Effects of ML-236B (Compactin) on Sterol Synthesis and Low Density Lipoprotein Receptor Activities in Fibroblasts of Patients with Homozygous Familial Hypercholesterolemia

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ABSTRACT We studied biochemical genetics of low density lipoprotein (LDL) receptor mutations in fibroblasts from six homozygous and five heterozygous patients with familial hypercholesterolemia (FH). Three of six homozygotes are receptor-negative type and the other three homozygotes are receptor-defective type. In the cells from three receptor-negative homozygotes, the receptor binding, internalization, and degradation of ^{125}I-LDL were 0.5±0.3 ng/mg protein (mean±SEM), 14±8 and 8±6 ng/mg protein per 6 h (four normal cells; 44±3, 386±32, and 1,335 ±214 ng/mg protein per 6 h), respectively. In the cells from three receptor-defective homozygotes, the receptor binding, internalization, and degradation of ^{125}I-LDL were 6±2, 29±8, and 90±32 ng/mg protein per 6 h, respectively. In these six homozygotes, two pairs of siblings are included. Two siblings in the same family were classified as receptor-negative and two siblings in another family were classified as receptor-defective. The receptor-negative phenotypes and the receptor-defective phenotypes bred true in individual families. The cells from five heterozygotes showed ~46% of the normal activities of receptor.

ML-236B, competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), completely inhibited the incorporation of [^{14}C]acetate into digitonin-precipitable sterols in fibroblasts from normal subjects and heterozygous and homozygous patients with FH with the concentration of 0.5 μg/ml. However, at 0.05 μg/ml of ML-236B sterol synthesis in fibroblasts from homozygotes was not completely suppressed in contrast to normal and heterozygous cells. Moreover, after preincubation with 0.05 μg/ml of ML-236B for 24 h in medium containing lipoproteins, sterol synthesis in the cells from receptor-negative homozygote showed 75% of the initial activity compared with that of 25% without preincubation. In the cells from a normal subject and a heterozygote, sterol synthesis was inhibited even after preincubation. These results suggest that (*a*) the inhibitory effect of ML-236B is overcome in homozygote cells by their high intracellular levels of HMG-CoA reductase and (*b*) that a higher dose of ML-236B may be required to lower serum cholesterol levels in FH homozygotes than in heterozygotes.

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INTRODUCTION

Familial hypercholesterolemia (FH)[1] is an autosomal dominant disease characterized by high levels of serum and low density lipoprotein (LDL) cholesterol, xanthomas, and premature coronary heart disease (1, 2). Although the serum cholesterol level of the general population in Japan is much lower than that in the Western countries, the serum cholesterol level of homozygous and heterozygous patients with FH in Japan are as high as those in the Western countries

[1] *Abbreviations used in this paper*: FH, familial hypercholesterolemia; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase; LDL, low density lipoprotein; LPDS, lipoprotein-deficient serum.

TABLE I

Clinical Data of Control Subjects and Patients with Heterozygous or Homozygous FH Whose Fibroblasts Were Used in This Study

Name	Age	Sex	Cholesterol	LDL-cholesterol	Triglyceride	Achilles tendon thickness*	Xanthoma	Coronary heart disease
	yr		*mg/dl*	*mg/dl*	*mg/dl*	*mm*		
Normal Subjects								
R.M.	25	m	175	ND	40	ND	–	–
R.T.	28	m	168	ND	60	ND	–	–
I.M.	36	f	94	ND	ND	ND	–	–
K.O.‡	62	m	208	122	78	ND	–	–
Mean±SEM	38±8		161±24		59±11			
Heterozygotes								
H.K.	23	f	441	367	59	27	+	–
S.M.	26	f	425	336	92	12	–	–
M.O.‡	27	m	290	229	63	25	+	–
T.Y.	53	m	361	314	164	15	–	Myocardial infarction
T.O.‡	60	f	323	237	100	34	+	–
Mean±SEM	38±8		368±29	297±27	96±19	23±4		
Homozygotes								
M.S.§	4	f	890	ND	160	ND	++	–
J.S.§	5	m	853	ND	123	ND	++	–
M.T.	16	m	478	401	67	8	++	–
T.S.	19	f	604	426	105	ND	++	–
Y.Y.‖	25	m	538	378	183	42	++	Angina pectoris
K.M.‖	39	f	586	503	107	24	++	Sudden death (42 yr)
Mean±SEM	18±5		658±70	427±27	124±17	25±10		

ND, not determined; m, male; f, female.
* Normal value (mean±SEM) of Japanese subjects: 6.3 ±0.2 mm.
‡ K.O.: father of M.O., T.O.: mother of M.O.
§ M.S. and J.S. are siblings.
‖ Y.Y. and K.M. are siblings.

(3–6). Moreover, the frequency of homozygotes in Japan (1 in 1,450,000) is close to that of America (1 in 1,000,000) (4, 6).

Recent studies in cultured human skin fibroblasts have led to the identification of a cell surface receptor that binds serum LDL (1). Goldstein and Brown (7, 8) have demonstrated that mutant cells from homozygotes have a deficiency in the LDL receptor on the cell surface and at least three different mutations (i.e., receptor-negative, receptor-defective, and internalization defect) can cause homozygous FH in the Western countries. However, there are no detailed studies of the molecular mechanism of homozygous FH in Japan. Thus, it is interesting to have such information about biochemical phenotypes of FH in Japan. We have analyzed the LDL receptor activities of the fibroblasts from six homozygotes and five heterozygotes by measurement of uptake, internalization, and degradation of ^{125}I-LDL.

