Rapid Identification of Smith-Lemli-Opitz Syndrome Homozygotes and Heterozygotes (Carriers) by Measurement of Deficient 7-Dehydrocholesterol-Δ^7-Reductase Activity in Fibroblasts

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To extend the enzyme deficiency in Smith-Lemli-Opitz syndrome (SLOS) to extrahepatic tissues, 7-dehydrocholesterol-Δ^7-reductase activity was measured in fibroblasts from 10 controls, five SLOS homozygotes, and five obligate heterozygotes. In cells grown almost to confluence in cholesterol-containing medium (4 mg/dL), the conversion of [1,2-^{3}H]7-dehydrocholesterol to cholesterol (7-dehydrocholesterol-Δ^7-reductase activity) was 3.8 times higher in control than in homozygote cells and 2.2 times higher than in heterozygote cells. After 24 hours' exposure of the fibroblasts to cholesterol-deficient medium supplemented with lovastatin, 7-dehydrocholesterol-Δ^7-reductase activity increased twofold in controls, but did not change significantly in either heterozygous or homozygous cells. In contrast, the activities of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and lathosterol 5-dehydrogenase, two key enzymes that precede 7-dehydrocholesterol-Δ^7-reductase in the cholesterol biosynthetic pathway, and low-density lipoprotein (LDL) receptor–mediated binding were equal in control, homozygote, and heterozygote fibroblasts. Further, HMG-CoA reductase activity and LDL receptor–mediated binding increased after exposure of the cells to cholesterol-deficient medium. Fibroblast cholesterol concentrations were approximately equal, although homozygote cells contained 30 times more 7-dehydrocholesterol. Thus, markedly reduced 7-dehydrocholesterol-Δ^7-reductase activity that cannot be upregulated after exposure of the cells to cholesterol-deficient medium is diagnostic for the biochemical defect in SLOS. Significantly reduced enzyme activity between the levels in controls and homozygotes without accumulation of 7-dehydrocholesterol in fibroblasts identified heterozygotes.

ACCORDING TO CURRENT KNOWLEDGE, cholesterol is essential in every mammalian cell.[1] It is incorporated into membranes to provide cell structure and is the sole precursor for bile acids and steroid hormones. Approximately 25% of myelin in the central and peripheral nervous systems is composed of cholesterol.[2]

The cholesterol biosynthetic pathway (Fig 1) begins with mevalonic acid that is formed from 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA). This reaction is catalyzed by HMG-CoA reductase, which is under negative-feedback control by dietary cholesterol and hepatic bile acid flux and is considered rate-controling for cholesterol biosynthesis.[3,4] Six molecules of mevalonic acid are condensed to form squalene, which is cyclized to yield lanosterol, the first sterol in the pathway that contains 30 carbons. To form cholesterol, three carbons are eliminated from lanosterol, and the double bond located at C-8(9) is transferred to C-5(6) in ring B.[5]

Recently, we have discovered a major abnormality in cholesterol biosynthesis in Smith-Lemli-Opitz syndrome (SLOS). In this recessively inherited birth disorder, tissues showed reduced cholesterol concentrations and large quantities of the precursor 7-dehydrocholesterol and its derivatives.[6-8] These findings suggested that the final reaction in the cholesterol biosynthetic pathway (Fig 1) involving conversion of 7-dehydrocholesterol to cholesterol that is catalyzed by the microsomal enzyme 7-dehydrocholesterol-Δ^7-reductase was blocked.[8,9] In support, we reported that 7-dehydrocholesterol-Δ^7-reductase activity was significantly reduced in the liver of four SLOS homozygotes, and suggested that 7-dehydrocholesterol-Δ^7-reductase was inherited abnormally.[10] Since liver is rarely available, and to extend the enzyme diagnosis to extrahepatic tissues, we investigated cholesterol biosynthesis in fibroblasts grown almost to confluence in 10% fetal bovine serum (FBS) containing cholesterol (4 mg/dL) and after exposure of these cells for 24 hours to cholesterol-deficient medium (0.02 mg/dL) supplemented with lovastatin to stimulate biosynthesis. Previously, Honda et al[9] and Lund et al[11] measured the conversion of [^{3}H]lathosterol, the precursor that immediately precedes 7-dehydrocholesterol, to cholesterol in fibroblasts from SLOS homozygotes and heterozygotes, and found that little cholesterol was formed and 7-dehydrocholesterol accumulated only in homozygote fibroblasts. In contrast, heterozygote fibroblasts produced cholesterol, but only trace amounts of the precursor 7-dehydrocholesterol were present, so the transformation of [^{3}H]lathosterol to cholesterol did not distinguish SLOS heterozygote from control cells. To directly assay 7-dehydrocholesterol-Δ^7-reductase activity, conversion of the penultimate precursor, [1,2-^{3}H]7-dehydrocholesterol, to cholesterol was measured in fibroblasts from controls, SLOS homozygotes, and heterozygotes. After 30 minutes' incubation, virtually no cholesterol was produced in homozygote cells, while intermediate amounts were formed in heterozygote cells. Thus, markedly reduced fibroblast 7-dehydrocholesterol-Δ^7-reductase activity was a sensitive indicator of SLOS homozygotes, whereas intermediate activity levels could detect heterozygotes who are carriers of a partial enzyme deficiency.

