Regulation of 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase Activity and the Esterification of Cholesterol in Human Long Term Lymphoid Cell Lines†

Herbert J. Kayden,* Lynda Hatam, and Nicholas G. Beratis‡

ABSTRACT: The regulation of the rate-controlling enzyme in cholesterol biosynthesis and of the incorporation of [^{14}C]oleate into cholesterol esters were studied in established lymphoid cell lines from normal subjects and compared with that of eight patients with genetic abnormalities of lipid metabolism. The activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase, the rate-controlling enzyme in cholesterol biosynthesis, increases in lymphoid cell lines derived from normal subjects after the culture medium is changed to a lipid deficient medium and reaches peak activity after 48 hr. The addition of whole serum and of low density lipoproteins to cell lines derived from normal subjects suppressed 3-hydroxy-3-methylglutaryl coenzyme A reductase activity by 50%, but failed (almost completely) to suppress the activity in the lymphoid cell lines derived from two patients with homozygous familial hypercholesterolemia. When 7-ketocholesterol was added, the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase was markedly suppressed in both normal and abnormal lymphoid cell lines. Lymphoid cell lines derived from patients presumably heterozygous for familial hypercholesterolemia were difficult to distinguish from normal cells in these studies. The incorporation of [^{14}C]oleate into the fatty acid fraction of cholesteryl esters was stimulated by the addition of the low density lipoproteins to the culture media of the lymphoid cell lines derived from the normal human subjects. The lymphoid cell lines derived from the patients with homozygous familial hypercholesterolemia showed no increase in [^{14}C]oleate incorporation into cholesteryl esters even when a fourfold amount of low density lipoprotein was added to the media; a modest increase in [^{14}C]oleate incorporation was observed in lymphoid cell lines from patients with heterozygous familial hypercholesterolemia. The results of these studies in lymphocyte cell lines are compared with the findings in cultured human fibroblasts obtained from normal subjects and from patients with homozygous familial hypercholesterolemia. Studies of the regulation of cholesterol biosynthesis in the apparently permanent lymphoid cell line maintained in suspension culture offer certain advantages over cultured skin fibroblasts, and, in addition, provide a second tissue for the study of genetic abnormalities from the same patient.

Studies on cultured human fibroblasts have demonstrated that the rate of cholesterol synthesis is proportional to the activity of the rate-limiting enzyme in the cholesterol biosynthetic pathway, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase)[1] (Brown et al., 1973). The observations of Brown, Dana, and Goldstein on the regulation of HMG CoA reductase in cultured fibroblasts from normal subjects indicated that the enzyme was inhibited by cholesterol, and, more particularly, from that lipoprotein-bound cholesterol present in low density lipoproteins (LDL) and very low density lipoproteins (VLDL), suggesting the presence of a receptor for apolipoprotein B on the surface of the cell (Brown et al., 1973). In fibroblasts grown from a skin biopsy from a patient homozygous for the autosomally inherited disorder familial hypercholesterolemia, no suppression of the activity of HMG CoA reductase occurred in the presence of LDL, presumably due to the absence of the receptor for LDL, since cholesterol in a non-lipoprotein form when added to the culture medium did suppress the activity of the enzyme (Brown et al., 1974; Brown and Goldstein, 1974a). In a recent publication Goldstein, Dana, and Brown demonstrated that there was a striking increase in the rate of incorporation of [^{14}C]oleate into the fatty acids of cholesteryl esters when cultured fibroblasts from normal subjects were incubated with LDL; this stimulation did not occur with cultured fibroblasts derived from a patient with homozygous familial hypercholesterolemia (Goldstein et al., 1974).

The present report focuses on human long term lymphoid cell lines obtained from normal subjects and from patients with genetic disorders of lipid metabolism; including two patients with homozygous familial hypercholesterolemia, F.H.(hom); two patients with heterozygous familial hypercholesterolemia, F.H.(het); two patients with abetalipoproteinemia (ABL); and two patients with combined hyperlipidemia (Com. Hyp.). The activity of HMG CoA reductase was measured in growing cells, under conditions of induction and repression of the enzyme, and the results in cells derived from normal and genetically abnormal subjects were compared. The incorporation of oleic acid into cholesteryl esters of the lymphoid cells was studied in the presence

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‡ Present address: Department of Pediatrics, Division of Medical Genetics, Mt. Sinai School of Medicine, New York, N.Y. 10029.

[1] Abbreviations used are: HMG CoA, 3-hydroxy-3-methylglutaryl coenzyme A; [^{14}C]HMG CoA, DL-3-hydroxy-3-methyl[3-^{14}C]glutaryl coenzyme A; F.H.(het), familial hypercholesterolemia heterozygous state; F.H.(hom), familial hypercholesterolemia, homozygous state; ABL, abetalipoproteinemia; Com.Hyp., combined hyperlipidemia; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; LPDS, lipoprotein deficient serum, i.e., infranatant fraction of human plasma centrifuged 40 h in an ultracentrifuge at 170 000*g* at a density of 1.21 g/ml; H.S., human serum; FCS, fetal calf serum; PBS, phosphate-buffered saline.

and absence of low density lipoproteins.