Recently, Endo et al. (9) discovered a fungal metabolite, ML-236B (CS-500, mevastatin, compactin), which is a potent inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), the rate-limiting enzyme in cholesterol synthesis. Inhibitory effects of ML-236B have also been shown both in vitro (9–11) and in vivo (12–15).[2] We have studied the effects of ML-236B on sterol synthesis and LDL receptor activities in cultured skin fibroblasts from

[2] Mabuchi, H., T. Haba, R. Tatami, S. Miyamoto, T. Kametani, K. Ueda, R. Ueda, T. Wakasugi, A. Watanabe, J. Koizumi, and R. Takeda. Effects of ML-236B, a competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase, on serum lipoproteins of familial hypercholesterolemic patients. Submitted for publication.

normal subjects, heterozygous and homozygous patients with FH.

METHODS

Materials. Sodium [2-^{14}C]acetate (59 mCi/mmol) was obtained from The Radiochemical Centre, Amersham, England. DL-3-[3-^{14}C]-hydroxy-3-methylglutaryl coenzyme A (49.5 mCi/mmol) was obtained from New England Nuclear, Boston, Mass. D-glucose 6-phosphate, NADP, and dithiothreitol were obtained from P-L Biochemicals, Inc., Milwaukee, Wis. Glucose 6-phosphate dehydrogenase was obtained from Boehringer Mannheim Biochemical, Indianapolis, Ind. Dextran sulfate (M_r = 500,000) was obtained from Sigma Chemical Co., St. Louis, Mo. ML-236B was kindly supplied by Sankyo Co., Japan. Fetal calf serum and nonessential amino acid solution were obtained from Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y. Eagle's minimum essential medium was obtained from Nissui Co., Japan. Tissue culture dishes (60 × 15 mm) and flasks (75 cm^2, 250 ml) were obtained from Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif.

Patients. Table I summarizes the clinical data of control subjects and patients with heterozygous or homozygous FH whose fibroblasts were used in this study. Subjects were assigned on the basis of family history. The control group includes four healthy subjects. Five heterozygotes showed elevated serum cholesterol levels and tendinous xanthoma. Achilles' tendon xanthoma was detected by radiological examination of Achilles' tendon (16, 17). Six homozygotes had juvenile xanthomatosis and high serum cholesterol levels about twice those of the parents with heterozygous FH. The pedigrees of the families are shown in Fig. 1. The data of M.T. and Y.Y. were partially published in our earlier paper (4).

Cells. Human fibroblasts were established from the skin of the forearm. The receptor-negative homozygous FH fibroblast (GM-486) was obtained from the (U.S.) National Institute of Medical Research. Skin fibroblasts were maintained in a humidified incubator (5% CO_2) at 37°C in 250 ml flasks containing 12 ml of growth medium supplemented with kanamycin (100 μg/ml), 17 mM $NaHCO_3$, 1% (vol/vol) nonessential amino acid, and 10% (vol/vol) fetal calf serum. Cells were used between the 5th and 20th passage. Cells from stock flasks were dissociated with 0.05% trypsin-0.01% EDTA solution and were seeded at a concentration of 1.5–2.0 × 10^5 cells per dish into dishes containing 3 ml of growth medium. On day 3 the medium was replaced with a fresh medium. On the 5th or 6th day, when cells grew nearly confluent, the experiments were initiated.

Lipoproteins. Human LDL (d = 1.020–1.050 g/ml) and lipoprotein-deficient serum (LPDS) (d > 1.215 g/ml) were obtained from the serum of healthy subjects and prepared by differential ultracentrifugation according to the method of Havel et al. (18). The concentration of LDL is expressed in terms of its protein content. ^{125}I-LDL was prepared by modification of the iodine monochloride method of MacFarlane (19, 20). 95% of the radioactivity was precipitated by incubation with trichloroacetic acid, and 3% was extractable into chloroform:methanol. For each experiment, the ^{125}I-LDL was diluted with unlabeled LDL to give the final specific activity (33–100 cpm/ng of protein).

Determination of surface-bound, intracellular, and degraded ^{125}I-LDL in fibroblast monlayers. Fibroblast mono-