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Submitted October 25, 1996; accepted January 22, 1997.

Supported in part by Grants No. DK 26756 and HL 17818 from the National Institutes of Health and the Research Service, Department of Veterans Affairs.

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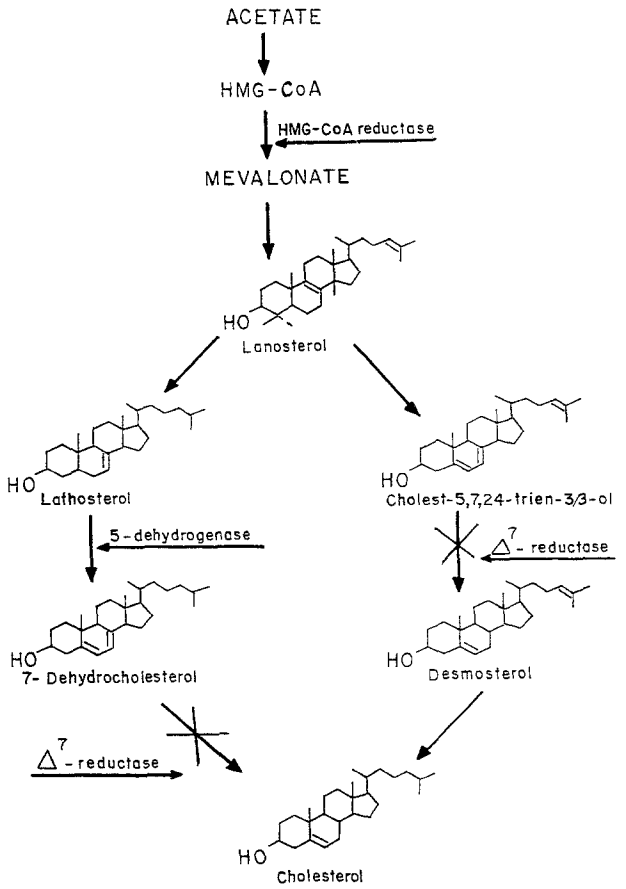

Fig 1. Flow diagram of the cholesterol biosynthetic pathway. Formation of mevalonate from HMG-CoA catalyzed by HMG-CoA reductase is considered the rate-controling reaction. Lanosterol is the first sterol produced in the pathway and is transformed to cholesterol by 2 possible routes. Lathosterol is converted to 7-dehydrocholesterol by lathosterol 5-dehydrogenase, which is then reduced to cholesterol by 7-dehydrocholesterol-Δ^7-reductase. The last reaction, denoted by an "X," is considered abnormal in SLOS. No desmosterol or its Δ^7-unsaturated precursor have been detected in SLOS homozygotes.

SUBJECTS AND METHODS

Fibroblast Cultures

Skin biopsies were obtained from five SLOS homozygotes and five parents who were obligate heterozygotes. All homozygotes showed a typical clinical phenotype (microcephaly, flat nares, low-set posteriorly rotated ears, micrognathia, and mental retardation) with reduced plasma cholesterol and elevated 7-dehydrocholesterol concentrations.[6] Three patients were classified as type I and two patients as type II phenotypes.[7] Parents were clinically normal. The fibroblasts were grown and maintained as monolayers in Dulbecco's modified Eagle's medium (DME) (Life Technologies, New York, NY) supplemented with 10% FBS that contained 4 mg/dL cholesterol. Control fibroblasts were obtained from 10 patients without abnormalities in cholesterol metabolism. All cells were used before the fifteenth passage. Approximately 1×10^6 cells were seeded per flask (75 cm^2) and grown in a humidified incubator at 37°C in 5% CO_2/95% air for 7 days to reach near-confluence with approximately 4×10^6 cells per flask. The cells were harvested with a cell scraper, washed twice with phosphate-buffered saline (PBS), and stored at −70°C.

To investigate the effect of delipidated medium supplemented with lovastatin on cholesterol biosynthesis, the original growth medium was removed from the near-confluent fibroblast cultures. The attached cells were washed twice with PBS, and the medium was replaced with DME 6 mL per flask that contained 5% delipidated calf serum (Sigma Chemical, St Louis, MO), with a final cholesterol concentration of 0.02 mg/dL and lovastatin 5 μg/mL (Merck, West Point, PA) added in 50 μL ethanol to the medium. Lovastatin was added to the medium to maximally stimulate the formation of HMG-CoA reductase and expression of low-density lipoprotein (LDL) receptors. The flasks were incubated for 24 hours at 37°C in a humidified incubator containing 5% CO_2/95% air, after which the cells were harvested with a cell scraper, washed twice with PBS, and stored at −70°C.

The experimental protocol was approved by the human studies committees at the Veterans Affairs Medical Center, East Orange, NJ, and UMD-New Jersey Medical School, Newark, NJ.

