27-Hydroxylated Low Density Lipoprotein (LDL) Cholesterol Can Be Converted to 7α,27-Dihydroxy-4-cholesten-3-one (Cytochrome) before Suppressing Cholesterol Production in Normal Human Fibroblasts

EVIDENCE THAT AN ALTERED METABOLISM OF LDL CHOLESTEROL CAN UNDERLIE A DEFECTIVE FEEDBACK CONTROL IN MALIGNANT CELLS*

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The formation of oxysterols in cultured human fibroblasts and their physiological roles as intracellular regulators of cholesterol production have been investigated. In the presence of low density lipoproteins (LDL), normal fibroblasts converted LDL cholesterol to 27-hydroxycholesterol, which was further metabolized to 7α,27-dihydroxycholesterol, 7α,27-dihydroxy-4-cholesten-3-one, and 7α-hydroxy-3-oxo-4-cholestenolic acid. Autooxidation products of cholesterol contaminating the lipoproteins were also metabolized in the cells. 7α-Hydroxycholesterol was converted to 7α-hydroxy-4-cholesten-3-one prior to 27-hydroxylation and further oxidation to 7α-hydroxy-3-oxo-4-cholestenolic acid. 7β-Hydroxycholesterol and 7α-cholesterol were 27-hydroxylated and then oxidized to C27-acids. Oxidation of the 7β-hydroxy group also occurred. 25-Hydroxycholesterol was 7α-hydroxylated and further oxidized to 7α,25-dihydroxy-4-cholesten-3-one. 25-Hydroxylation of sterols was observed only under specific conditions. In contrast, only small amounts of oxysterols were formed in virus-transformed human fibroblasts when incubated with lipoproteins. This was due to very low activities of the 27- and 7α-hydroxylating enzymes. The rate of oxidation of C-3 was also decreased moderately.

A defective suppression of 3-hydroxy-3-methylglutaryl coenzyme A reductase by LDL and autooxidation products of cholesterol observed in the transformed fibroblasts could be caused by the deficiencies of the sterol-metabolizing enzymes, since these cells responded normally to the sterol metabolites 7α,27-dihydroxycholesterol, 7α,25-dihydroxy-4-cholesten-3-one, and 27-hydroxy-7α-cholesterol. These metabolites, which all possessed an oxo group with a conjugated double bond in the steroid nucleus and a hydroxyl group in the side chain, did not seem to require further metabolism in order to be active. An impaired response to LDL was also observed in other human tumor cells, including breast carcinoma, colon carcinoma, and malignant melanoma cells. Common to all the malignant cells was an intracellular shortage of 7α,27-dihydroxycholesterol induced by a decreased formation or an increased metabolism.

Low density lipoprotein (LDL) is the only regulator of cellular cholesterol production whose physiological role has been established (1–5). Internalized LDL suppresses 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme of cholesterol synthesis, but the underlying mechanisms are not fully understood. Recently, we reported that LDL cholesterol is converted to 27-hydroxycholesterol in human fibroblasts and that this oxysterol is an important intracellular mediator of the LDL-induced suppression of HMG-CoA reductase (6). The metabolism of LDL cholesterol and the biological effects of its metabolites in normal human fibroblasts have now been investigated further. Virus-transformed fibroblasts were included in the study, since a defective regulatory response to LDL has been observed previously in several different malignant cells (7–10). This impaired cholesterol feedback system, the causes of which are unknown, seems to appear early in the development of cancer and has been considered to promote cell growth by increasing the cellular supply of cholesterol and intermediates in the mevalonate pathway (11). The two fibroblastic cell lines permitted us to make a direct comparison between the handling of LDL cholesterol and the suppression of HMG-CoA reductase in the normal and the corresponding tumor-transformed cell. Here we report that in normal human fibroblasts 27-hydroxylated LDL cholesterol is converted to 7α,27-dihydroxycholesterol, which does not seem to be metabolized further before suppressing HMG-CoA reductase. The study also shows that the metabolism of LDL cholesterol is markedly changed following transformation of fibroblasts and that these cells display a defective regulatory response to LDL. An altered metabolism of LDL cholesterol and an impaired suppression of HMG-CoA reductase were also noted in other human neoplastic cells.