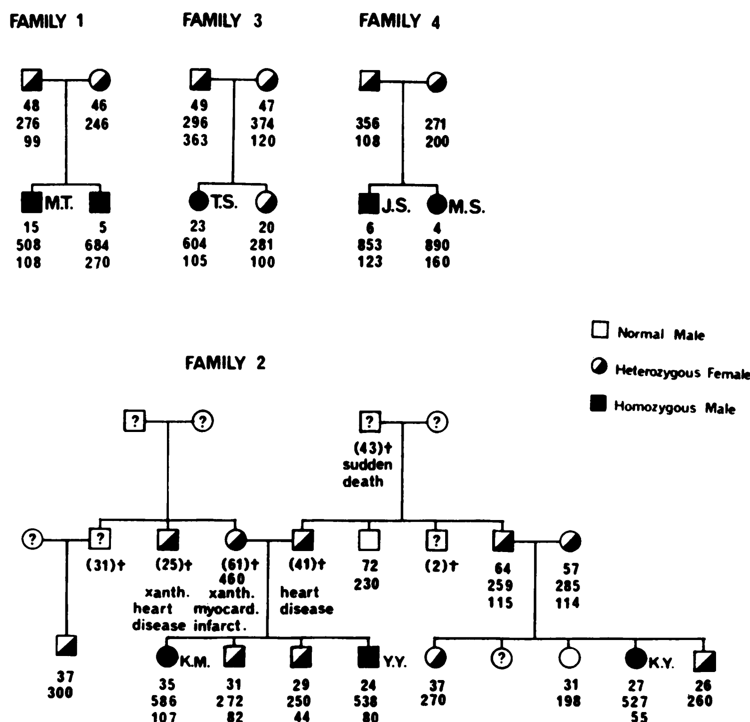

FIGURE 1 Pedigrees of six homozygous familial hypercholesterolemic patients. The numbers under the symbols represent: age (top No.), plasma cholesterol level in milligrams per deciliter (middle No.), and plasma triglyceride level in milligrams per deciliter (bottom No.).

layers were incubated with ^{125}I-LDL in 2 ml of medium containing 5% LPDS for 6 h. Surface-bound, intracellular, and degraded ^{125}I-LDL in fibroblasts monolayers were measured by the method of Goldstein et al. (8, 21, 22). High-affinity LDL receptor activity values were determined by subtracting the radioactivity in the presence of 200 μg/ml of unlabeled LDL from the radioactivity in the absence of unlabeled LDL.

Assay of HMG-CoA reductase activity. HMG-CoA reductase activity was measured by slight modification (12) of the method of Brown et al. (23). [^{14}C]Mevalonolactone formed was isolated by silica thin-layer chromatography in acetone-benzene (1:1, vol/vol) and counted (24).

Incorporation of [^{14}C]acetate into digitonin-precipitable sterols. Cell monolayers were incubated at 37°C in 2 ml of medium containing either 10% fetal calf serum or 5% LPDS and supplemented with 2.7 μCi of [^{14}C]acetate (59 mCi/mmol). Digitonin-precipitable ^{14}C-labeled sterols were isolated and counted by the method of Brown et al. (10).

Assays of protein. Protein concentration was determined

TABLE II
High-Affinity Binding, Internalization, and Degradation of ^{125}I-LDL in Fibroblasts from Normal Subjects and Patients with Heterozygous or Homozygous FH

Cell strain	Receptor-bound ^{125}I-LDL (B)	Internalized ^{125}I-LDL (I)	Degraded ^{125}I-LDL (D)	$\frac{I+D}{B}$
	ng/mg	*ng/mg*	*ng/6 h per mg*	
Normal Subjects				
R.M.	36	305	831	31.6
R.T.	45	459	1,774	49.6
I.M.	47	394	1,141	32.7
K.O.*	46	385	1,594	43.0
Mean±SEM	44±3	386±32	1,335±214	39.2±4.3
Heterozygotes				
H.K.	13	96	368	32.6
S.M.	22	160	733	40.6
M.O.*	28	197	624	29.3
T.K.	33	173	636	24.5
T.O.*	25	108	711	32.8
Mean±SEM	24±3	147±19	614±65	32.6±2.7
Homozygotes, receptor-defective				
M.T.	11	42	142	16.7
Y.Y.‡	5	29	96	25.0
K.M.‡	3	16	33	16.3
Mean±SEM	6±2	29±8	90±32	19.3±2.8
Homozygotes, receptor-negative				
M.S.‖	0	0	0	—
J.S.‖	0.4	29	21	—
T.S.	1	14	4	—
GM-486	0.3	13	2	—
Mean±SEM	0.4±0.2	14±6	7±5	

Cell monolayers were grown in dishes containing 3 ml of growth medium. On the 5th or 6th day, the monolayers received 3 ml of medium containing 5% LPDS. After incubation for 48 h, the cells received 2 ml of fresh medium containing 5% LPDS and 10 μg protein/ml of ^{125}I-LDL (33–100 cpm/ng). After incubation for 6 h, surface-bound, internalized, and degraded ^{125}I-LDL were determined. Each value represents the average of duplicate or triplicate incubations.

* K.O., father of M.O.; T.O.; mother of M.O.

‖ M.S. and J.S. are siblings.

‡ Y.Y. and K.M. are siblings.

by the method of Lowry et al. (25) with bovine serum albumin as a standard.

RESULTS

When monolayers of normal fibroblasts were incubated with ^{125}I-LDL at concentration of LDL between 5 and 40 μg/ml, the receptor binding, internalization, and degradation of ^{125}I-LDL in normal cells showed saturation kinetics. Saturation occurred at ~10–20 μg/ml of LDL (data not shown). In contrast to normal cells, fibroblasts from homozygotes showed tiny amounts of LDL binding, internalization, and degradation (data not shown). The time-course of ^{125}I-LDL binding and internalization of normal cells reached a plateau at ~3 h, whereas degradation continued in a linear fashion for at least 6 h (data not shown).

