td>FH homozygote, receptor-negative</td>
</tr>
<tr>
<td>A.C.</td>
<td>F</td>
<td>23</td>
<td>FH homozygote, receptor-negative</td>
</tr>
<tr>
<td>K.M.</td>
<td>M</td>
<td>18</td>
<td>FH homozygote, receptor-defective</td>
</tr>
<tr>
<td>A.B.</td>
<td>F</td>
<td>25</td>
<td>FH homozygote, receptor-defective</td>
</tr>
<tr>
<td>R.S.</td>
<td>F</td>
<td>35</td>
<td>FH homozygote, receptor-defective</td>
</tr>
</tbody>
</table>

* Siblings; † Parents of J.P.; ‡ Mother of R.S.
hydroxymethylglutaryl-CoA reductase activity (7), and rates of proteolytic degradation of cholesteryl [14C]esters (2), ii, and receptor-negative FH homozygotes (m), M.C. and J.P.

RESULTS

Five assays are now available to measure the action of the LDL receptor in human fibroblasts: i, 125I-labeled LDL binding (2), ii, proteolytic degradation of 125I-labeled LDL (2), iii, LDL-mediated accumulation of free and esterified cholesterol within cells (4), iv, LDL-mediated suppression of hydroxymethylglutaryl-CoA reductase activity (7), and v, LDL-stimulated esterification of cholesterol (5). Of these, the most sensitive is the measurement of the ability of LDL to stimulate the incorporation of [14C]oleate into cellular cholesteryl [14C]esters. Fig. 1 shows that when human fibroblasts were incubated in the absence of LDL, they failed to incorporate [14C]oleate into cholesteryl [14C]esters. However, after cells had been incubated with LDL for 12 hr so as to achieve equilibrium for binding and maximum capacity for esterification (5), the rate of cholesteryl ester formation was proportional to the amount of LDL bound. Cells from normal subjects and from the two subjects with CHL showed similar rates of cholesteryl ester formation, indicating that the levels of LDL binding were similar. Cells from two FH heterozygotes, who have previously been shown to possess about 40% of the normal number of LDL receptors (3, 8), showed a maximal rate of cholesteryl esterification that was about 40% of normal. However, although quantitatively different, the LDL response in the FH heterozygotes was qualitatively similar to normal cells in that the LDL concentration necessary to produce one-half the maximal response was the same in both groups of cells. Cells from two receptor-negative FH homozygotes showed no detectable incorporation of [14C]oleate into cholesteryl [14C]esters at any concentration of LDL, a finding consistent with their demonstrated lack of functional LDL receptor sites (1-3).

When cholesterol esterification assays were done on cells from a larger series of FH homozygotes, we found that three previously unreported patients with the clinical phenotype of homozygous FH showed a low but detectable incorporation of [14C]oleate into cholesteryl [14C]esters. The kinetic data for two of these three patients are shown in Fig. 2A. Whereas at 6 hr the normal cells reached a maximal response at an LDL concentration between 100 and 200 µg of protein per ml, the receptor-defective FH homozygotes showed a low rate of esterification that continued to increase as the concentration of LDL was raised up to at least 400 µg of protein per ml (Fig. 2A, inset). In contrast, the five previously described receptor-negative homozygotes (3, 5) plus one additional homozygote (O.C.) all showed no detectable incorporation of [14C]oleate into cholesteryl [14C]esters at any LDL concentration.

The receptor-defective FH homozygotes also differed from the receptor-negative homozygotes in that their cells showed clear-cut suppression of hydroxymethylglutaryl-CoA reductase activity by LDL. Fig. 2B shows the lack of suppression of this enzyme in a typical receptor-negative homozygote (J.P.). A similar degree of resistance to LDL has been reported in the four other receptor-negative homozygotes (3). In contrast, normal cells showed maximal suppression of the enzyme (>95%) at an LDL concentration of 40 µg of protein per ml. Under the same conditions, the receptor-defective homozygotes continued to show an increasing suppression of enzyme activity at levels of LDL up to 400 µg of protein per ml, reaching a maximal suppression of about 80%.

To confirm that the diminished response to LDL-mediated stimulation of cholesteryl esterification and suppression of reductase activity in the receptor-defective FH homozygotes'
cells reflected a defect in their LDL receptors, we compared $^{125}$I-labeled LDL binding in cells from one of these patients, one receptor-negative FH homozygote, and one normal subject (Fig. 3). Consistent with previous findings, the normal cells (D.S.) took up $^{125}$I-labeled LDL by two processes (2). One reached saturation at an LDL concentration of about 100 $\mu$g of protein per ml; this represented high affinity binding to the LDL receptor. The second was nonsaturable and appeared to represent a nonspecific endocytosis of LDL (2). As reported (1-3) and as shown in Fig. 3, the high affinity component of LDL binding was absent in cells from the receptor-negative FH homozygotes (as in J.P.), and the only binding process observed in their cells was a nonsaturable component identical with the nonspecific component of the normal cells. From the data of Fig. 2, it can be calculated that in the receptor-defective cells the degree of stimulation of esterification and suppression of hydroxymethylglutaryl-CoA reductase activity that was observed would have occurred if the binding were only about 10% of normal, which is too low to be reliably detected by the present $^{125}$I-labeled LDL binding assay. Thus, although at each LDL concentration, binding in the receptor-defective homozygote’s cells (K.M.) was slightly and consistently higher than in the receptor-negative homozygote’s cells (J.P.), the presence of a normal nonspecific component in these receptor-defective cells prevented a clear-cut demonstration of the defective receptor sites that are presumed to be present. This lack of detectable specific binding sites in these mutant cells does, however, serve to distinguish them from FH heterozygotes in whom the half normal number of receptors can easily be demonstrated (3).