MATERIALS AND METHODS

Steroids, Chemicals, and Sera—Diosgenin ((25R)-5-spirosten-3β-ol) was from Sigma and was used as the starting material for the synthesis of 27-oxygenated steroids (12–14). In addition, 5-cholostene-3β,7α,25-triol (7α,25-dihydroxycholesterol) was prepared from the 3-acetoate,25-trimethylsilyl ether derivative of 25-hydroxycholesterol, and, after hydrolysis, this steroid was further oxidized to 7α,25-dihydroxycholesterol as described for the corresponding 27-hydroxysteroids (14). 25-Hydroxycholesterol was oxidized in the same way to 25-hydroxycholesterol-3-one. Other unlabeled steroids were those used in a previous study (15). [1α,2α-3H]Cholesterol (44 Ci/mmol) and [1α,2α-3H] hydroxysteroids. 25-Hydroxycholesterol was oxidized in the same way to 25-hydroxycholesterol-3-one. Other unlabeled steroids were those used in a previous study (15). [1α,2α-3H]Cholesterol (44 Ci/mmol) and [1α,2α-3H]cholesterol were purchased from Amersham, and [1α,2α-3H]cholesterol was purchased from New England Nuclear.

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1 The abbreviations used are: LDL, low density lipoprotein; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; FCS, fetal calf serum; LDS, lipoprotein-deficient serum; CsA, cyclosporin A; HPLC, high performance liquid chromatography.
25-[3H]hydroxycholesterol (86 Ci/mmol) was from Du Pont de Nemours NV, NEN Products (Brussels, Belgium). Radioactivity was determined in an LKB/Wallac 1215 Rackbeta Scintillation Counter with OptiPhase "HiSafe" (Wallac) as the scintillation liquid. Cyto-ponin A (CSA) and ketoconazole were kind gifts from Sandoz Pharma Ltd. (Budapest, Hungary) and Janssen Pharmaceutica (Beerse, Belgium), respectively. Fetal calf serum (FCS) having a total cholesterol concentration of 1.2 mM (12% free and 88% esterified cholesterol) was from Life Technologies, Inc. (St. Louis, MO, USA). Parallel experiments in the absence of lipoproteins (media containing 5% FCS) were performed with normal fibroblasts, and growth was not affected negatively when this serum was used. LDL particles containing [3H]labeled cholesterol or 25-hydroxycholesterol were obtained by incubating the radioactive steroid with FCS overnight at 20 °C (6).

Cell Culture Conditions—Normal human fibroblasts (line GM 08333) were obtained from NIGMS, Coriell Institute for Medical Research (Camden, NJ) and SV40 virus-transformed human fibroblasts (90-VA IV) were a kind gift from Dr. Stein (University of Colorado, Boulder, CO). Human colonic carcinoma (WiDr), breast carcinoma (MDA 231), and malignant melanoma (SK-MEL-2) cell lines were from American Type Culture Collection.