Plasma and Cell Sterols

Cholesterol, 7-dehydrocholesterol, and 8-dehydrocholesterol concentrations were determined in plasma by capillary column gas-liquid chromatography as described previously.[7,12]

Fibroblast sterol concentrations and composition were measured according to a modification of the method described recently by Kelley.[13] After addition of 1 μg coprostanol (5β-cholestan-3β-ol) as an internal recovery standard, lipids were extracted from about 50% of the cells in one flask with chloroform:methanol 2:1.[14] The extracts were hydrolyzed in 1N ethanolic NaOH for 1 hour at 70°C, extracted with *n*-hexane, and converted into trimethylsilyl (TMS)-ether derivatives before analysis by gas chromatography–mass spectrometry using a Hewlett-Packard model 5988 gas chromatograph–mass spectrometer (Hewlett Packard, Palo Alto, CA). The TMS-ether derivatives were separated on a nonpolar CP-Sil 5CB (25 m × 0.25 ID) capillary column (Chrompack, Raritan, NJ) that uses helium as a carrier gas at a flow rate of 1.0 mL/min. The column oven was programed to change from 100° to 265°C at 35°C/minute, after a 2-minute delay from the start time. The mass spectral resolution was about 1,000. The retention time relative to 5α-cholestane of the TMS-ether of cholesterol was 1.66; TMS-ether of 7-dehydrocholesterol, 2.26; and TMS-ether of 8-dehydrocholesterol, 1.89. The multiple ion detector was focused on *m/z* 325 for 7-dehydrocholesterol, *m/z* 329 for cholesterol, and *m/z* 370 for coprostanol.

Preparation of [^{3}H] Substrates

[3α-^{3}H]lathosterol was prepared from unlabeled lathosterol (Aldrich Chemical, Milwaukee, WI) by chromium trioxide oxidation and reduced with [^{3}H]sodium borohydride (DuPont-NEN Research Products, Boston, MA) to form [3α-^{3}H]lathosterol. The labeled sterol was 98% pure; specific activity was 830 cpm/nmol.[10]

[1,2-^{3}H]7-dehydrocholesterol was synthesized from [1,2-^{3}H]cholesterol (DuPont-NEN Research Products) as reported by Shefer et al.[10] The specific activity was 330 cpm/nmol. Aliquots of [1,2-^{3}H]7-dehydrocholesterol were stored under argon in sealed tinfoil-covered ampules at −70°C until used for enzyme assay.

Preparation of Cell-Free Homogenates

To obtain solubilized enzyme preparations, the following procedure was used. Cells from two flasks were pooled and homogenized at 0°C (five strokes at 500 rpm; Thomas Scientific, Swedesboro, NJ) in 0.1 mL buffer containing 50 mmol/L K_2HPO_4, 5 mmol/L DTT, 5 mmol/L EDTA, 20 mmol/L KCl, and 0.25% Brij 96 (Sigma Chemical), pH 7.4. After incubation of the homogenate at 37°C for 5 minutes, cell debris was removed by centrifugation at 12,000 × *g* for 1 minute. Protein concentrations were determined on aliquots of the supernatant by the method of Lowry et al.[15] Approximately 0.05 to 0.2 mg solubilized protein was used in each assay.

Assay of 7-Dehydrocholesterol-Δ^7-Reductase Activity

[1,2-^{3}H]7-dehydrocholesterol (30 nmol, 10,000 cpm) was solubilized in 6 μL 33% aqueous cyclodextrin (2-Hydroxypropyl-β-cyclodextrin; Pharmatec, Alachua, FL) and incubated in a final volume of 150 μL containing phosphate buffer, 100 mmol/L K_2HPO_4, pH 7.3, 1 mmol/L DTT, 30 mmol/L nicotinamide, 0.1 mmol/L EDTA, and the NADPH-generating system, 3.4 mmol/L $NADP^+$, 30 mmol/L glucose-6-phosphate, and 0.3 IU glucose-6-phosphate dehydrogenase. The reaction was initiated by addition of 0.1 mg fibroblast solubilized supernatant protein supplemented with 0.5 mg hepatic cytosolic protein (100,000 × *g* supernatant)[10] and continued for 30 minutes. The reaction was stopped by adding 50 μL 50% aqueous NaOH. The final product, cholesterol (R_f 0.31), was separated from 7-dehydrocholesterol (R_f 0.09) by argentation thin-layer chromatography.[10] Radioactivity was measured by liquid scintillation spectroscopy. Enzyme-specific activity was calculated from the mass of (cholesterol) formed after 30 minutes of incubation and was expressed as picomoles of cholesterol formed per milligram of protein per minute.

