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Familial Hypercholesterolemia: Identification of a Defect in the Regulation of 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Activity Associated with Overproduction of Cholesterol

(cholesterol synthesis/hyperlipidemia/low-density lipoproteins/enzyme regulation/coronary heart disease)

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ABSTRACT **The homozygous form of the autosomal dominant disorder, familial hypercholesterolemia, is characterized by the presence in children of profound hypercholesterolemia, cutaneous planar xanthomas, and rapidly progressive coronary vascular disease that usually results in death before age 30 years. Cultured skin fibroblasts from three unrelated subjects with this disorder showed 40- to 60-fold higher activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (EC 1.1.1.34), the rate-controlling enzyme in cholesterol biosynthesis, when compared with fibroblasts of seven control subjects. Enhanced enzyme activity resulted from a complete absence of normal feedback suppression by low-density lipoproteins, which led to a marked overproduction of cholesterol by the mutant cells. The demonstration of apparently identical kinetic properties of the reductase activity of control and mutant cells, coupled with the evidence that this enzyme is normally regulated not by allosteric effectors but by alterations in enzyme synthesis and degradation, suggests that the primary genetic abnormality does not involve the structural gene for the enzyme itself, but a hitherto unidentified gene whose product is necessary for mediation of feedback control by lipoproteins. The fibroblasts of two obligate heterozygotes, the parents of one of the homozygotes, showed a pattern of enzyme regulation intermediate between that of controls and homozygotes.**

Familial hypercholesterolemia is transmitted in humans as an autosomal dominant trait (1–4). Although heterozygotes are born with an elevated level of plasma cholesterol, they usually remain clinically asymptomatic until the third–sixth decade of life, at which time they may manifest tendinous xanthomas and coronary heart disease of varying severity (1–5). Subjects who are homozygous for the familial hypercholesterolemia gene uniformly develop: (*a*) a profound elevation in plasma cholesterol often exceeding 800 mg/dl; (*b*) cutaneous planar xanthomas appearing in the first several years of life; (*c*) rapidly progressive coronary, cerebral, and peripheral vascular occlusive disease occurring in childhood and associated with widespread accumulation of cholesterol in atheromatous plaques; and (*d*) death from myocardial infarction, often before the age of 30 (1, 2, 5).

Despite the unique phenotype of the homozygous form of familial hypercholesterolemia and the relatively high population frequency of individuals with the heterozygous form of the disease (6), very little is known about the fundamental physiological or biochemical defect in this disorder. For example, there is no agreement as to whether cholesterol accumulates in familial hypercholesterolemia because of increased synthesis (7–9) or because of defective degradation either of cholesterol itself (10) or of the plasma low-density lipoprotein (LDL) to which it is bound (11).

Studies of Siperstein and other workers have demonstrated that the rate of cholesterol synthesis in mammalian liver is controlled by the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase; EC 1.1.1.34), the enzyme catalyzing the first step unique to the cholesterol biosynthetic pathway (reviewed in ref. 12). We have recently developed a method for studying regulation of the activity of this enzyme in cultured human-skin fibroblasts and have found that the enzyme activity in normal cells is under a sensitive form of feedback control mediated specifically by LDL and very low-density lipoproteins (13).

In the present studies, we used this model system to characterize regulation of HMG CoA reductase activity in fibroblasts from subjects with the homozygous and heterozygous forms of familial hypercholesterolemia. Our studies indicate that cells of these patients have a genetic defect in regulation of HMG CoA reductase activity by lipoproteins and that this abnormality results in a marked increase in their rate of cholesterol synthesis.

MATERIALS AND METHODS

Human Subjects. J.P. is a 12-year-old Caucasian girl with the classic phenotypic and genetic features of the homozygous form of familial hypercholesterolemia (1, 2, 5). She has diffuse cutaneous planar xanthomas present since age 2 years, tendinous xanthomas, bilateral arcus corneae, and generalized atherosclerosis manifest by angina pectoris, aortic stenosis, and a myocardial infarction at age 11 years. Her total plasma cholestrol has ranged between 700 and 1000 mg/dl, and her fasting plasma triglyceride has been repeatedly below 100 mg/dl. Both her father (M.P., age 40 years) and her mother (P.C., age 38 years) have the phenotypic features of the heterozygous form of familial hypercholesterolemia (1–5). Her father's total plasma cholesterol has averaged 350 mg/dl, and his fasting plasma triglyceride has been consistently below 140 mg/dl. Her mother, who had a documented myocardial infarction at age 31 years, has a total plasma cholesterol ranging between

Abbreviations: HMG CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase; LDL, low-density lipoproteins.