For screening of the abnormalities of LDL receptor, we have established a standardized assay in which fibroblasts were incubated with 10 μg/ml of ^{125}I-LDL for 6 h. In cells from four normal subjects, the average amount of receptor binding was 44±3 ng of ^{125}I-LDL/mg of cellular protein (mean±SEM). The cells contained an average of 386±32 ng of ^{125}I-LDL/mg of cellular protein and a total of 1,335±214 ng of ^{125}I-LDL/mg of cellular protein had been degraded for 6 h (Table II). In the cells from five heterozygotes, the receptor binding, internalization, and degradation of LDL were found to be 24±3 ng/mg of cellular protein, 147±19 ng/mg of cellular protein, and 614±65 ng/ml of cellular protein/6 h, respectively (Table II). In short, these cells showed about one-half the normal amount of LDL receptor. In the cells from three homozygotes, the receptor binding, internalization, and degradation were found to be 6±2 ng/mg, 29±8 ng/mg, and 90±32 ng/mg per 6 h, respectively (Table II), whereas, in the cells from the other three homozygotes and GM-486, the receptor binding, internalization, and degradation of LDL were barely detectable (Table II). The data suggest that these patients were receptor-negative homozygotes and the former were receptor-defective homozygotes according to the criteria of Goldstein and Brown (8). The cells from three receptor-defective homozygotes showed ~8% of the normal activity of the LDL receptor. The internalization index (the index of the rate of intracellular + degraded ^{125}I-LDL relative to the amount of receptor-bound ^{125}I-LDL) of the normal cells and heterozygous cells were 39.2 and 32.6, respectively.

The HMG-CoA reductase activity of the cultured fibroblasts is shown in Table III. In the growth medium containing 10% fetal calf serum, HMG-CoA reductase activities of fibroblasts from normal subjects were very low (Table III). Enzyme activities in the cells from the receptor-negative homozygotes were higher than those of the three receptor-defective homozygotes (Table III). The fibroblasts of five heterozygotes showed an intermediate level of enzyme activity between normal subjects and the receptor-defective homozygotes (Table III).

When the fetal calf serum was replaced by human LPDS, the enyzme activities of the normal cells, heterozygote cells, and receptor-defective homozygote cells increased by ~13-, 5-, and 4-fold, respectively, but the cells from receptor-negative homozygotes showed only a slight increase of activity (Table III).

Fig. 2 shows the results of 6 h LDL-mediated suppression of HMG-CoA reductase activity in the fibroblasts after 36–48 h growth in medium containing 10% LPDS. The receptor-negative homozygotes showed no suppression of enzyme activity, whereas the receptor-defective homozygotes showed partial suppression of enzyme activity (Fig. 2). Heterozygotes showed an intermediate suppression between the controls and the homozygotes (Fig. 2).

TABLE III
The HMG-CoA Reductase Activity in Fibroblasts from Normal Subjects and Patients with Heterozygous or Homozygous FH

Cell strain	HMG-CoA reductase activity 10% Fetal calf serum (F)	HMG-CoA reductase activity 10% Lipoprotein-deficient serum (L)	L/F
	pmol/min/mg		
Normal subjects (n = 5)	2.8±0.9	36.0±10.7	12.9
Heterozygotes (n = 5)	7.2±2.8	34.0±6.7	4.7
Homozygotes			
Receptor-defective (n = 3)	11.3±3.7	40.4±6.9	3.6
Receptor-negative (n = 1)	36.0	41.6	1.2

Cell monolayers were grown in dishes containing 3 ml of growth medium. On day 5 or 6, one group of cells received 3 ml of fresh growth medium and the other group of cells received 3 ml of 10% LPDS. After incubation for 36–48 h, the cells were scraped and cellular HMG-CoA reductase activity was determined. Each value was determined by the average of duplicate assays.

Effects of ML-236B on the LDL receptor and sterol synthesis were studied using fibroblasts from our patients. Fig. 3 shows the inhibitory effects of ML-236B on the sterol synthesis in fibroblasts from a normal subject, a heterozygote and a receptor-negative homozygote with FH. Sterol synthesis in these fibroblasts was inhibited >90% at a concentration of 0.5 μg/ml of ML-236B. At a concentration of 0.01 and 0.05 μg/ml, the fibroblasts from a normal subject and heterozygote were more sensitive to this drug than the fibroblasts from a receptor-negative homozygote. Sterol synthesis in fibroblasts from three receptor-defective homozygotes was also sensitive to this drug at concentration of 0.5 μg/ml (Fig. 4).

After the cells had been preincubated in growth medium containing 10% fetal calf serum supplemented with 0.05 μg/ml of ML-236B, sterol synthesis in the cells from receptor-negative homozygote showed 75% of initial activity compared with 25% without preincubation (Fig. 5). In the cells from a normal subject and a heterozygote, sterol synthesis was inhibited even after preincubation of 24 h (Fig. 5).

When ML-236B was removed from the medium, the rate of sterol synthesis from [^{14}C]acetate in the cells that had been preincubated with 0.05 μg/ml of the drug for 24 h increased by more than 2.8-fold and was 1.5-fold higher than in cells that were not preincubated with the drug (Table IV).

Table V shows the effect of ML-236B on the LDL receptor activities in the cells from a normal subject and two homozygotes. At the medium concentration of 0.5 μg/ml of ML-236B, there was no change of LDL binding, internalization, and degradation.