Experimental Procedure

Materials. DL-3-Hydroxy-3-methyl[3-^{14}C]glutaryl coenzyme A (11.7 mCi/mmol), [*methyl*-^{3}H]thymidine, 6.7 Ci/mmol, aquasol, and liquifluor were purchased from the New England Nuclear Corp. Nonradioactive 3-hydroxy-3-methylglutaryl coenzyme A was obtained from P-L Biochemicals, Inc., DL-[2-^{3}H]mevalonic acid lactone (500 mCi/mmol) was purchased from Amersham/Searle Corp. Dithiothreitol, glucose 6-phosphate (monosodium salt), NAD^+ (nicotinamide adenine dinucleotide), DL-mevalonic acid lactone, oleic acid, and bovine serum albumin (fraction V, essentially fatty acid free) were obtained from Sigma Chemical Co. L-[^{14}C]Leucine, 312 mCi/mmol, and 5-cholesten-3-β-ol-7-one (7-ketocholesterol) were purchased from Schwarz/Mann. Cholesteryl oleate was purchased from Applied Science. [1-^{14}C]Oleic acid, 32.5 mCi/mmol, and [1α-T]cholesterol, 7150 mCi/mmol, were obtained from Nuclear Chicago. The detergent Kyro EOB was a gift from Dr. D. H. Hughes, Miami Valley Research Laboratories, Proctor and Gamble Co. Topical thrombin (bovine origin) was purchased from Parke, Davis and Co. Human albumin (fraction V) was obtained from Pentex Biochemicals. Silica gel G thin-layer plates were purchased from Analtech, Inc. Glucose-6-phosphate dehydrogenase, 350 units/mg, was obtained from Boehringer Mannheim. Cholesterol was purchased from Fisher and recrystallized in our laboratory. Falcon flasks, 75 and 25 cm^2, were obtained from Bioquest and RPMI 1640 culture medium was purchased from Associated Biomedic Systems, Inc. Fetal calf serum, glutamine, and penicillin (10 000 units/ml)-streptomycin (10 000 μg/ml) solution were obtained from GIBCO.

Cells. Lymphoid lines were established by a modification of the method of Hirschhorn as follows: 30–50 ml of heparinized blood was collected by venipucture into a plastic syringe. Erythocytes were separated by gravity for 1–2 h, and the supernatant plasma, rich in white blood cells, was drawn off the syringe and centrifuged at 150*g* for 15 min. The cell pellet was disrupted gently and washed once with RPMI 1640 medium, and replicate cultures containing approximately 1–2 $\times$ 10^7 lymphocytes were prepared. Purified phytohemagglutinin (PHA- Burroughs Wellcome MR68 and 69) at a concentration of 1 μg/ml of media was added to each flask, and the flasks were incubated at 37 °C and at 5% CO_2 tension for 48 h. Cultures were then transferred to tubes and centrifuged at 150*g* for 15 min, and the supernatant fractions were removed and discarded. Cell pellets were resuspended in complete media and returned to the original culture flasks. Cultures were fed biweekly until they were established and were then maintained in 25-cm^2 flasks with RPMI medium containing 20% fetal calf serum (FCS), 2 mM glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml) (Beratis and Hirschhorn, 1973). Incubation of the lymphocytes was at 37 °C and in a 5% CO_2 environment. Viability of cell suspensions during labeling experiments was 90–100% as determined by trypan blue exclusion. Growth characteristics were typical of lymphoid cell lines: the cells grow in suspension, form clusters, and double their number every 24–48 h, to a maximum concentration of about 1–2 $\times$ 10^6 cells/ml of media. When examined microscopically they appear to be homogeneous, and have the appearance of immature blast-like transformed cells, similar to PHA stimulated (in vitro) peripheral small lymphocytes (Glade and Hirschhorn, 1970).

For experimental studies, the cells were centrifuged at 365*g* for 10 min, washed once with phosphate-buffered saline (PBS, 0.01 M sodium phosphate–0.15 M NaCl, pH 7.5), suspended at a density of 0.9–1.5 $\times$ 10^6 cells/ml in RPMI 1640 medium containing no FCS or HS but containing 10% lipoprotein-deficient serum (LPDS) at a final protein concentration of 5 mg/ml, and divided into 25-cm^2 flasks in 10-ml volumes. After 48-h growth, additions were made as indicated in the legends. All materials were sterilized through a Millipore Swinnex filter unit, 0.45 μ, or a 0.2-μ nalgene filter unit before additon to culture flasks.

Measurement of DNA and Protein Synthesis. The rates of DNA and protein synthesis were determined by incubation at 37 °C of 1 ml of cell suspension (10^6 cells) with 4 μCi of [*methyl*-^{3}H]thymidine, 6.7 mCi/mmol, and 1 μCi of L-[^{14}C]leucine, 312 mCi/mmol. Final specific activity of leucine in the medium was 2.62 μCi/mmol. After 20 h, the cells were centrifuged at 365*g* for 10 min and washed once with ice-cold PBS and the wash was discarded. The protein was then precipitated with 4 ml of ice-cold 5% trichloroacetic acid containing 130 mg of unlabeled leucine per l. After standing 30 min or overnight with refrigeration, the precipitate was collected on glass fiber filters by vacuum filtration. The filters were washed three times with 3 ml of 5% trichloroacetic acid, air dried, and placed in counting vials. Distilled water (1 ml) and 15 ml of aquasol were added to each vial which was then counted in a Packard Model 3375 liquid scintillation counter with double label settings; automatic external standardization was used to calculate DPM after quench correction calculations.