400 and 490 mg/dl and a fasting plasma triglyceride that has always been below 140 mg/dl except for one value of 190 mg/dl.

Fibroblasts derived from two other homozygotes were also studied. L.L., a 10-year-old boy, and A.C., a 23-year-old woman, each had a clinical phenotype identical to that of J.P., and both had pedigree evidence consistent with homozygosity for the familial hypercholesterolemia gene.

The control subjects consisted of six healthy individuals of various ages (D.S., newborn boy; D.C., 6-year-old boy; T.L., 9-year-old girl; G.W., 24-year-old woman; G.C., 25-year-old woman; L.G., 28-year-old woman) and one patient with a nonfamilial form of hyperlipidemia (E.S., 44-year-old woman) whose disorder was manifest clinically by a type-V lipoprotein pattern (1), total plasma cholesterol of 463 mg/dl, and fasting plasma triglyceride of 2173 mg/dl.

Cells. Skin biopsies were obtained with informed consent, and fibroblast cultures were established in our laboratory for all subjects except for T.L., a control subject whose cells were obtained from the American Type Culture Collection, Rockville, Md. Cell lines were maintained in a humidified CO_2 incubator at 37° in 75-cm² flasks (Falcon) containing 10 ml of Eagle's minimum essential medium (Gibco, Cat. no. F-11), supplemented with penicillin (100 units/ml), streptomycin sulfate (100 μg/ml), 50 mM Tricine (pH 7.4) (Sigma), 0.05 g/100 ml of $NaHCO_3$, 1% (v/v) nonessential amino acids (Gibco), and 10% (v/v) fetal-calf serum (Flow Laboratories). For all experiments, cells from the stock flasks were dissociated with trypsin–EDTA solution (13) and were seeded (day 1) at about 2.5×10^5 cells per dish into 60 × 15-mm dishes (Falcon) containing 3 ml of the above growth medium with 10% fetal-calf serum. On day 3 the medium was replaced with fresh growth medium containing 10% fetal-calf serum. On day 6 when the cells were confluent, the medium was removed and the cellular monolayer was washed with 1.5 ml of Puck's saline A (Gibco), after which 3 ml of fresh medium containing either 10% fetal-calf serum or 5% lipoprotein-deficient human plasma was added as indicated.

Extracts. Cells were harvested by scraping, collected by centrifugation, washed, and frozen at −196° as described (13). Cell extracts were prepared by dissolving the thawed pellet in 0.1 ml of buffer containing 50 mM potassium phosphate (pH 7.4)–5 mM dithiothreitol–5 mM EDTA–0.2 M KCL–0.25% Kyro EOB (13). After incubation for 10 min at 37°, the suspension was centrifuged for 1 min at 12,000 rpm in a Beckman Microfuge, and aliquots of the supernatant were assayed for HMG CoA reductase activity and protein content.

HMG CoA Reductase Activity was measured by a described method (13). Aliquots of the cell extract (20–100 μg of protein) were incubated for 120 min at 37° in a final volume of 0.2 ml containing 0.1 M potassium phosphate (pH 7.5), 20 mM glucose-6-phosphate, 2.5 mM TPN, 0.7 unit of glucose-6-phosphate dehydrogenase, 5 mM dithiothreitol, and 30 μM DL-[3-^{14}C]HMG CoA (5.26 Ci/mol) (14). The [^{14}C]-mevalonate formed was isolated by thin-layer chromatography and counted, with an internal standard of [^{3}H]mevalonate to correct for incomplete recovery (13, 14). The amount of extract was adjusted so that mevalonate formation was always linear with time and protein concentration (13). The mean variation in HMG CoA reductase activity between duplicate dishes was ±5.1%.