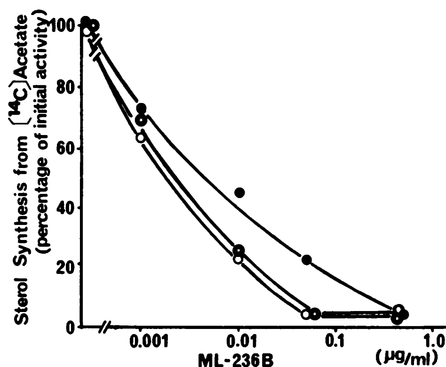

FIGURE 3 Effect of ML-236B on sterol synthesis from [^{14}C]-acetate in fibroblasts from normal subjects and patients with heterozygous or homozygous FH. Cell monolayers were grown in dishes containing 3 ml of growth medium. On day 5 or 6, the monolayers received 2 ml of fresh growth medium containing 2.7 μCi of [^{14}C]acetate and indicated concentrations of ML-236B. After incubation for 4 h, sterol synthesis from [^{14}C]acetate were determined. The data are expressed as a percentage of synthesized digitonin-precipitable sterols (disintegrations per min per hour per milligram of protein) without ML-236B. These control value was 4,107 dpm/h per mg in normal cells (○), 5,543 dpm/h per mg in heterozygous cells (◉), and 51,453 dpm/h per mg in receptor-negative homozygous cells (●). Each value was determined by the average of duplicate incubations.

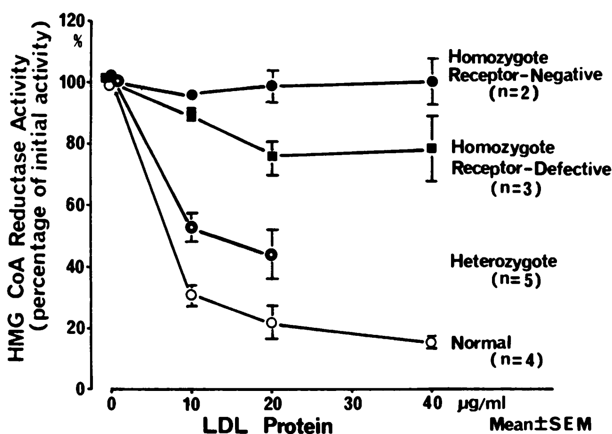

FIGURE 2 Effects of various concentrations of LDL on HMG-CoA reductase activity was determined. The mean values for the "100% of initial activity" for the normal, heterozygote, receptor-defective homozygote, and receptor-negative homo-medium. On day 5 or 6, the monolayers received 3 ml of fresh medium containing 10% LPDS. After incubation of 36–48 h, the cells received 2 ml of fresh medium containing 10% LPDS and indicated concentration of LDL. After a further 6 h of incubation, the cells were scraped, and cellular HMG-CoA reductase activity was determined. The mean values for the "100% of initial activity" for the normal, heterozygote, receptor-defective homozygote, and receptor-negative homozygote cells were 34.8, 34.0, 40.4, and 38.3 pmol/min per mg of protein, respectively.

DISCUSSION

FH is a common inherited disorder characterized by an elevated concentration of LDL-cholesterol. Hyper-

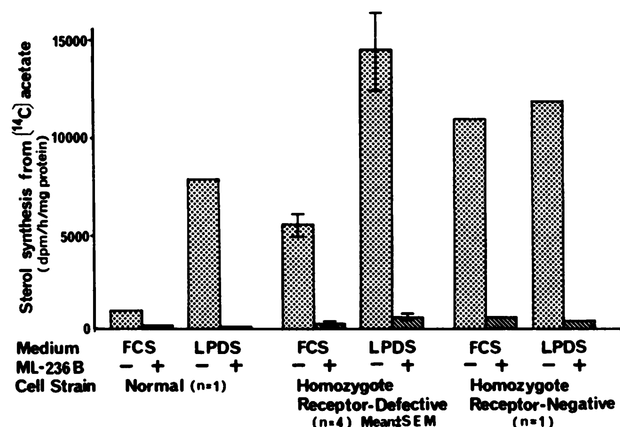

FIGURE 4 Inhibition of sterol synthesis from [^{14}C]acetate in fibroblasts from subjects with different genotypes at concentration of 0.5 μg/ml of ML-236B. On day 5 or 6, one group of cells received 3 ml of fresh growth medium and the other group of cells received 3 ml of 5% LPDS. After incubation for 48 h, the cells received 2 ml of either growth medium or 5% LPDS containing 2.7 μCi of [^{14}C]acetate with or without 0.5 μg/ml (1.17 μM) of ML-236B. After further incubation of 4 h, sterol synthesis from [^{14}C]acetate were determined. Each value was determined by the average of duplicate incubations.

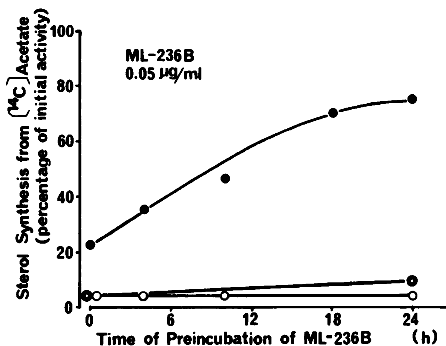

FIGURE 5 Changes of inhibition of ML-236B on sterol synthesis from [^{14}C]acetate in fibroblasts after preincubation with 0.05 μg/ml of ML-236B. Cell monolayers were grown in dishes containing 3 ml of growth medium. On day 6, each monolayer received 2 ml of growth medium containing 2.7 μCi of [^{14}C]acetate and 0.05 μg/ml (0.12 μM) of ML-236B. Before incubation with [^{14}C]acetate, cells received 0.05 μg/ml of ML-236B for indicated hours. After incubation for 4 h, sterol synthesis from [^{14}C]acetate were determined. The data are expressed as a percentage of synthesized digitonin-precipitable sterols (disintegrations per minute per hour per milligram of cellular protein) without ML-236B. The control value was 3,665 dpm/h per mg in normal cells (○), 4,375 dpm/h per mg in heterozygous cells (◉), and 18,142 dpm/h per mg in receptor-negative homozygous cells (●). Each value was determined by the average of duplicate incubations.