The sensitivity of the measurement of LDL-stimulated cholesterol esterification permitted the development of a standardized assay that could distinguish between fibroblasts from patients in the various genetic groups. Several important variables had to be standardized so that the widest range of discrimination could be obtained. First, the cells were used while in the mid-range of logarithmic growth when normal cells displayed their highest rates of esterification. Second, to widen the separation between the genetic groups we pulse-labeled the cells with $[^{14}$C]oleate after only 5 hr incubation with LDL, at which time steady-state conditions are not achieved and the rate of esterification is increasing more rapidly in normal controls than in FH heterozygotes and more rapidly in FH heterozygotes than in receptor-defective FH homozygotes. To rule out any defect in the cellular cholesterol esterification process itself, we took advantage of the fact that cholesterol esterification can be stimulated in fibroblasts not only by LDL but also by addition to the medium of either nonlipoprotein cholesterol (5) or one of its more polar derivatives, such as 25-hydroxycholesterol or 7-ketocholesterol, either of which can stimulate esterification in the absence of the LDL receptor. These polar derivatives of cholesterol, which are more potent than cholesterol in suppressing hydroxymethylglutaryl-CoA reductase activity (18), are also more potent in their ability to stimulate incorporation of $[^{14}$C]oleate into cholesteryl $[^{14}$C]esters (manuscript in preparation). Fig. 4 shows a comparison of the ability of LDL and 25-hydroxycholesterol to stimulate cholesterol esterification under these standardized assay conditions in fibroblasts from 14 subjects with various levels of LDL receptor function. In the presence of LDL, each of the four genetic groups exhibited mean rates of esterification that were at least 4-fold different from any other group and there was no overlap between individuals comprising a given group. In contrast, there was no significant difference among the four genetic groups in their response to 25-hydroxycholesterol which does not require the LDL receptor for its action (18). These data indicate that cells from subjects with FH have no defect in the cholesterol esterification process itself, but rather their response is limited by their ability to bind LDL. Thus, this
standardized quantitative assay of LDL-dependent cellular cholesterol esterification should prove useful in assigning patients to genotypeic categories.

**DISCUSSION**

The data presented in this paper indicate that within the group of patients classified clinically as homozygous FH there exist at least two biochemically distinct subgroups. One of these, designated *receptor-negative* FH, comprises individuals whose fibroblasts fail to show specific LDL binding at any concentration of LDL and thus manifest virtually complete absence of the four known secondary biochemical functions mediated by the LDL receptor. These mutant cells appear to lack functional LDL receptors. The cells of the other group of FH homozygotes, designated *receptor-defective* FH, possess a low but detectable level of LDL receptor function. At high levels of LDL, this class of mutant cells, unlike the *receptor-negative* cells, is capable of esterifying cholesterol and suppressing hydroxymethylglutaryl-CoA reductase activity.

The observation that in the *receptor-defective* cells, stimulation of cholesterol esterification and suppression of reductase activity continue to increase at LDL concentrations up to 400 \( \mu g \) of protein per ml, whereas in normal cells a plateau is reached at levels less than 200 \( \mu g \)/ml, is consistent with the possibility that the LDL receptor in *receptor-defective* cells has reduced binding affinity. However, the exact relation between the amount of LDL bound, the stimulation of esterification, and the suppression of the enzyme is a complicated function that is dependent on the time of incubation of cells with LDL. In normal cells and at low levels of LDL, even though binding reaches a constant equilibrium value at 3 hr (1-3), reductase activity continues to fall and esterification continues to increase over the ensuing 3-24 hr, each process increasing at a different relative rate. Thus, the shape of the LDL saturation curve for these processes does not simply reflect the affinity of the receptor sites for LDL. The low level of binding in *receptor-defective* FH cells prevents the direct examination of binding kinetics needed to determine whether the alteration in these mutant cells is due to a reduction in the affinity of the receptors, a reduction in the number of receptors, or a combination of both. However, the fact that at high levels of LDL, suppression of reductase activity and stimulation of cholesterol esterification occur in these cells but not in *receptor-negative* FH cells, implies that *receptor-defective* cells must possess some finite number of functional LDL receptors. It is of interest that Breslow et al., in a study of hydroxymethylglutaryl-CoA reductase activity in fibroblasts from four FH homozygous, found one cell line that showed partial enzyme suppression while the other three were resistant (19). It is likely that their partially responsive FH homozygote represents an example of the *receptor-defective* FH subgroup.

The biochemical observations in the six *receptor-negative* FH homozygotes and in their heterozygous parents are compatible with the genetic hypothesis that these patients are homozygous for one type of mutant FH allele that either fails to specify the production of an LDL receptor or specifies the production of a functionless receptor molecule. In contrast, the *receptor-defective* FH homozygotes would appear to possess at least one different type of mutant FH allele, i.e., an allele that specifies the production of an LDL receptor capable of a low level of function. Further studies of the cells of the heterozygous parents of these *receptor-defective* FH subjects may help to clarify the precise genetic constitution of these homozygotes, i.e., whether they have inherited two doses of the *receptor-defective* FH allele; whether they have inherited one dose of the *receptor-defective* FH allele and one dose of the *receptor-negative* FH allele; or whether some more complex genetic situation exists.

At present, no obvious clinical differences are apparent either between the two genetic subgroups of FH homozygotes or between their heterozygous parents. However, a detailed comparison of atherosclerotic involvement and therapeutic responsiveness in these FH families has not yet been completed.

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