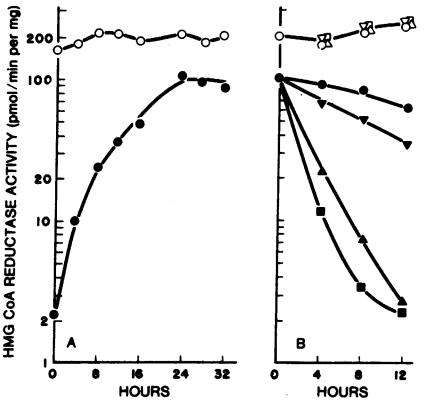

FIG. 1. HMG CoA reductase activity in fibroblasts of a control subject (*closed symbols*) and a patient with homozygous familial hypercholesterolemia, J.P. (*open symbols*). (*A*) Cells were grown in dishes containing 10% fetal-calf serum. On day 6 (0 time), the medium was replaced with 3 ml of fresh medium containing 5% human lipoprotein-deficient plasma. At the indicated time, extracts were prepared and HMG CoA reductase activity was measured. (*B*) 24 hr after addition of 5% human lipoprotein-deficient plasma, 0.1 ml of buffer A containing human LDL was added to give the indicated concentration: (○,●) None; (▽,▼) 2 μg/ml; (△,▲) 10 μg/ml; (□,■) 20 μg/ml. HMG CoA reductase activity was measured at the indicated time.

Measurement of Cholesterol Synthesis. Fibroblasts were grown to confluence, and the growth medium was replaced with 2 ml of Krebs–Ringer phosphate buffer (pH 7.0) containing either 0.6 mM [2-^{14}C]sodium acetate (New England Nuclear Corp., 53.4 Ci/mol) or 0.1 mM [2-^{14}C]potassium mevalonate (Amersham Searle, 10 Ci/mol). Each dish was incubated 2 hr at 37° in a humidified atmosphere of 95% air–5% CO_2. The medium was then removed, and the cells were scraped with a rubber policeman into 1 ml of water. Each dish was further washed with 2 ml of water; the medium, the cells, and both washes were pooled in a final volume of 5 ml, to which was added 0.5 ml of 90% KOH and 1 mg of nonradioactive cholesterol. [^{14}C]Cholesterol was isolated and quantitated by digitonin precipitation followed by thin-layer chromatography (15, 16).

Lipoproteins from normolipidemic subjects and from J.P. were isolated from plasma collected in EDTA (1 mg/ml) after a 15-hr fast. They were fractionated by sequential flotation and dialyzed into buffer A containing 0.15 M NaCl–0.3 mM EDTA (pH 7.4) (13). The fraction of density 1.019–1.063 g/ml is referred to as LDL. The fraction of density >1.215 g/ml is referred to as lipoprotein-deficient plasma.

Protein concentrations were determined by a modification of the method of Lowry *et al.* (17), with bovine-serum albumin as a standard.

RESULTS

In control fibroblasts grown to confluence in medium containing 10% fetal-calf serum, HMG CoA reductase activity was relatively low (Fig. 1*A*, 0 time). We previously showed that this low enzyme activity is due to suppression of the enzyme by lipoproteins present in fetal-calf serum (13). When fetal-calf serum was replaced with human lipoprotein-

TABLE 1. *Synthesis of [^{14}C]cholesterol from [^{14}C]acetate and [^{14}C]mevalonate by fibroblasts from a control subject and a patient with homozygous familial hypercholesterolemia (J.P.)*

Growth conditions	[^{14}C]Acetate → [^{14}C]Cholesterol (pmol/2 hr per mg of protein)		[^{14}C]Mevalonate → [^{14}C]Cholesterol (pmol/2 hr per mg of protein)		HMG CoA reductase activity (pmol/min per mg of protein)	
	Control	Homozygote	Control	Homozygote	Control	Homozygote
A. 10% Fetal-calf serum	12.2	960	149 (446)	266 (798)	4.2	123
B. 5% Lipoprotein-deficient human plasma	251	878	188 (564)	208 (624)	74.0	158
C. 5% Lipoprotein-deficient human plasma + LDL	96	1828	187 (562)	195 (584)	—	—