Lipoproteins. The lipoproteins were prepared from normal human plasma (collected in ACD vacuum bag solutions) by ultracentrifugation in a Beckman Model L ultracentrifuge using standard techniques and KBr for density adjustments (Havel et al., 1955). The plasma was fractionated as follows: an initial centrifugation at density of 1.005 g/ml at 100 000*g* for 20 h yielded a supernatant fraction, the very low density lipoprotein or VLDL. The density of the infranatant was adjusted to 1.019 g/ml and centrifuged for 20 h at 100 000*g*; the supernatant portion was removed and discarded. The density of the infranatant fraction was adjusted to 1.063 g/ml, centrifuged for 20 h at 100 000*g*, and separated into a supernatant and infranatant fraction. The supernatant fraction was the low density lipoprotein fraction, or LDL. The density of the infranatant fraction was adjusted to 1.21 g/ml and centrifuged for 40 h at 170 000*g*; the supernatant fraction (of density 1.063–1.21 g/ml) is the high density lipoprotein fraction, HDL; the infranatant fraction (>1.21 g/ml) is the lipoprotein deficient plasma. All fractions were dialyzed 36–48 h at 4 °C against 0.15 M NaCl–0.3 mM EDTA (pH 7.4). The density of the dialyzed HDL fraction was then adjusted to 1.063 g/ml, and the solution centrifuged again for 24 h at 100 000*g*; the supernatant portion was discarded and the infranatant fraction again dialyzed for 48 h; this recentrifuged preparation of HDL was used in additions to culture media. The purity of the LDL and HDL fractions was confirmed by immunoelectrophoresis and reactions with specific antisera in Ouchterlony double diffusion agarose gels. No contamination by other lipoprotein fractions was found in HDL. The lipoprotein deficient plasma (d > 1.21 g/ml) was incubated 10 min at room temperature in the presence of topical thrombin (20 units/ml of plasma) and then centrifuged at 78 000*g* for 2 h at 4 °C: this is the lipoprotein deficient serum, LPDS. The cholesterol content of each fraction was

determined by the method of Franey and Amador (Franey and Amador, 1968); the protein content was assayed according to Lowry et al. (Lowry et al., 1951).

Steroids. A 50-μg/ml solution of 7-ketocholesterol was prepared by evaporation of the sterol from an ethanol solution (3 mg/ml) and the addition of 0.15 M NaCl (pH 7.0) containing 5% human albumin. This mixture was sonicated 1 min in a Branson sonifier with the output control at tap 5 and diluted tenfold by addition to culture media. Final concentration of the 7-ketocholesterol was 5 μg/ml of medium. Non-lipoprotein cholesterol, purified by recrystallization, was added to the culture medium in 0.1 ml of ethanol. The purity of both 7-ketocholesterol and the recrystallized cholesterol was greater than 98% as determined by thin-layer chromatography and gas–liquid chromatography.

Heat-Inactivated Serum. Normal human plasma collected in ACD solution was incubated at 56 °C for 1 hr. The resulting serum was centrifuged at 80 000*g* for 2 h and the supernatant fraction filtered through a 0.2-μ nalgene filter unit before addition to culture medium.

Measurement of HMG CoA Reductase. Cell cultures were harvested by centrifugation at 365*g* for 10 min; cell pellets were washed three times with PBS and were stored frozen (−80 °C) until used.

Cell extracts were prepared and assayed according to the method of Brown, Dana, and Goldstein which involved dissolving the thawed pellet in 0.1–0.3 ml of 50 mM potassium phosphate buffer (pH 7.4) containing 5 mM EDTA, 5 mM dithiothreitol, 0.2 M KCl, and 0.25% Kyro EOB (Brown et al., 1973). The homogeneous suspension formed was incubated 10 min at 37 °C and centrifuged at 10 000*g* for 1 min, and the supernatant was used for the assay. Protein content was also determined.

Aliquots of cell extract containing 0.02–0.1 mg of protein were incubated 10 min at 37 °C in a total volume of 0.19 ml containing 20 mM glucose 6-phosphate, 2.5 mM NAD^+, 5 mM dithiothreitol, 100 mM potassium phosphate, and 0.7 unit of glucose-6-phosphate dehydrogenase.

Each extract was analyzed in duplicate or at two different protein concentrations while control samples were carried out for each analysis by adding HCl prior to the addition of substrate. The reaction was started by the addition of 0.01 ml of [^{14}C]HMG CoA, (5.08 mCi/mmol) at a final concentration of 30 μM. [^{14}C]HMG CoA, 11.7 mCi/mmol, was diluted with unlabeled HMG CoA to the desired specific activity. The amount of free CoA present was less than 5% as determined by a modification of the method of Ellman (Ellman, 1959). After 2 h of incubation at 37 °C, the reaction was stopped by the addition of 0.02 ml of 5 N HCl. An internal standard of 110 000 dpm of [^{3}H]mevalonolactone, (20 μCi/mmol) was added and the tubes were incubated 15 min at 37 °C to assure lactonization of the mevalonic acid. The reaction mixture was extracted three times with 2 ml of diethyl ether in the presence of sodium sulfate and the extracts were combined, evaporated under N_2, and applied to silica gel G thin-layer plates with acetone. Unlabeled mevalonolactone (0.2 mg) was spotted as carrier and the plates were developed in benzene–acetone 1:1. The mevalonolactone was located by iodine vapor and the spots were scraped into a counting vial and counted in aquasol. The recovery of the mevalonolactone averaged 75%. The amount of enzyme protein that catalyzes the formation of 1 pmol of mevalonate/min under these assay conditions corresponds to 1 unit of HMG CoA reductase.

Preparation of [^{14}C]Oleate. [1-^{14}C]Oleic acid in benzene and nonradioactive oleic acid in benzene (both 98% pure as determined by thin-layer chromatography) were combined to a specific activity of 1 μCi/μmol and evaporated to dryness under N_2, 1 ml of 95% ethanol was added, and the solution was titrated to a phenolphthalein end point (pH 8.5) with 1 M NaOH. This solution was reevaporated, 10% bovine serum albumin in 0.9% NaCl (pH 7.5) was added, and the mixture was incubated 1 hr with shaking at 37 °C. The final concentration of the sodium oleate was 5.5 mM.

Table I: Age, Sex, Serum Cholesterol, and Triglyceride Values, and Clinical Diagnosis of Eight Patients from Whom Lymphoid Cell Lines were Derived.