TABLE IV
Changes of Inhibition on Sterol Synthesis from [^{14}C]Acetate in Homozygote Cells after Preincubation with ML-236B

Preincubation of 24 h ML-236B	Incubation of 2 h ML-236B	Sterol synthesis from [^{14}C]acetate	
0.05 μg/ml	*0.05 μg/ml*	*dpm/h/mg protein*	*%*
(−)	(−)	17,845	100
(−)	(+)	3,539	19.8
(+)	(+)	9,580	53.7
(+)	(−)	27,039	151.5

Cell monolayers were grown in dishes containing 3 ml of growth medium. On day 6, the monolayers received 2 ml of growth medium containing 0.05 μg/ml of ML-236B. After preincubation at 37°C for 24 h, the medium was removed and washed. Then, each monolayer received 2 ml of fresh medium containing 2.7 μCi of [^{14}C]acetate with or without 0.05 μg/dl of ML-236B. After incubation for 2 h, sterol synthesis from [^{14}C]acetate was determined. Each value was determined by the average of duplicate incubations.

cholesterolemia is the earliest manifestation of the mutant gene and produces both xanthomas and coronary heart disease (1, 2). Brown and Goldstein (1) have demonstrated that skin fibroblasts from homozygotes have a deficiency of the cell surface receptor that binds serum LDL and regulates both LDL degradation and cholesterol synthesis. Studies in fibroblasts using patients with a clinical phenotype of homozygous FH have also disclosed the existence of at least three different mutations affecting the cellular LDL receptor. These three classes of mutant alleles are described as receptor-negative (R^{b0}), receptor-defective (R^{b-}), and internalization-defective ($R^{b+,i0}$) (7, 8). The receptor-negative class of alleles exhibits no functional receptors (<1% of normal). The receptor-defective class of alleles exhibit markedly reduced LDL receptors (1–10% of normal). The third mutant allele, internalization-defective, is a receptor that can bind LDL normally but cannot mediate the internalization of the receptor-bound LDL. These previous studies were done in FH patients from Western countries. There are no detailed studies of FH in Japan.

The current data showed that among the homozygotes of FH in Japan there exist at least two genetic subgroups, i.e., receptor-negative type and receptor-defective type. Fibroblasts from three homozygotes showed no specific LDL uptake and degradation and no LDL-mediated suppression of HMG-CoA reductase activity. These data are good agreement with those of receptor-negative homozygous FH fibroblast GM-486. These homozygotes are thought of as receptor-negative (genotype, R^{b0}/R^{b0}) and the other three homozygotes were receptor-defective (genotype, R^{b0}/R^{b-} or R^{b-}/R^{b-}) (Table II). In these homozygotes, two pairs of siblings are included. The cells of one of these sibling pairs (M.S. and J.S.) have inherited the receptor-negative alleles and the other pair (Y.Y. and K.M.) have inherited the receptor-defective alleles (Table II). These findings provide validation for the concept that receptor-negative and receptor-defective homozygotes are different genetic entities. Goldstein and Brown (29) studied genetic analysis of fibroblasts strains from 50 patients with homozygous FH and showed that there are 29 receptor-negative homozygotes, 20 receptor-defective homozygotes, and only one internalization-defective homozygote among them (29). It is interesting that although in Japan the serum cholesterol level and the frequency of ischemic heart disease of the general population is much lower than that in the Western countries, the incidence and subgroups of mutant genes of FH are very close to that in the West (3, 4). In the fibroblasts from five heterozygotes, the amount of receptor binding, internalization, and degradation of ^{125}I-LDL was reduced by ~50% (Table II). These heterozygotes are thought to possess one mutant allele R^{b0} or R^{b-}, but we cannot distinguish between the heterozygotes with receptor-negative allele and those with receptor-defective allele.

Goldstein and Brown (29) reported that there are no obvious clinical differences between the three genetic subgroups of FH homozygotes or between their heterozygous parents. In the present study, the three receptor-negative homozygotes showed high

TABLE V

No Effect of ML-236B on High-Affinity Binding, Internalization, and Degradation of ^{125}I-LDL in Fibroblasts from Normal Subjects and Patients with Homozygous FH

	Receptor-bound ^{125}I-LDL (B)	Internalized ^{125}I-LDL (I)	Degraded ^{125}I-LDL (D)	(I) + (D) / (B)
	ng/mg	*ng/mg*	*ng/mg/6 h*	
Normal subjects (K.O.)				
None	41	311	1,845	52.6
ML-236B	46	337	1,777	46.0
Homozygote, receptor-defective (Y.Y.)				
None	6	53	96	24.8
ML-236B	6	57	120	29.8
Homozygote, receptor-negative (T.S.)				
None	0	13	7	—
ML-236B	0	15	2	—

Cell monolayers were grown in dishes containing 3 ml of growth medium. On day 6, the monolayers received 3 ml of medium containing 5% LPDS and were incubated 48 h. On day 8, the cells received 2 ml of fresh medium containing 5% LPDS and 10 μg/dl of ^{125}I-LDL (51 cpm/ng) with or without 0.5 μg/ml of ML-236B. After incubation for 6 h, surface-bound, internalized, and degraded ^{125}I-LDL were determined. Each value represents the average of duplicate incubations.

serum cholesterol levels between 604 and 890 mg/dl and xanthoma appeared immediately after birth. However, three receptor-defective homozygotes showed serum cholesterol levels between 478 and 586 mg/dl and xanthoma appeared several years after birth, and two patients survived to >30 yr old. Thus, receptor-defective homozygotes seemed to be a milder phenotype than receptor-negative homozygotes.