Cells were grown in dishes containing 10% fetal-calf serum. On day 6 (0 time), the medium was removed, the cells were washed, and 3 ml of fresh medium containing either 10% fetal-calf serum (*A*) or 5% lipoprotein-deficient human plasma (*B* and *C*) was added. 24 hr later cells in group *C* received LDL at a final protein concentration of 12 μg/ml (20 μg/ml of cholesterol). Incorporation of [^{14}C]acetate and [^{14}C]mevalonate into [^{14}C]cholesterol and measurement of HMG CoA reductase activity were determined at either 24 hr (*A* and *B*) or 30 hr (*C*). Each value represents the mean of duplicate determinations on duplicate dishes. The protein content of the dishes ranged between 0.16 and 0.24 mg per dish. Since each molecule of mevalonate originates from three molecules of acetate, the values in parentheses indicate the number of pmol of acetate represented by the [^{14}C]mevalonate incorporated into [^{14}C]cholesterol.

deficient plasma, the specific activity of HMG CoA reductase progressively increased by about 40-fold, reaching a plateau at 24 hr (Fig. 1*A*). At this point addition of human LDL resulted in a time- and concentration-dependent decrease in HMG CoA reductase activity (Fig. 1*B*). Fibroblasts from a patient with the homozygous form of familial hypercholesterolemia (J.P.) showed no such regulation (Fig. 1). In the presence of 10% fetal-calf serum, HMG CoA reductase activity in the mutant cells was about 60-fold higher than in normal cells grown under identical conditions (Fig. 1*A*, 0 time). Moreover, this activity did not change either when fetal-calf serum was replaced with lipoprotein-deficient plasma (Fig. 1*A*) or when LDL was added (Fig. 1*B*). When extracts from normal and mutant cells, both grown in the presence of fetal-calf serum, were mixed, the activity of HMG CoA reductase was additive, indicating that the mutant cells did not lack an intracellular inhibitor of this enzyme.

If HMG CoA reductase activity is the rate-controlling step in cholesterol synthesis in human fibroblasts as it is in other mammalian tissues (12), then the failure of regulation of this enzyme should result in defective regulation of cholesterol synthesis from acetate but not mevalonate. That this was indeed the case is shown by the data in Table 1. Cholesterol synthesis from acetate in control fibroblasts increased by about 20-fold when fetal-calf serum was replaced with lipoprotein-deficient plasma and it decreased by 62% when LDL was added. In contrast, in the presence of fetal-calf serum, mutant fibroblasts synthesized cholesterol from acetate at a rate that was nearly 80-times greater than that of control cells; in addition, the mutant cells showed no response to alterations in the lipoprotein content of the medium. Mevalonic acid, the product of the HMG CoA reductase reaction, was incorporated into cholesterol at a similar rate in control and mutant cells and this rate was unaffected by alterations in lipoprotein content of the medium (Table 1). Although the experiments in Table 1 represent a valid comparison of the relative rates of cholesterol synthesis at one arbitrary concentration of either acetate or mevalonate, these substrate concentrations were such that maximal rates were not necessarily obtained. Consequently, stoichiometric comparisons between the rates of cholesterol synthesis from acetate and mevalonate in intact cells or a comparison of either of these rates with HMG CoA reductase activity in cell-free extracts was not possible.

After we demonstrated that normal plasma LDL had no effect on cells of the homozygote, it was of interest to ascertain whether plasma LDL of the homozygote was capable of depressing HMG CoA reductase activity in normal cells. When equal concentrations of LDL isolated from a normolipidemic subject and from the homozygote J.P. were added to normal fibroblasts that had been previously grown in lipoprotein-deficient plasma, both LDL preparations reduced HMG CoA reductase activity in an identical manner (Fig. 2). In other experiments not shown, both types of LDL were identical in their inability to depress the HMG CoA reductase activity of the cells of J.P.

HMG CoA reductase activity from mutant and control cells showed identical Michaelis constants for the two substrates, HMG CoA and TPNH (Fig. 3). These kinetic data suggest that the increased HMG CoA reductase activity in the cells of the homozygote is not due to an enhanced affinity of the enzyme for one of its substrates.