Patient	Age	Sex	Cholesterol (mg/100 ml)	Triglycerides (mg/100 ml)	Diagnosis
O.C	18	F	685	<100	F.H.(hom)
A.C.	23	F	656	136	F.H.(hom)
J.Z.	43	M	480	99	F.H.(het)
A.G.	16	F	384	56	F.H.(het)
J.S.	32	M	584	1733	Com.Hyp.
A.S.	36	F	460	2434	Com.Hyp.
M.S.	22	M	37	6	ABL
A.M.V.	18	F	32	2	ABL

Measurement of [^{14}C]Oleate Incorporation. Aliquots of the [^{14}C]oleate–albumin complex (0.2–0.4 ml) were added to the culture flasks to give the desired concentration. After 3–6 h of incubation, the cells were harvested by centrifugation at 365*g* for 10 min and the supernatant media was removed. The cells were washed twice with ice-cold PBS and suspended in 0.25 ml of cold PBS buffer. Aliquots (0.01 ml) were removed for protein determinations and 0.2 ml of cell suspension was added to 6 ml of chloroform–methanol (2:1) containing 100 000 dpm of [^{3}H]cholesterol, 50 μg of cholesterol, and 50 μg of cholesterol oleate. This mixture was shaken vigorously, then allowed to stand at least 30 min. After the addition of 6 ml of 0.03 N HCl, the mixture was inverted slowly three times and allowed to stand at room temperature overnight for separation of phases. The lower phase was evaporated to dryness, applied to silica gel G thin-layer plates with chloroform, and developed in petroleum ether–diethyl ether–acetic acid, 70:30:0.4. Lipid spots were located with iodine vapor, scraped into counting vials, and counted in liquifluor toluene scintillation fluid. Automatic external standardization was used for quench correction of the double label counting. All results are expressed as picomoles of [^{14}C]oleate incorporated per hour per milligram of protein and are corrected for the recovery of the internal standard, [^{3}H]cholesterol, which averaged 75%.

Results

Lymphoid cell lines were established in long term culture from five normal subjects, and from eight patients: two F.H.(hom), two F.H.(het), two ABL, and two Com.Hyp. (Table I). The two F.H.(hom) patients had markedly elevated serum cholesterol levels since childhood, had extensive xanthomata and cardiovascular disease, and fibroblasts in cultures from skin biopsies have been shown to be receptor negative (Goldstein et al., 1975). The two F.H.(het) patients were unrelated and were members of families in which hypercholesterolemia and characteristic features of this disorder were present for three generations. The two

Table II: Effect of Lipoprotein Fractions LDL, VLDL, and HDL and of 7-Ketocholesterol on the Activity of HMG CoA Reductase in Lymphoid Cell Lines from Normal Subjects and from Patients with Genetic Abnormalities.[a]

Additions	Normal Subjects A	B	C	D	E	Mean (pmol per min per mg of protein)	Patients F.H.(hom) 1	2	F.H.(het) 1	2	ABL 1	Com.Hyp. 1
None (control)	95	75	135	123	126	(113)	104	90	100	135	147	80
						% of control						
LDL	26	29	70	40	59	(45)	87	89	51	47	57	40
VLDL	70	63	70	71	70	(70)	83	100	89	65	79	66
HDL	64	69	86	80	53	(70)	85	110	86	76	76	81
7-Keto	19	11	30	32	19	(22)	36	30	30	10	20	33

[a] Lymphoid cell lines were derived from five unrelated normal subjects (A–E), from two unrelated patients with F.H.(hom), two unrelated patients with F.H.(het), one patient with ABL, and one patient with Com.Hyp. The activity of HMG CoA reductase of cells grown for 48 h in LPDS media is given in pmol per min per mg of protein and serves as the control value. The effect of the addition of four separate compounds to the media for 16 h of additional incubation is expressed as percent of the control value. The cholesterol content was adjusted for each addition to be 100 μg/ml of media. 7-Ketocholesterol was added at a concentration of 5.6 μg/ml of media. The mean value for the cultures from the normal subjects is given in parentheses.

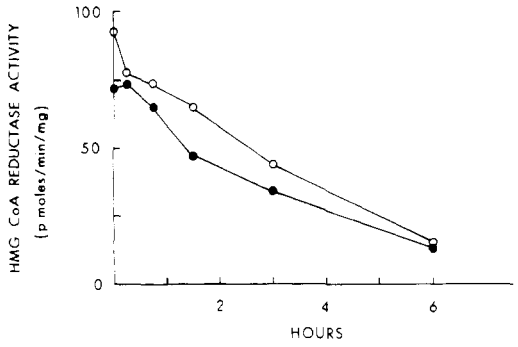

FIGURE 1: Time course of suppression of HMG CoA reductase activity by 7-ketocholesterol. Cells (normal, ●, F.H.(hom), ○) were grown for 48 h in flasks containing 20 ml of LPDS medium/flask. 7-Ketocholesterol was prepared as described and 2.5 ml was added to each flask. Final concentration of 7-ketocholesterol in the media was 5.6 μg/ml. Cells were harvested at the indicated intervals and assayed as described.

patients with ABL were classical examples of that disorder and the two patients with combined hyperlipemia had markedly elevated serum triglyceride and cholesterol levels, but insufficient family data were available to make any genetic diagnosis.

The average value of HMG CoA reductase in lymphoid cell lines derived from normal subjects and maintained in media that contained 10 to 20% FCS or 1% HS, 24 h after the addition of the appropriate fresh media, was 50 pmol per min per mg of protein. In normal cell lines grown in LPDS media, HMG CoA reductase activity increased and reached peak levels 48 h after lipoprotein deprivation. Peak activity of HMG CoA reductase averaged 113 pmol per min per mg of protein in five cell lines derived from normal subjects. Cell growth was not impaired by lipoprotein deprivation since no diminution in the rate of incorporation of labeled thymidine into DNA or leucine into protein was observed, and cell viability, monitored by trypan blue exclusion, was not affected. In some experiments the incubation in LPDS media was continued for a total of 64 h and no significant change in HMG CoA reductase activity, thymidine or leucine incorporation, or in cell viability was found.