Assay of Lathosterol 5-Dehydrogenase Activity

[3α-^{3}H]lathosterol (30 nmol, 25,000 cpm) was solubilized with 6 μL of a 33% solution of β-cyclodextrin, and incubated in a final volume of 150 μL that contained phosphate buffer, 100 mmol/L K_2HPO_4, pH 7.3, 0.1 mmol/L EDTA, 1 mmol/L DTT, 30 mmol/L nicotinamide, and a NADH-generating system, 3.4 mmol/L NADH, 30 mmol/L glucose-6-phosphate, and 0.3 IU glucose-6-phosphate dehydrogenase. The reaction was initiated by addition of 0.1 mg fibroblast supernatant protein supplemented with 0.5 mg hepatic cytosolic protein (100,000 × *g* supernatant). The mixture was incubated in air at 37°C for 10 minutes. The reaction was stopped by adding 50 μL aqueous NaOH (50%) so that the final mixture contained 12.5% NaOH. The reaction product 7-dehydrocholesterol (Rf 0.09) was extracted with ethylacetate and separated from lathosterol (Rf 0.37) by argentation thin-layer chromatography.[10] Enzyme-specific activity was calculated from the mass of 7-dehydrocholesterol formed after 10 minutes of incubation and was expressed as picomoles of 7-dehydrocholesterol formed per milligram of protein per minute.

To study the conversion of [^{3}H]lathosterol to cholesterol via 7-dehydrocholesterol, a NADPH-generating system was substituted for the NADH-generating system. This provides NADPH, which is necessary for the reduction of 7-dehydrocholesterol to cholesterol.

Assay of Total HMG-CoA Reductase Activity

Approximately 50 μg solubilized fibroblast supernatant protein was preincubated at 37°C for 5 minutes in a total volume of 150 μL buffer (50 mmol/L Tris, 68 mmol/L EDTA, 5 mmol/L DTT, and 70 mmol/L KCl, pH 7.5) containing a NADPH-generating system (34 mmol/L $NADP^+$, 30 mmol/L glucose-6-phosphate, and 0.3 IU glucose-6-phosphate dehydrogenase) and 40,000 dpm [^{3}H]mevalonolactone as internal recovery standard. The reaction was started with addition of 30 nmol [^{14}C]HMG-CoA (Amersham, Arlington Heights, IL) diluted with unlabeled HMG-CoA to yield a specific activity of 30 dpm/pmol. The reaction was stopped after 10 minutes at 37°C with addition of 20 μL 6N HCl. After lactonization of the product at 37°C for 30 minutes, the precipitates were pelleted by centrifugation (10,000 rpm) in a microfuge (Beckman, Palo Alto, CA), and the product mevalonolactone in the supernatant fraction was separated by TLC on 0.25-mm Silica Gel 6O plates (Merck, Darmstadt, Germany) developed with benzene:acetone 1:1 (vol/vol) and radioactivity was determined by liquid scintillation counting.

Measurements of LDL Receptor Function in Fibroblasts

LDLs ($1.019 < d < 1.063$) from healthy volunteer subjects were prepared from venous blood by the sequential flotation method of Havel et al.[16] The protein concentration of LDL was determined by the method of Lowry et al[15] after dialysis in 2,000 vol buffer (150 mmol/L NaCl and 0.01% disodium EDTA, pH 7.4). LDL was labeled with ^{125}I (Amersham) by the iodine monochloride method.[17] After removal of unbound label through a G-25 column (prepacked PD-10; Pharmacia, Piscataway, NJ) and dialysis in the above-mentioned buffer, the filtered labeled LDL had specific activities of 150 to 300 cpm/ng. Ninety-nine percent of the label was in the trichloroacetic acid–precipitable protein fraction.

Receptor-mediated binding of ^{125}I-labeled LDL by fibroblasts was assayed by methods previously published.[17-19] Fibroblasts were obtained from two 25-cm^2 incubation flasks from controls and SLOS subjects that were grown to near-confluence before and after exposure to delipidated medium for 24 hours. On the day of assay, the cells were washed with 3 mL Tris-buffered saline (50 mmol/L Tris and 150 mmol/L NaCl, pH 7.4) and warmed to 37°C with 0.2% bovine albumin (essential fatty acid–free; Sigma Chemical). The washed cells were incubated with 10 μg/mL ^{125}I-LDL in the presence and absence of 400 μg/mL unlabeled LDL in a total volume of 1 mL assay buffer (DME containing 10% delipidated calf serum and 2% bovine albumin). After 4 hours of incubation at 37°C in a 5% CO_2/95% air atmosphere, the media were collected. Assay buffer (1 mL) was used to wash the cells and added to the medium for analysis of ^{125}I-labeled, trichloroacetic acid–soluble degradation products, which contained no free iodide.[18]

Receptor-mediated binding was determined as the difference between total binding (in the absence of unlabeled LDL) and nonspecific binding (in the presence of 40-fold excess unlabeled LDL). The binding measurements include both surface-bound and internalized LDL.

Statistics

Data are expressed as the mean ± SEM. Differences were analyzed statistically by the unpaired Student's *t* test and by comparing confidence intervals for the means.[20] When more than one comparison was made to the same value, Bonferroni correction was applied to estimate statistical significance using the BMDP statistical software package (BMDP Statistical Software, Los Angeles, CA).