To determine whether regulation of HMG CoA reductase activity in fibroblasts from heterozygotes with familial hypercholesterolemia was abnormal, the enzyme activity was studied in cultured cells of the parents of J.P., who are obligate heterozygotes for this disorder. In the presence of fetal-calf serum, the enzyme activity of the two heterozygotes resembled that of seven control lines (Fig. 4). However, when lipoproteins were removed, enzyme activity of the two heterozygotes became abnormally elevated and approached the levels seen in three unrelated homozygotes. A more clear-cut abnormality in these heterozygotes was demonstrated by the experiments in Fig. 5, in which the inhibitory response to LDL was compared in cell lines from two controls, two heterozygotes, and a homozygote. The heterozygotes showed a partial and intermediate defect in regulation of HMG CoA reductase activity by LDL.

DISCUSSION

In the experiments reported in this paper, cultured skin fibroblasts from three subjects with the homozygous form of familial hypercholesterolemia were shown to have a marked increase in the activity of the rate-controlling enzyme in cholesterol biosynthesis, HMG CoA reductase, when compared with cells from seven control subjects. The enhanced activity of the enzyme, which resulted from a complete absence of its normal regulation by lipoproteins, led to overproduction of cholesterol by the mutant cells. In fibroblasts

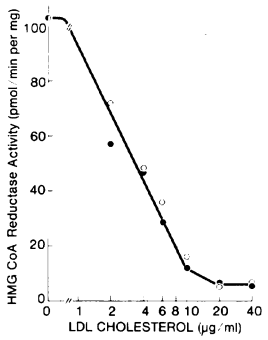

FIG. 2. Effect of LDL from a control subject and LDL from a patient with homozygous familial hypercholesterolemia (J.P.) on HMG CoA reductase activity of control fibroblasts. Cells were grown in dishes containing 10% fetal-calf serum; on day 6 the medium was replaced with 3 ml of fresh medium containing 5% human lipoprotein-deficient plasma. After 24 hr 0.1 ml of buffer A containing LDL from a control subject (●) or LDL from the homozygote J.P. (○) was added to give the indicated concentration. 6 hr later extracts were prepared and assayed for HMG CoA reductase activity.

from two obligate heterozygotes, HMG CoA reductase activity was higher than in controls and these cells showed a partial defect in enzyme regulation by LDL.

The failure of regulation of HMG CoA reductase activity in the mutant cells could be due either to a mutation in a structural gene for the enzyme itself or to a mutation in a gene that specifies an hitherto unidentified protein critical for normal regulation of the enzyme activity by lipoproteins. Two lines of evidence suggest that a mutation in a structural gene for the enzyme is not responsible for the observed defect. First, the normal process of regulation of this enzyme does not appear to

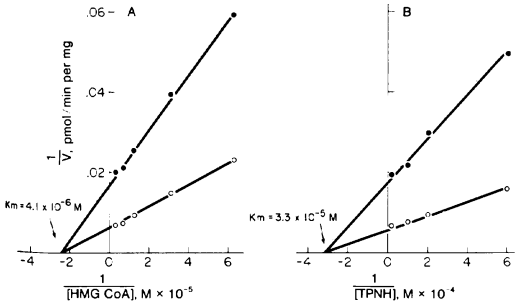

FIG. 3. Kinetic properties of HMG CoA reductase activity from fibroblasts of a control subject and a patient with homozygous familial hypercholesterolemia (J.P.). Cells were grown in dishes containing 10% fetal-calf serum; on day 6 the medium was removed and 3 ml of fresh medium containing 10% fetal-calf serum was added to the cells of the homozygote (○) and medium containing 5% human lipoprotein-deficient plasma was added to the cells of the control subject (●). 24 hr later, extracts were prepared and aliquots containing 22 μg of protein (homozygote) or 45 μg of protein (control) were assayed for HMG CoA reductase activity as described in *Methods* except that the concentrations of HMG CoA (*A*) and TPNH (*B*) were varied as indicated.