Table II presents data obtained from five cultures derived from five normal subjects (A–E), from two unrelated patients with F.H.(hom), two unrelated patients with F.H.(het), one patient with ABL, and one patient with Com.Hyp.

Control HMG CoA reductase activity of cultures from five normal subjects (grown in LPDS) media ranged between 75 and 135 pmol per min per mg of protein and averaged 113 pmol per min per mg. The activity in cultures derived from the six patients was in the same range (80–147 pmol per min per mg of protein). The addition of LDL (containing 100 μg of cholesterol/ml of media) to the cultures of five normal subjects caused a sharp fall in reductase activity to an average of 45% of the control value (range 26–70%). In the two cultures derived from the two F.H.(hom) patients, the addition of LDL had minimal effect, 87 and 89% of control values. The effect of the addition of LDL to cultures from the other four patients was similar to that seen in cultures from normal subjects, 40–57% of control values. VLDL and HDL added to cultures of normal subjects caused modest suppression of HMG CoA reductase activity (53–86% of control value). VLDL and HDL did not suppress significantly the activity of the reductase when added to cultures from the patients. The addition of the oxygenated analogue of cholesterol, 7-ketocholesterol, markedly suppressed HMG CoA reductase activity in the cultures derived from normal subjects and from the six patients. The rate and extent of suppression of HMG CoA reductase activity in a culture from one normal subject and from one F.H.(hom) are shown in Figure 1. A parallel response is noted in the two experiments with the final values at the end of 6 h of incubation representing 17% of control in normal cultures and 17% of control in cultures from F.H.(hom).

The effect of increasing concentrations of LDL (as microgram of cholesterol per milliliter of media) on the activity of HMG CoA reductase of two cultures from normal subjects, and of two lines established from a patient with F.H.(hom) was compared and is shown in Figure 2. Enzyme activity of normal cultures was suppressed to levels below 50 pmol per min per mg of protein by as little as 12.5 μg of LDL cholesterol per ml of media and maximal suppression exceeded 75% with 100 μg/ml of media; in contrast, the activity of HMG CoA reductase in the two cultures derived from the F.H.(hom) patient was almost en-

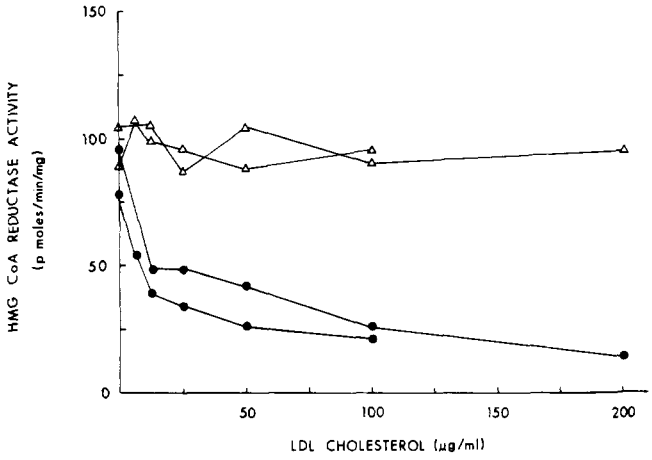

FIGURE 2: Comparison of the effect of varying concentrations of LDL cholesterol on the HMG CoA reductase activity of normal and hypercholesterolemic lymphoid lines. Cells were incubated in LPDS media as described under Experimental Procedure. The low density lipoprotein (LDL) isolated as described was diluted with 0.01 M sodium phosphate–0.15 M NaCl (pH 7.5) so that 1 ml contained the necessary amount of cholesterol to give the desired concentration in 10 ml of culture media. After 48-h growth in LPDS media, the LDL was added and incubation continued for 17 h. Cells were harvested and assayed for HMG CoA reductase activity as described under Experimental Procedure. All values represent the mean of duplicate flasks. The results of two different experiments each of the normal cells (●), and of the cells from a homozygote for familial hypercholesterolemia (△) are shown.

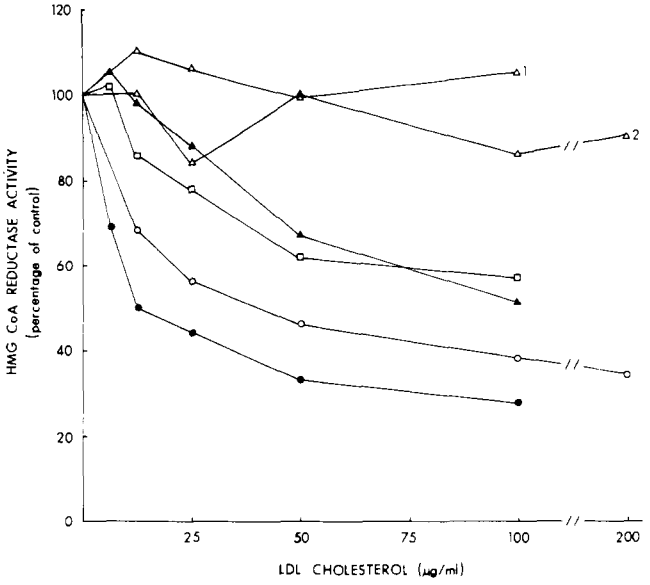

FIGURE 3: Effect of varying concentrations of LDL cholesterol on HMG CoA reductase activity of normal cells and cells from patients with four different disorders of lipid metabolism. Cell lines were established as described under Experimental Procedure from normal subjects (●), a patient with ABL (□), a patient with Com.Hyp. (○), a patient with F.H.(het) (▲) and a patient with F.H.(hom) (△). Cells were grown 48 h in 10 ml of media containing LPDS (5 mg/ml). Additions of 1 ml of LDL were made giving the desired cholesterol concentration in the media. After 16 h, cells were harvested and assayed for HMG CoA reductase as described under Experimental Procedure. Results are expressed as percentage of activity of the cells grown in LPDS media with LDL. These control activities (pmol per min per mg of protein) were (●) 78; (□) 147; (○) 85; (▲) 100; (Δ_1) 89; (Δ_2) 104. All values represent the mean of duplicate flasks and Δ_1 and Δ_2 represent two different lines established from the same patient.