RESULTS

Table 1 shows sterol concentrations in control, SLOS homozygote, and heterozygote fibroblasts grown to near-confluence in medium with 10% FBS that contained 4.0 mg/dL cholesterol. Cholesterol concentrations tended to be higher in heterozygote and homozygote fibroblasts than in control cells, but because of individual variation in concentrations, the differences were not statistically significant. In contrast, 7-dehydrocholesterol concentrations were 30 times greater in homozygote than in control cells and 20 times higher than in heterozygote cells. However,

Table 1. Sterol Concentrations in Fibroblasts

Group (n)	Sterol Concentrations in Fibroblasts (nmol/mg protein)	
	7-Dehydrocholesterol	Cholesterol
FBS		
SLOS homozygotes (5)	5.1 ± 1.1	173 ± 32
SLOS heterozygotes (5)	0.25 ± 0.06	167 ± 24
Controls (10)	0.16 ± 0.04	130 ± 17
Cholesterol-deficient serum + lovastatin		
SLOS homozygotes (3)	4.2 ± 1.3	170 ± 43
SLOS heterozygotes (3)	0.35 ± 0.12	162 ± 37
Controls (3)	0.19 ± 0.08	144 ± 17

*$P < .001$ *v* heterozygotes and controls.

7-dehydrocholesterol was only present in trace amounts in heterozygote and control cells, and the differences were not significant. Thus, concentrations of cholesterol and 7-dehydrocholesterol in fibroblasts cannot be used to distinguish SLOS heterozygotes from controls, although increased concentrations of 7-dehydrocholesterol indicated SLOS homozygosity. Moreover, differences in fibroblast sterol concentrations were not accentuated after exposing the almost-confluent cell cultures to cholesterol-deficient medium that contained less than 0.02 mg/dL cholesterol supplemented with lovastatin for 24 hours (Table 1). 7-Dehydrocholesterol concentrations remained elevated in homozygote fibroblasts and did not increase significantly in either heterozygote or control cells. Thus, stimulating cholesterol biosynthesis did not increase 7-dehydrocholesterol levels in fibroblasts and did not help with the detection of heterozygotes. 8-Dehydrocholesterol (data not shown) was detected in only trace amounts in these fibroblast preparations.[12]

Figure 2 presents the conversion of the precursor [3α-^{3}H]lathosterol to cholesterol via 7-dehydrocholesterol in control and SLOS homozygote and heterozygote fibroblasts. To study the transformation pathway of newly formed 7-dehydrocholesterol to cholesterol, a NADPH-generating system was substituted for the NADH system that was used when only an assay of lathosterol 5-dehydrogenase activity was intended. In control cells, lathosterol was rapidly transformed to 7-dehydrocholesterol during the first 5 minutes of incubation, after which 7-dehydrocholesterol was converted efficiently to cholesterol over the remaining 25 minutes. In fibroblasts from SLOS homozygotes, 7-dehydrocholesterol was produced abundantly, but virtually no cholesterol was formed. Unlike in the liver, 8-dehydrocholesterol was not detected.[10] In SLOS heterozygote fibroblasts, 7-dehydrocholesterol was also produced efficiently from lathosterol. However, in contrast to homozygote cells, cholesterol was synthesized from newly formed 7-dehydrocholesterol, but at a much slower rate than in controls.

Figure 3 presents measurements of cholesterol formed when the final precursor [1,2-^{3}H]7-dehydrocholesterol was incubated for 30 minutes with control, homozygote, and heterozygote

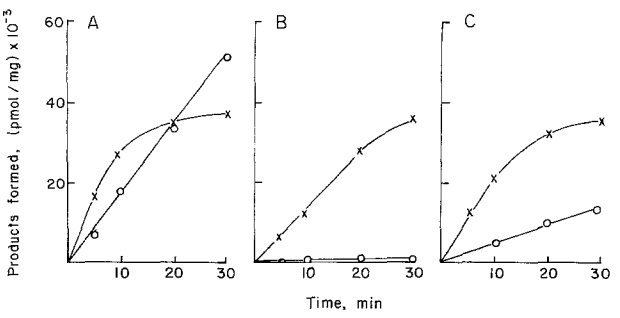

Fig 2. Cholesterol formation from lathosterol. [3α-^{3}H]lathosterol was incubated with representative fibroblast cultures grown almost to confluence with cofactors and a NADPH-generating system. The average of 2 measurements per point is shown. In control fibroblasts (A), 7-dehydrocholesterol (x) was formed and rapidly converted to cholesterol (○). In SLOS homozygote fibroblasts (B), 7-dehydrocholesterol was produced abundantly, but virtually no cholesterol was formed. In SLOS heterozygote fibroblasts (C), 7-dehydrocholesterol was synthesized and converted to cholesterol, but at a slower rate than in control cells.