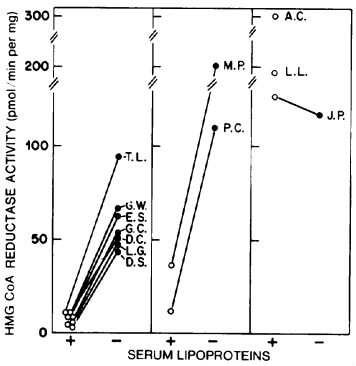

FIG. 4. Regulation of HMG CoA reductase activity in fibroblasts from control subjects (*left*) and patients with the heterozygous (*middle*) and homozygous (*right*) forms of familial hypercholesterolemia. Cells were grown in dishes containing 10% fetal-calf serum; on day 6 the medium was replaced with 3 ml of fresh medium containing either 10% fetal-calf serum (0) or 5% human lipoprotein-deficient plasma (●). 18 hr later, extracts were prepared and assayed for HMG CoA reductase activity. Each value represents the mean enzyme activity of duplicate dishes. The initials of each subject correspond to those given in *Methods*.

depend upon a property of the enzyme itself since no direct allosteric effects on the isolated enzyme can be demonstrated (13, 14); rather, its regulation appears to be mediated by alterations in enzyme synthesis and degradation (13). This observation implies that a genetically determined abnormality in the structure of HMG CoA reductase would produce a result different from that reported here in that it would lead to altered activity of the enzyme without affecting the process of its regulation (18). Second, the demonstration of apparently identical kinetic properties of the enzyme from normal and mutant cells also suggests that the enzyme itself may not be

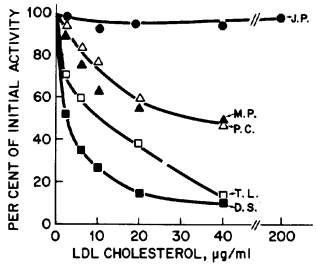

FIG. 5. Effect of LDL on HMG CoA reductase activity in fibroblasts from control subjects (*squares*), heterozygotes (*triangles*), and a homozygote (*circles*). Cells were grown in dishes containing 10% fetal-calf serum; on day 6 the medium was replaced with 3 ml of fresh medium containing 5% human lipoprotein-deficient plasma. After 24 hr, 0.1 ml of buffer A containing LDL from a control subject was added to give the indicated concentration. 6 hr later extracts were prepared and assayed for HMG CoA reductase. Results are plotted as the percentage of HMG CoA reductase activity of cells receiving only buffer A without LDL (initial activity). These values, expressed in pmol/min per mg were: J.P., 227; M.P., 211; P.C., 162; D.S., 65 4; T.L., 93.3.

altered. If further studies support the hypothesis that the mutation in these patients' cells involves a protein other than HMG CoA reductase, then identification of this putative gene product should provide new insight into the normal mechanism of regulation of cholesterol biosynthesis.

Our studies of cultured fibroblasts indicate that subjects with familial hypercholesterolemia of the type reported in this paper carry in all cells of the body an abnormal gene that has the potential to induce excessive cholesterol production. The tissues in which this abnormal gene is expressed *in vivo* and the conditions governing its expression cannot be determined from these cell-culture studies, since human cells of a given type in culture may express genes *in vitro* that they do not express *in vivo* (19). However, our data would seem to warrant the suggestion that accumulation of cholesterol in subjects with this disorder is due to a genetically determined defect in regulation of cholesterol synthesis at the level of HMG CoA reductase.

Dr. H. Peter Chase of the John F. Kennedy Child Development Center, the B. F. Stolinsky Research Laboratories, University of Colorado Medical Center, Denver, Colo., is the physician of J.P.; we thank him for calling our attention to this patient and for allowing us to obtain a skin biopsy from her and her parents. Dr. Jean D'Avignon of the Institut de Recherches Cliniques, Montreal, Canada, is the physician of L.L. and A.C.; we thank him for allowing us to obtain skin biopsies from them. Dr. Jean D. Wilson was of invaluable aid in the experiments in which cholesterol synthesis was measured. Suzanna E. Dana, Gwendolyn Fidler, Mary Jo Harrod, and Jean Helgerson provided excellent assistance. This research was supported by grants from the American Heart Association (72629) and the National Institutes of Health (GM 19258, CA 08501, and 5 TO1 AM05490). J.L.G. is the recipient of a USPHS Research Career Development Award 1K4-GM-70, 227-01.

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