Recently, Endo et al. reported that a fungl metabolite, ML-236B, is a potent inhibitor of cholesterol synthesis both in vitro (9–11) and in vivo (12–15).[2] They suggested that hypocholesterolemic activity of the drug in rats is due to inhibition of hepatic cholesterol synthesis (11). Yamamoto et al. (15) reported that the administration of ML-236B produced a 27% decrease in serum cholesterol in cases of heterozygous FH and combined hyperlipidemia. Mabuchi et al. found that ML-236B reduced intermediate density lipoprotein cholesterol and triglyceride as well as LDL cholesterol and triglyceride in heterozygous patients with FH.[2]

Brown et al. (10) reported that the inhibition of HMG-CoA reductase activity by ML-236B led to the production of large amounts of enzyme that was not active in the cell because it was competitively inhibited by the drug. They assumed that complete inhibition of HMG-CoA reductase by ML-236B would prevent the synthesis of small amount of mevalonate for the synthesis of an essential substance and this in turn would lead to an enhanced synthesis of HMG-CoA reductase molecules. In the present study, at 0.05 μg/ml, sterol synthesis in fibroblasts from a receptor-negative homozygote increased from 25 to 75% of the control value after 24 h of preincubation with the drug. When ML-236B was removed from the culture medium, the rate of cholesterol synthesis in homozygous cells that had been exposed to the drug was 1.5-fold higher than it was in the cells that were never exposed to the drug. This increase in sterol synthesis of homozygous cells in probably due to the increased intra-cellular HMG-CoA reductase activity. Although ML-236B is a potent inhibitor of HMG-CoA reductase, in homozygous fibroblasts the inhibitory effect of the drug at 0.05 μg/ml might be overcome by the increased HMG-CoA reductase after 24 h of preincubation of the drug. Therefore, a much higher dose of ML-236B may be necessary for lowering serum cholesterol levels in the homozygotes. ML-236B showed no effects on LDL binding, internalization, and degradation in the cultured cells from heterozygous and homozygous patients with FH as well as normal subjects reported by Goldstein and Brown (10).

ML-236B was shown to lower serum cholesterol level in patients with FH (15). Mabuchi et al. showed that this drug is effective in lowering serum cholesterol levels in heterozygous patients with FH, but not in the homozygotes.[2] Thus, the present data of LDL receptor activity in vitro may provide a reasonable basis for the clinical evaluation of this drug in FH.