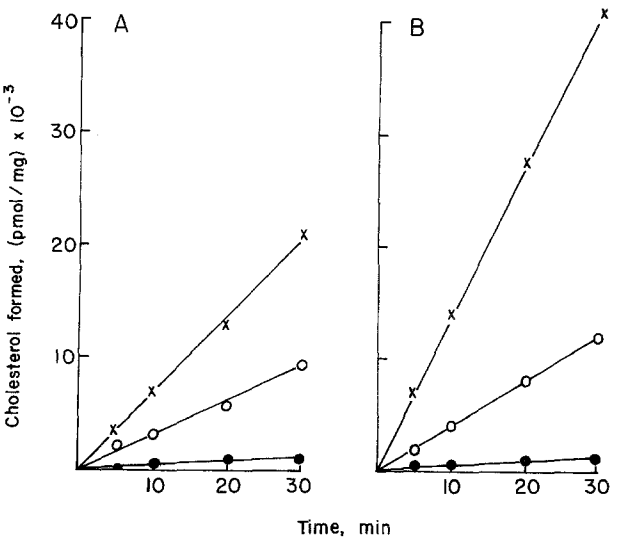

Fig 3. Formation of cholesterol from [1,2-^{3}H]7-dehydrocholesterol by representative fibroblast cultures. The average of 2 measurements per point is shown. In fibroblasts grown almost to confluence in 10% FBS (A), 7-dehydrocholesterol was efficiently converted to cholesterol by control cells (x), but little cholesterol was produced by SLOS homozygote cells (•). SLOS heterozygote cells (○) formed cholesterol at an intermediate rate. After exposure of the fibroblasts to cholesterol-deficient medium supplemented with lovastatin for 24 hours (B), cholesterol was synthesized twice as rapidly by control cells, but at the same rates by SLOS homozygote and heterozygote fibroblasts.

fibroblasts grown in medium that contained 10% FBS and after exposure of the cells for 24 hours to cholesterol-deficient medium plus lovastatin. Cholesterol was rapidly formed from 7-dehydrocholesterol in control fibroblasts. In contrast, little cholesterol was produced over 30 minutes when incubations were performed with SLOS homozygote fibroblasts. In comparison, SLOS heterozygote fibroblasts converted 7-dehydrocholesterol to cholesterol at an intermediate rate that was slower than control cells but more than twice as rapid as homozygote cells. When fibroblasts were exposed for 24 hours to cholesterol-deficient medium plus lovastatin, twice as much cholesterol was formed in control cells, but the rates of cholesterol formation did not change significantly in either SLOS homozygote or heterozygote cells. These findings suggested that 7-dehydrocholesterol-Δ^7-reductase that catalyzes the conversion of 7-dehydrocholesterol to cholesterol can be upregulated after exposure of control fibroblasts to cholesterol-deficient medium with lovastatin, but in neither SLOS homozygote nor heterozygote cells can 7-dehydrocholesterol-Δ^7-reductase be stimulated further. As a result, 7-dehydrocholesterol-Δ^7-reductase appears maximally expressed in SLOS homozygote and heterozygote fibroblasts.

The specific activity of 7-dehydrocholesterol-Δ^7-reductase (Fig 4A) was 3.8 times greater in control than in homozygote cells ($P < .001$) and 2.2 times greater in control than in heterozygote cells ($P < .001$) when the fibroblasts were grown in medium with 10% FBS. Moreover, in heterozygote cells, 7-dehydrocholesterol-Δ^7-reductase activity was 1.6 times higher than in homozygote fibroblasts ($P < .05$). After stimulating cholesterol biosynthesis (Fig 4B), mean 7-dehydrocholesterol-Δ^7-reductase activity increased in control cells, so enzyme

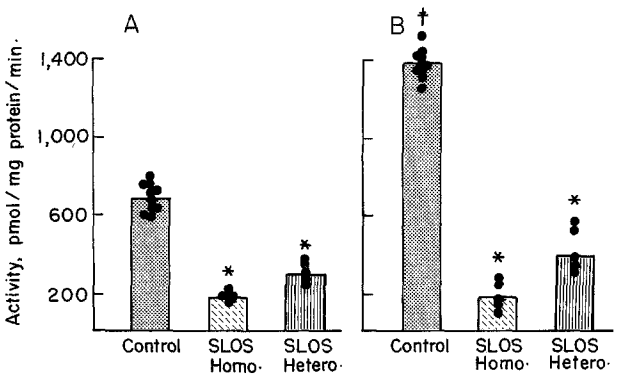

Fig 4. 7-Dehydrocholesterol-Δ^7-reductase activities in SLOS homozygote, heterozygote, and control cells. In fibroblasts grown to confluence in 10% FBS (A), 7-dehydrocholesterol-Δ^7-reductase activity was 3.8 times higher in control than in homozygote cells (*$P < .001$) and 2.2 times greater than in heterozygote fibroblasts (*$P < .001$). After exposure of the confluent fibroblast cultures to cholesterol-deficient medium for 24 hours (B), 7-dehydrocholesterol-Δ^7-reductase activity increased twofold in control cells (‡$P < .0001$); however, there was only a slight increase in 7-dehydrocholesterol-Δ^7-reductase activity in SLOS heterozygote cells and no change in SLOS homozygote cells (B *v* A). In heterozygote cells (B), 7-dehydrocholesterol-Δ^7-reductase activity was now 2.3 times higher than in homozygote cells ($P < .01$).

activity was 7.6 times greater than the mean for homozygotes and 4.4 times higher than the mean for heterozygotes. 7-Dehydrocholesterol-Δ^7-reductase activity increased slightly in heterozygote cells, and was now 2.3-fold higher than in homozygote cells ($P < .01$).