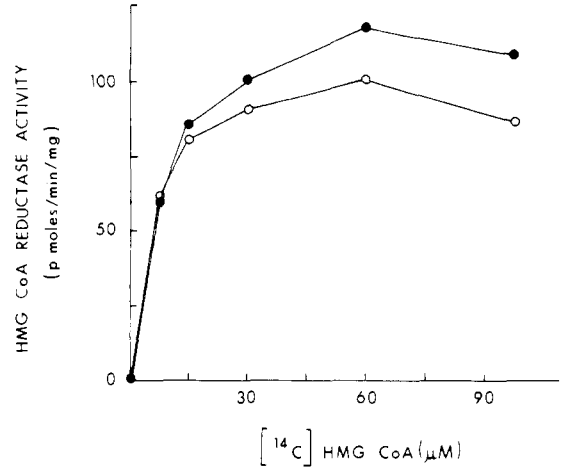

FIGURE 4: The effect of varying concentrations of [^{14}C]HMG CoA on HMG CoA reductase activity. Normal (●) and F.H.(hom) (○) cells were harvested after 64-h growth in LPDS media. Extracts were prepared and assayed as described using the indicated amount of [^{14}C]HMG CoA. Each value represents the mean of duplicate flasks.

tirely unaffected by the addition of 100 or 200 μg/ml of media of LDL cholesterol.

The effect of increasing concentrations of LDL cholesterol added to the growth media, upon the activity of HMG CoA reductase in lymphocyte cultures obtained from a patient with F.H.(het), a patient with ABL, a patient with Com.Hyp., from a normal subject, and in two separate cultures from a patient with F.H.(hom) is shown in Figure 3. As LDL cholesterol concentration was increased, the initial value of HMG CoA reductase activity (expressed as 100%) was reduced in the culture from the normal subject and in the cultures from the patients with ABL and Com.Hyp. (Figures 2 and 3). In the two cultures from the patient with F.H.(hom) there was no effect upon the activity of the reductase even when the concentration of cholesterol was increased to 200 μg/ml of media of LDL; at this level of addition HMG CoA reductase activity was 96 pmol per min per mg of protein compared with the control value of 104 pmol per min per mg of protein. The activity of HMG CoA reductase in the culture from the patient with F.H.(het) showed little change with the addition of low levels of LDL cholesterol to the media, but at higher concentrations was not different from cultures from normal subjects or other patients.

In the above studies the concentration of HMG CoA used was 30 μM. This substrate concentration was not rate limiting, as shown in the next figure. In Figure 4, HMG CoA concentration was varied from 7.5 to 90 μM and the enzyme activity measured in cell extracts from cultures of a normal subject and of a patient with F.H.(hom).

The stimulatory effect of LDL upon cholesterol esterification was studied in lymphoid cell lines. The cells were incubated in the presence of sodium [^{14}C]oleate and the incorporation of the radioactive fatty acid into ^{14}C-labeled cholesteryl esters was studied as a function of increasing concentration of LDL and of the addition of other lipoprotein fractions to the media. To determine the optimal concentrations of oleate for the study of oleate incorporation into cholesteryl esters, the study in Figure 5 was carried out. In each of the two cultures, one from a normal subject and one from a patient with F.H.(het), 0.1 mM oleate gave maximal activity of incorporation into cholesteryl ester in the presence of 200 μg of LDL cholesterol/ml of medium. The linearity of the cholesterol esterification reaction was established by studying two cell lines, one from a normal

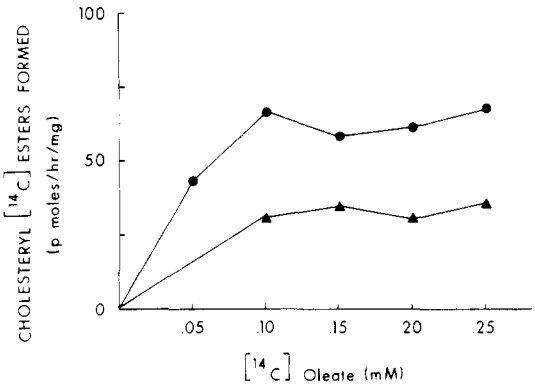

FIGURE 5: Effect of oleate concentration on the incorporation of [^{14}C]oleate into cholesteryl esters. Cells from a normal subject (●), and a patient with F.H.(het) (▲), were established as described under Experimental Procedure and maintained in media containing FCS. On day 1 this media was removed and replaced as described with media containing LPDS (5 mg/ml). After 48 h (day 3), LDL was added at a final cholesterol concentration of 200 μg/ml and incubation continued for 17 h. The [^{14}C]oleate-albumin complex (1 μCi/μmol) was added (day 4) to 10 ml of culture media giving the indicated concentration. Cells were harvested after 6 h and the cellular content of cholesteryl ^{14}C esters was determined as described under Experimental Procedure.

subject and one from a patient with ABL over a 6-h period in the presence of 0.1 mM [^{14}C]oleate and at a concentration of 200 μg/ml of media of LDL cholesterol. The incorporation into ^{14}C-labeled cholesteryl esters was linear for the 6-h period; in the experimental studies most of the incubations were carried out for only 4 h.