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REFERENCES

1. Fredrickson, D. S., J. L. Goldstein, and M. S. Brown. 1978. The familial hyperlipoproteinemias. *In* The Metabolic Basis of Inherited Disease. J. B. Stanbury, J. B. Wyngaarden, and D. S. Fredrickson, editors. McGraw-Hill, Inc., New York. 4th edition. 604-655.
2. Schrott, H. G., J. L. Goldstein, and W. R. Hazzard, M. M. McGoodwin, and A. G. Motulsky. 1972. Familial hypercholesterolemia in a large kindred. Evidence for a monogenic mechanism. *Ann. Intern. Med.* **76:** 711–720.
3. Mabuchi, H., T. Haba, K. Ueda, R. Ueda, R. Tatami, S. Ito, T. Kametane, J. Koizumi, S. Miyamoto, M. Ohta, R. Takeda, T. Takegoshi, and H. Takeshita. 1977. Serum lipids and coronary heart disease in heterozygous familial hypercholesterolemia in the Hokuriku district of Japan. *Atherosclerosis.* **28:** 417–423.
4. Mabuchi, H., R. Tatami, T. Haba, K. Ueda, R. Ueda, T. Kametani, S. Ito, J. Koizumi, M. Oota, S. Miyamoto, R. Takeda, and R. Takeshita. 1978. Homozygous familial hypercholesterolemia in Japan. *Am. J. Med.* **65:** 290–297.
5. Mabuchi, H., R. Tatmi, K. Ueda, R. Ueda, T. Haba, T. Kametani, A. Watanabe, T. Wakasugi, S. Ito, J. Koizumi, M. Ohta, S. Miyamoto, and R. Takeda. 1979. Serum lipid and lipoprotein levels in Japanese patients with familial hypercholesterolemia. *Atherosclerosis.* **32:** 435–444.
6. Brown, M. S., and J. L. Goldstein. 1975. Familial hypercholesterolemia: genetic, biochemical, and pathophysiologic considerations. *Adv. Intern. Med.* **20:** 273–296.
7. Goldstein, J. L., S. E. Dana, G. Y. Brunschede, and M. S. Brown. 1975. Genetic heterogeneity in familial hypercholesterolemia: evidence for two different mutations affecting functions of low density lipoprotein receptor. *Proc. Natl. Acad. Sci. U. S. A.* **72:** 1092–1096.
8. Goldstein, J. L., M. S. Brown, and N. J. Stone. 1977. Genetics of the LDL receptor: evidence that the mutations affecting binding and internalization are allelic. *Cell.* **12:** 629–641.
9. Endo, A., M. Kuroda, and K. Tanzawa. 1976. Competitive inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase by ML-236B and ML-236B fungal metabolites, having hypocholesterolemic activity. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **72:** 323–326.
10. Brown, M. S., J. R. Faust, J. L. Goldstein, I. Kaneko, and A. Endo. 1978. Induction of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in human fibroblasts incubated with compactin (ML-236B), a competitive inhibitor of the reductase. *J. Biol. Chem.* **253:** 1121–1128.
11. Endo, A., Y. Tsujita, M. Yuroda, and K. Tanzawa. 1977. Inhibition of cholesterol synthesis in vitro and in vivo by ML-236A and ML-236B, competitive inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A reductase. *Eur. J. Biochem.* **77:** 31–36.
12. Kaneko, I., Y. Hazama-Shimada, and A. Endo. 1978. Inhibitory effects of lipid metabolism in cultured cells of ML-236B, a potent inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme-A reductase. *Eur. J. Biochem.* **87:** 313–321.
13. Tsujita, Y., M. Kuroda, K. Tanzawa, N. Kitano, and A. Endo. 1979. Hypolipidemic effects in dogs of ML-236B, a competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase. *Atherosclerosis.* **32:** 307–313.
14. Kuroda, M., Y. Tsujita, K. Tanzawa, and A. Endo. 1979. Hypolipidemic effects in monkeys of ML-236B, a competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase. *Lipids.* **14:** 585–589.
15. Yamamoto, A., H. Sudo, and A. Endo. 1980. Therapeutic effects of ML-236B in primary hypercholesterolemia. *Atherosclerosis.* **35:** 259–266.
16. Mabuchi, H., S. Ito, T. Haba, K. Ueda, R. Ueda, R. Tatami, T. Kametani, J. Koizumi, M. Ohta, S. Miyamoto, R. Takeda, and T. Takegoshi. 1977. Discrimination of familial hypercholesterolemia and secondary hypercholesterolemia by Achilles' tendon thickness. *Atherosclerosis.* **28:** 61–68.
17. Mabuchi, H., R. Tatami, T. Haba, K. Ueda, R. Ueda, S. Ito, T. Kametani, J. Koizumi, S. Miyamoto, M. Ohta, R. Takeda, T. Takegoshi, and H. Takeshita. 1978. Achilles tendon thickness and ischemic heart disease in familial hypercholesterolemia. *Metab. Clin. Exp.* **27:** 1672–1679.
18. Havel, R. J., E. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1345–1353.
19. MacFarlane, A. S. 1958. Efficient trace-labelling of proteins with iodine. *Nature (Lond.).* **182:** 53.
20. Shepherd, J., D. K. Bedford, and H. G. Morgan. 1976. Radioiodination of human low density lipoprotein: a comparison of four methods. *Clin. Chim. Acta.* **66:** 97–109.
21. Goldstein, J. L., and M. S. Brown. 1974. Binding and degradation of low density lipoproteins by cultured human fibroblasts: comparison of cells from a normal subject and from a patient with homozygous familial hypercholesterolemia. *J. Biol. Chem.* **249:** 5153–5162.
22. Goldstein, J. L., S. K. Basu, G. Y. Brunschede, and M. S. Brown. 1976. Release of low density lipoprotein from its surface receptor by sulfated glycosaminoglycans. *Cell.* **7:** 85–95.
23. Brown, M. S., S. E. Dana, and J. L. Goldstein. 1974. Regulation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase activity in cultured human fibroblasts: comparison of cells from a normal subject and from a patient with homozygous familial hypercholesterolemia. *J. Biol. Chem.* **249:** 789–796.
24. Shapiro, D. J., J. L. Norstrom, J. J. Mitschelen, V. W. Rodwell, and R. T. Schimke. 1974. Micro assay for 3-hydroxy-3-methylglutaryl-CoA reductase in rat liver and in L-cell fibroblasts. *Biochem. Biophys. Acta.* **370:** 369–377.
25. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
26. Goldstein, J. L., and M. S. Brown. 1973. Familial hypercholesterolemia: identification of a defect in the regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity associated with overproduction of cholesterol. *Proc. Natl. Acad. Sci. U. S. A.* **70:** 2804–2808.
27. Khachadurian, A. K., M. Lipson, and F. S. Kawahara. 1975. Diagnosis of familial hypercholesterolemia by measurement of sterol synthesis in cultured skin fibroblasts. *Atherosclerosis.* **21:** 235–244.
28. Goldstein, J. L., S. E. Dana, and M. S. Brown. 1974. Esterification of low density lipoprotein cholesterol in human fibroblasts and its absence in homozygous familial hypercholesterolemia. *Proc. Natl. Acad. Sci. U. S. A.* **71:** 4288–4292.
29. Goldstein, J. L., and M. S. Brown. 1979. The LDL receptor locus and the genetics of familial hypercholesterolemia. *Ann. Rev. Genet.* **13:** 259–89.