Table 2 lists the mean activities of HMG-CoA reductase and lathosterol 5-dehydrogenase. HMG-CoA reductase catalyzes the conversion of HMG-CoA to mevalonic acid and is considered rate-controling for cholesterol biosynthesis, since most molecules of mevalonic acid are committed to the cholesterol biosynthesis pathway (Fig 1). In control, heterozygote, and homozygote fibroblasts, HMG-CoA reductase activities were about equal, and increased almost four times when the cells were exposed to cholesterol-deficient medium supplemented with lovastatin for 24 hours. Lathosterol 5-dehydrogenase that catalyzes the conversion of lathosterol to 7-dehydrocholesterol was also similarly expressed in all three cell preparations. These results suggested that earlier enzymatic reactions in cholesterol biosynthesis that precede 7-dehydrocholesterol-Δ^7-reductase

Table 2. Fibroblast HMG-CoA Reductase and Lathosterol 5-Dehydrogenase Activities (pmol/mg/min)

Group (n)	HMG-CoA Reductase	Lathosterol 5-Dehydrogenase
10% FBS		
Controls (10)	15.8 ± 2.3	1,000 ± 83
SLOS homozygotes (5)	19.0 ± 3.1	1,200 ± 132
SLOS heterozygotes (5)	13.5 ± 3.5	1,050 ± 147
Cholesterol-deficient serum + lovastatin		
Controls (3)	58.2 ± 9.8	ND
SLOS homozygotes (3)	78.5 ± 11.6	ND
SLOS heterozygotes (3)	60.9 ± 9.0	ND

Abbreviation: ND, not determined.

Table 3. Receptor-Mediated LDL Binding to Fibroblasts

Group (n)	LDL Binding (ng/mg cell protein)
10% FBS	
Controls (4)	268.2 ± 46.2
SLOS homozygotes (4)	242.5 ± 52.8
SLOS heterozygotes (4)	215.1 ± 25.1
Cholesterol-deficient serum + lovastatin*	
Controls (4)	569.6 ± 113.3
SLOS homozygotes (4)	686.7 ± 60.9
SLOS heterozygotes (4)	370.4 ± 46.1

NOTE. Receptor-mediated binding was determined as the difference between total binding assayed in the absence of unlabeled LDL and nonspecific binding assayed in the presence of a 40-fold excess of unlabeled LDL. Values are the mean ± SEM.

*Fibroblasts exposed to delipidated bovine calf serum + lovastatin for 24 hours had significantly higher LDL receptor–mediated binding compared with cells grown with 10% FBS: $P < .05$ for controls and heterozygotes and $P < .01$ for homozygotes.

were equally active in the three cell preparations, and as expected, HMG-CoA reductase was upregulated when fibroblast cultures were exposed to cholesterol-deficient medium with lovastatin for 24 hours.

Table 3 lists measurements of receptor-mediated LDL binding in fibroblasts from controls, SLOS heterozygotes, and homozygotes. No differences in LDL receptor–mediated binding and degradation were observed in fibroblasts from the three groups. Moreover, exposure of these fibroblasts to cholesterol-deficient medium for 24 hours increased LDL receptor–mediated binding significantly in all three cell lines. Thus, LDL receptors were expressed in SLOS homozygote and heterozygote fibroblasts and, as expected, increased when the cells were exposed to cholesterol-deficient medium for 24 hours.

DISCUSSION

The results of this investigation demonstrated that 7-dehydrocholesterol-Δ^7-reductase activity was significantly decreased in fibroblasts from both SLOS homozygotes and heterozygotes compared with controls. However, because 7-dehydrocholesterol-Δ^7-reductase was significantly more active in fibroblasts from heterozygotes versus homozygotes, the precursor 7-dehydrocholesterol did not accumulate in heterozygote cells, whereas in homozygote fibroblasts 7-dehydrocholesterol was 30 times more abundant (Table 1). The formation of cholesterol by SLOS heterozygote fibroblasts as compared with virtually no cholesterol produced by homozygote fibroblasts (Figs 2 and 3) is consistent with the intermediate levels of 7-dehydrocholesterol-Δ^7-reductase activity measured in the heterozygote cells and the markedly inhibited enzyme activity found in homozygote cells (Fig 4). Moreover, when almost-confluent fibroblast cultures were exposed for 24 hours to cholesterol-deficient medium that contained lovastatin, 7-dehydrocholesterol-Δ^7-reductase activity doubled in the control cells but did not increase further in either homozygote or heterozygote fibroblasts. Thus, the first and last enzymatic reactions in the cholesterol biosynthetic pathway catalyzed by HMG-CoA reductase and 7-dehydrocholesterol-Δ^7-reductase, respectively, are coordinately regulated and respond similarly to cholesterol-deficient medium by

upregulation of enzyme function, as reported in rat liver cholesterol synthesis by Honda et al.[21] It is also important to realize that when the cells were grown almost to confluence in cholesterol-containing medium (10% FBS), they expressed receptors (Table 3) that bind LDL, so homozygote and heterozygote cells contained substantial cholesterol derived from the medium; yet 30 times more 7-dehydrocholesterol was present in homozygote cells. Thus, even in the presence of abundant cholesterol, where biosynthesis is limited, the abnormal pathway still contributed 7-dehydrocholesterol to SLOS homozygote fibroblasts because conversion of the precursor to cholesterol was blocked.