The rate of incorporation of oleate into cholesteryl esters as a function of LDL cholesterol concentration was studied in five cell lines: two normal cell lines, and one cell line each from a patient with Com.Hyp. and one with F.H.(hom). In each instance maximum incorporation appeared at a concentration of 50 μg/ml of LDL cholesterol, and no significant change occurred thereafter despite a fourfold increase in concentration in LDL. But although there was marked stimulation (approaching tenfold) of incorporation of [^{14}C]oleate into ^{14}C-labeled cholesteryl esters in the normal cell lines, and two- to fivefold stimulation in the cell lines of ABL and Com.Hyp., respectively, there was no stimulation of incorporation in the cell line derived from F.H.(hom).

To establish the specificity of the stimulatory effect of LDL upon cholesterol esterification, the effects of HDL, VLDL, and LDL were compared in cultures obtained from five normal subjects, two patients with F.H.(hom), two patients with F.H.(het), two patients with ABL, and two patients with Com.Hyp. In certain studies free cholesterol was added to the culture media at a concentration of 100 μg/ml of media. The data from the experiments are presented in Table III. Control values ranged from 13 to 61 pmol per mg of protein per hr (mean 26.6 pmol per mg of protein per hr) in cultures derived from normal subjects; the control values in cultures derived from patients with the genetic abnormalities were in the same range. The addition of LDL at a concentration of 100 μg/ml of media markedly stimulated incorporation of [^{14}C]oleate into ^{14}C-labeled cholesteryl esters to values of from 60 to 182 pmol per mg of protein per h (mean 138.4 pmol per ml of protein per h). The addition of LDL to the cultures derived from the two patients with F.H.(hom) showed no stimulation in formation of ^{14}C-labeled cholesteryl esters; the two cultures from F.H.(het) showed minimal stimulation, while the cultures from the two patients with ABL and Com.Hyp. responded in a manner similar to the cultures derived from normal subjects. VLDL and HDL were without effect in all the cultures of both normal and abnormal patients. When free cholesterol (100 μg/ml of media) was added to the cultures there was a marked increase in esterification in cultures derived from normal subjects and in the two cultures from F.H.(hom).

Discussion

These studies establish that human long term lymphoid cell lines can be used to study the regulation of cholesterol biosynthesis, and that genetic abnormalities in this regulation are expressed in lymphoid cell lines. A series of publications on the regulation of cholesterol biosynthesis in cultured fibroblasts derived from skin biopsies of normal and genetically abnormal humans provided useful background for methodology and data for a comparison of the two cell types (Brown et al., 1973, 1974; Brown and Goldstein, 1974a; Goldstein et al., 1974).

The activity of HMG CoA reductase in lymphoid cell lines derived from normal subjects, measured in rapidly growing cells maintained in suspension in optimal growth media, averaged 50 pmol per min per mg of protein, a value about ten times higher than the specific activity reported in fibroblasts from normal human subjects maintained in culture under similar optimal conditions (Brown et al., 1973). This difference in enzyme activity may be due to the faster rate of growth of lymphocytes in suspension culture in comparison with fibroblasts grown in monolayers. When the culture medium was changed to contain LPDS the activity of HMG CoA reductase in lymphoid cell lines from normal subjects was increased greater than twofold (to an average value of 113 pmol per min per mg of protein); when the culture medium for fibroblasts in culture was similarly altered, the increase in reductase activity was 20–30-fold, to values comparable to the activity of lymphoid cell lines. The activity of HMG CoA reductase of the normal lymphoid cell lines and of lymphoid cell lines derived from genetically abnormal patients was approximately the same after the cells had been grown in deprived media, containing LPDS, for 48 h.

The addition of various lipoprotein fractions to the LPDS culture media for an additional 16–18-h period of incubation resulted in suppression of HMG CoA reductase activity in normal cell lines; activity was suppressed by more than 50% when LDL was added. HDL and VLDL suppressed activity by approximately 30%. In contrast to these observations in normal cultures the lymphoid lines from the two patients with F.H.(hom) showed no suppression of enzyme activity when either LDL, HDL, or VLDL was returned to the culture media; however, lymphoid cultures from other genetically abnormal human subjects showed degrees of suppression of HMG CoA reductase activity similar to normal cultures. The failure of suppression of reductase activity in F.H.(hom) cell lines by LDL parallels the observations of Brown, Dana, and Goldstein in cultured fibroblasts from patients with this genetic disorder (Brown et al., 1974). These authors reported that HMG CoA reductase activity in cultured fibroblasts from F.H.(het) showed a response intermediate between cultures from normal subjects and from F.H.(hom); our observations of HMG CoA reductase activity in lymphoid cell lines from F.H.(het) patients have not clearly distinguished these lines from the lymphoid cell lines from normal subjects. HMG CoA reductase activity in all lymphoid cultures was promptly and markedly sup-

Table III: Effect of Lipoprotein Fractions LDL, VLDL, and HDL and of Free Cholesterol upon the Incorporation of [^{14}C]Oleate into Cholesteryl ^{14}C Esters in Lymphoid Cell Lines Derived from Normal Subjects and from Patients with Genetic Abnormalities.[a]

	Normals						Patients							
							F.H.(hom)		F.H.(het)		ABL		Com.Hyp.	
Additions	A	B	C	D	E	Mean	1	2	1	2	1	2	1	2
None	38	24	13	32	26	(26.6)	51	46	50	61	53	16	27	19
LDL	182	60	160	131	159	(138.4)	54	49	68	77	109	177	88	122
VLDL	26	16	21		37	(25)	45	55	58	81			32	17
HDL	36	18	16		42	(28)	33	29	42	12				
Free Chol.	270			118			250	246						