All cell lines were grown in monolayers in tissue culture flasks in a humidified atmosphere, 5% CO2, at 37 °C. Upon confluence, the monolayers were trypsinized and used for plating at a density of 3–6 x 104 in 5–15 cm2 dishes. The cells were incubated in either Dulbecco’s modified Eagle’s medium (DMEM) or Eagle’s medium (the other cells) supplemented with 10% FCS (v/v) and 10% FCS (with or without CsA, ketoconazole, or cholesterol or cholesteryl oleate) or were incubated with the oxysterol in 10% LDS. Control cells were incubated in the same way, but only for 15 min. Effects of CsA, ketoconazole, and oxysterols were tested on normal and transformed fibroblasts (cell number 3–6 x 105 in 57–143 cm2 dishes) were first precultured for 24 h in medium containing 10% LDS and were then incubated for 3–68 h with 7–10 ml of medium containing 10% FCS (with or without CsA, ketoconazole, or cholesterol or cholesteryl oleate) or were incubated with the oxysterol in 10% LDS for 24–48 h. Control cells were incubated in the same way, but only for 15 min. Effects of CsA, ketoconazole, and oxysterols were tested in normal and transformed fibroblasts at concentrations of 10–30 μM, 30 μM, and 0.12 μM, respectively, in cell media containing 0–10% FCS and 10–5% LDS. The substances were added to the incubation media in fresh, prepared ethanol solutions, and the ethanol concentrations of media became 0.1–0.5%. Control cells were incubated in the same way, but without CsA, ketoconazole, or oxysterols. The dish size and volume of media when HMG-CoA reductase activity was to be determined were 20 cm2 and 5 ml, respectively, and the incubations were carried out in duplicate for 3–24 h. Each oxysterol was tested in 2–5 separate experiments. The HMG-CoA reductase activity was then carried out as described previously (6, 17, 18).

Analysis of Oxyester and Steroid Acids—The procedure for extraction and purification of oxysterols present in incubation media and cells was essentially the same as described previously (6). Following the collection of a neutral oxysterol fraction from the lipophilic anion exchange (6), a fraction containing sterols with a free carbonyl group was eluted with 0.15 M acetic acid in 95% aqueous methanol prior to elution of a fraction containing stronger acids (including sterol sulfates) with 0.5 M potassium acetate/potassium hydroxide, apparent pH 10.0, in 72% aqueous methanol (19).

Trimethylsilyl ethers of oxysterols and methyl ester trimethylsilyl ethers of neutral and acidic metabolites of [4H]cholesterol or [3H]cholesterol deacylated from media and cells were isolated by gas chromatography-mass spectrometry (GC/MS) as described previously (6).

High Performance Liquid Chromatography (HPLC)—25-[3H]hydroxycholesterol, cholesterol, cholesteryl oleate, and 25-hydroxycholesterol and/or their radioactive metabolites were analyzed by HPLC prior to or after group fractionation as described above. 25-[3H]hydroxycholesterol and cholesteryl esters were extracted from small aliquots of the incubation media with mixtures of isopropyl alcohol and hexane and from cells with mixtures of ethanol and water prior to separation by straight-phase HPLC using hexane/isopropanol alcohol, 98:2 (v/v), as the mobile phase (6). Appropriate fractions from the HPLC effluent were collected in vials, and the radioactivity was then determined by scintillation counting. Radioactive neutral and acidic metabolites of [4H]cholesterol or [3H]cholesterol deacylated from media and cells were isolated by gas chromatography and were identified by HPLC. For this purpose, three HPLC systems were used in the following order. Reversed-phase HPLC was carried out on a column of LiChrospher (250 x 4 mm, Hiber, 100RP-18, 5 μm, Merck, Darmstadt, Germany) using a pump (Constametric III) and a variable wavelength detector (Spectra Monitor D from LDC/Milton Roy, Riviera Beach, FL) set at 220 or 240 nm and a Rheodyne Model 7125 injector with a 100-μl loop. The mobile phase was ethanol (70% in water, 80:20:10 (by volume, flow rate 1 ml min−1) and fractions were collected between 0 and 11 min (fraction 1; containing polar metabolites, e.g., 7α,27-dihydroxy-4-cholesten-3-one, retention time about 4.5 min) and between 11 and 14 min (fraction 2; containing 7α-hydroxy-4-cholesten-3-one and 27-hydroxycholesterol having retention times 11.5 and 12.5 min, respectively). The mobile phase was then changed to 85% aqueous methanol (flow rate 1 ml min−1) for separation of sterol fraction 1 or for separation of steroid acids (as methyl ester derivatives). In the former case, a fraction of the eluent containing 7α,27-dihydroxy-4-cholesten-3-one (retention time about 8.5 min) was collected between 8.0 and 9.0 min, and in the latter case a fraction of the eluent containing 7α-hydroxy-3-oxo-4-cholesten-3-one (retention time about 12 min) was collected between 11.0 and 13.0 min. These fractions and fraction 2 (containing 7α-hydroxy-4-cholesten-3-one and 27-hydroxycholesterol) were then reanalyzed by straight-phase HPLC. The latter was carried out with an instrument similar to that above, but with a column (250 x 4.5 mm) of LiChrospher (Hibar, Si 100, 5 μm, Merck). The mobile phase was hexane/isopropanol alcohol, 94:6 (v/v), when fractionating the fractions analyzed (retention times of 7α-hydroxy-4-cholesten-3-one and 27-hydroxycholesterol were about 8 and 9 min, respectively) and 90:10 (v/v), when the fractions containing 7α,27-dihydroxy-4-cholesten-3-one or 7α-hydroxy-3-oxo-4-cholestenone acid methyl ester were analyzed. The flow rate was 1.0 ml min−1 in all cases. The HPLC effluent during the latter analyses was collected in scintillation vials with 15–60 s-intervals, and, after addition of scintillation fluid, the radioactivity was determined.