One interesting point is that 7-dehydrocholesterol-Δ^7-reductase activity was similar in fibroblasts from SLOS subjects with both the type I and type II phenotypes. Although the latter phenotype is clinically more severe,[22] with lower cholesterol and higher 7-dehydrocholesterol levels in serum, 7-dehydrocholesterol-Δ^7-reductase activities in the fibroblasts were profoundly reduced and did not separate the two clinical phenotypes.[22,23]

It is also important to emphasize that both HMG-CoA reductase and lathosterol 5-dehydrogenase activities in SLOS homozygote, heterozygote, and control fibroblasts were similar. These two enzymes catalyze reactions earlier in the cholesterol biosynthetic pathway, and their normal activities in SLOS fibroblasts indicate that the cell preparations were viable. Moreover, HMG-CoA reductase activities were stimulated proportionately in control, SLOS homozygote, and heterozygote cells after exposure for 24 hours to cholesterol-deficient medium containing lovastatin. The upregulation of this enzyme in homozygote cells grown in cholesterol-deficient medium, in conjunction with the observation of similar stimulation of LDL receptor function (Table 3), indicated that the coordinate regulation of HMG-CoA reductase with LDL receptors was operationally intact. Thus, inhibited 7-dehydrocholesterol-Δ^7-reductase activity is inherited in SLOS homozygotes and heterozygotes. However, the higher levels of 7-dehydrocholesterol-Δ^7-reductase activity in heterozygote cells that are between the levels in control and homozygote cells serve to detect heterozygosity, especially since only trace amounts of the precursor 7-dehydrocholesterol are present in their fibroblasts and plasma.[7,8] Nevertheless, the definitive diagnosis of SLOS homozygotes and heterozygotes must await discovery of the 7-dehydrocholesterol-Δ^7-reductase gene, when mutations can be ascertained and correlated with enzyme activities. However, the assay of fibroblast 7-dehydrocholesterol-Δ^7-reductase activity provides a rapid and convenient method to identify heterozygotes. Although SLOS heterozygotes appear clinically normal and do not accumulate the precursor 7-dehydrocholesterol, detection of these individuals is critically important, since they are potential parents of future SLOS homozygotes. Moreover, SLOS heterozygotes probably synthesize less cholesterol, and this may explain the potential advantages and disadvantages of reduced cholesterol biosynthesis in this population. For example, there is now ample evidence that low plasma cholesterol concentrations may be associated with psychologic depressions and a possible increased tendency for suicide.[24,25] Alternatively, reduced cholesterol biosynthesis may actually show a biologic advantage. Because the inherited deficiency of 7-dehydrocholesterol-Δ^7-reductase may limit the formation of cholesterol from lathosterol, SLOS heterozygotes may show lower plasma cholesterol levels and develop less atherosclerosis, with a decreased risk for heart attacks and stroke.

Recently, Honda et al[9] and Lund et al[11] have reported the reduced formation of cholesterol from lathosterol by fibroblasts from SLOS homozygotes. However, neither group was able to use this assay method based on the conversion of the earlier precursor, lathosterol, to cholesterol to identify heterozygotes, because cholesterol was formed in these cells without the significant accumulation of 7-dehydrocholesterol seen in homozygote cells. This problem was overcome when the final precursor in the pathway, 7-dehydrocholesterol, was used as substrate. We have demonstrated that although [1,2-^{3}H]7-dehydrocholesterol is unstable, small amounts can be protected under argon in sealed tinfoil-covered ampules until used for 7-dehydrocholesterol-Δ^7-reductase activity assay.[26]

In summary, markedly inhibited fibroblast 7-dehydrocholesterol-Δ^7-reductase activity with the accumulation of 7-dehydrocholesterol in cells confirms the diagnosis for SLOS homozygotes. Intermediate levels of 7-dehydrocholesterol-Δ^7-reductase activity without accumulation of 7-dehydrocholesterol in fibroblasts detected SLOS heterozygotes. Stimulating cholesterol biosynthesis after exposure of fibroblasts to cholesterol-deficient medium supplemented with lovastatin for 24 hours accentuated the differences in 7-dehydrocholesterol-Δ^7-reductase activities between control and SLOS homozygote and heterozygote cells and increased the sensitivity of the assay.

ACKNOWLEDGMENT

The excellent technical assistance of B. Pcolinsky, E. Paroulek, and B. Rouse is greatly appreciated.

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