[a] Lymphoid cell lines were derived from five unrelated normal subjects (A–E) and from two unrelated patients with F.H.(hom), two with F.H.(het), two with ABL, and two with Com.Hyp. Cells were grown for 48 h in LPDS media; one set of flasks for each study was incubated for an additional 17-h period prior to the [^{14}C]oleate addition. [^{14}C]Oleate (0.1 mM) was added, and the extent of incorporation per hour was measured at the end of 6 h. The effect of four separate compounds added to the culture media for 17 h of further incubation, each in a final concentration of 100 μg/cholesterol per ml of media, was measured by adding [^{14}C]oleate (0.1 mM) for 4 h of incubation and measuring the rate of incorporation in pmol per mg per h. Results are the mean values of duplicate flasks. Mean values for the normal subjects are given in parentheses.

pressed when 7-ketocholesterol was added to the culture media for 6 h. Similar observations have been made in cultures of fibroblasts derived from human skin biopsies (Brown and Goldstein, 1974b).

The incorporation of radioactive oleic acid into ^{14}C-labeled cholesteryl esters of normal lymphoid cells in culture was significantly stimulated by the addition of LDL to LPDS media; while VLDL and HDL had no stimulatory effect. Lymphoid cell lines from two patients with Com.Hyp. and from two patients with ABL responded to LDL in the same manner as did normal cultures. However, lymphoid cell lines from two patients with F.H.(hom) showed no increase in the rate of incorporation when either LDL, VLDL, or HDL was added to the growth media, while the lymphoid cell cultures from two patients with F.H.(het) showed a small increase in the rate of incorporation of oleic acid into cholesteryl esters when LDL was added to the culture medium, and virtually no change when either HDL or VLDL was added. These findings in lymphoid cell cultures are qualitatively similar to the changes found in the rate of incorporation of oleic acid into cholesteryl esters of fibroblasts in culture. Maximal stimulation of incorporation of oleic acid of fibroblasts from normal subjects was greater than that of lymphoid cell lines. Virtually no stimulation occurred in fibroblast cultures from patients with F.H.(hom). The level in normal fibroblasts at peak rate of incorporation approached 3 pmol per mg of protein per h, a value almost 20 times greater than the values in the lymphoid cell lines (Goldstein et al., 1974).

Lymphoid cell lines and fibroblasts in culture respond to changes in the growth media by altering the rate of cholesterol biosynthesis, as measured by the activity of HMG CoA reductase. The mechanism whereby this control is exerted has been shown in human fibroblasts in culture to be related to the rate of synthesis of the enzyme (Brown et al., 1974). This control has been postulated to be mediated by receptors for LDL on the surface of the fibroblast as reported by Goldstein and Brown (Goldstein and Brown, 1973, 1974). Their studies on receptors, carried out by binding experiments with radioactive LDL, indicated that enzymatic degradation of the protein of LDL takes place, and that free cholesterol enters the cell and undergoes esterification into cholesteryl ester which presumably serves as the feedback inhibitor for HMG CoA reductase. The absence of these receptors in fibroblasts from F.H.(hom) patients, and a reduced number of receptors for LDL in fibroblasts from F.H.(het) are considered to be the cardinal genetic abnormalities in fibroblasts derived from these patients (Goldstein and Brown, 1974).

The demonstration that lymphoid cell lines from normal subjects respond with a decreased activity of HMG CoA reductase to changes in growth media involving the addition of LDL implies that a similar receptor might be present on their cell surface. Studies of such receptors and the estimation of the number of binding sites are in progress. It was not possible to clearly distinguish lymphoid cell lines derived from normal and F.H.(het) by measuring HMG CoA reductase activity under several experimental conditions. By contrast, changes in the rate of incorporation of [^{14}C]oleic acid into ^{14}C-labeled cholesteryl esters, in response to changes in the concentration of LDL in the media demonstrated that differences between normal cell lines and those derived from F.H.(het) could be determined. This parameter of study appears therefore to be more sensitive in reflecting genetic differences than the changes in HMG CoA reductase activity in the lymphoid lines; additional experiments are in process to elucidate the reason for these differences. In addition to the different rates of growth of lymphoid cells and fibroblasts, the greater area of cell surface in contact with the media of cells growing in suspension in comparison with cells grown in monolayer may contribute to the observed differences in response. There may also be a difference in the nature or number of the postulated receptors on the lymphocyte compared with the fibroblast. One possible example that such differences exist has been reported by Brannan, Goldstein, and Brown (Brannan et al., 1975). They observed that the activity of HMG CoA reductase measured in hair roots in normal individuals does not change, despite manipulations of plasma cholesterol levels by medications that strikingly alter the activity of the enzyme in human liver. Furthermore, the same study could not demonstrate any difference in the activity of the reductase in the hair roots of normal subjects or in genetically abnormal patients including those with F.H.(hom).

Evidence has been presented that abnormal induction of HMG CoA reductase activity occurs in leukocytes from patients with F.H.(het) (Fogelman et al., 1975). In short term incubation periods of 6 h, peripheral leukocytes grown in

lipid-free media showed increased sterol biosynthesis from acetate in comparison with the rate of biosynthesis when leukocytes were incubated in medium containing full serum. The utilization of acetate by leukocytes of F.H.(het) patients, grown in media containing lipid-free media, increased threefold, while leukocytes from normal subjects increased only twofold. The increased acetate utilization was correlated with a rise in HMG CoA reductase activity, attesting to the induction of enzyme activity. This method of study, however, is limited by the short survival time of peripheral blood leukocytes.

The vigorous proliferation and apparent permanence of the long term lymphoid cell lines make them suitable for detailed in vitro studies of lipid metabolism in normal and genetically abnormal individuals. As a second cell type they offer an advantage over cultured skin fibroblasts, especially in studies where large volumes of cellular material are required.

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