25-[3H]hydroxycholesterol and its metabolites in media and cells were analyzed by HPLC following extraction. Mobile phase was extracted with ethanol, and, after centrifugation and removal of the supernatant, the eluate was re-extracted with ethanol/isopropanol alcohol, 1:1 (v/v). The extracts were combined and the solvent was then evaporated. Nonpolar compounds were dissolved in hexane, and, after removal, the solid residue was dissolved in 60% aqueous methanol, which was passed through a column (1.5 x 0.8 cm) of octadecyhydrosilane-bonded silica (Preparative C18 Waters Associates Inc., Milford, MA) and collected. The methanol in the eluate was then removed in vacuo, and the aqueous solution was re-extracted on the same column. After washing the column with water, sorbed sterols (polar metabolites) were eluted with methanol/chloroform, 1:1 (v/v), and were combined with the nonpolar metabolites present in the hexane fraction. This combined extract was evaporated to dryness and dissolved in methanol or hexane/isopropanol alcohol, 90:10 (v/v), prior to analysis by reversed-phase HPLC (mobile phase: methanol/ethanol/water, 80:20:10 (v/v) or straight-phase HPLC (mobile phase: hexane/isopropanol alcohol, 97:3 (v/v)).

RESULTS

Formation of Oxygenated Cholesterol Derivatives in Human Fibroblasts—The formation of oxygenated cholesterol derivatives in normal and virus-transformed human fibroblasts has been investigated in detail. A general method for the isolation of C27-steroids from media and cells was used, so that no major steroid was expected to escape detection. The final analysis was based on GC/MS.

When normal fibroblasts were incubated for 48 h in the absence of lipoproteins (media containing 10% LDS), only trace amounts (<1–10 pmol/mg of cell protein) of oxysterols could be detected in the medium and cells, with the exception of 7α-hydroxycholesterol, 7β-hydroxycholesterol, and 7-oxocholesterol (amounts about 5–50 pmol/mg of cell protein). Most of these steroids were probably formed by autoxidation of cholesterol during the purification of the samples, since their amounts did not differ significantly from those of the controls (15-min incubations). In contrast, when normal fibroblasts were incubated for 24–48 h with lipoproteins (media containing 10% FCS), 13 neutral and 5 acidic C27-steroids were found in the media. Steroids identified are listed in Table I. The identification was
Table I
Oxysterols in medium after incubating normal fibroblasts with lipoproteins
Oxygenated cholesterol derivatives identified in the neutral and acidic fractions from media (containing 10% FCS) after incubation with normal human fibroblasts and their gas chromatographic-mass spectrometric characteristics as trimethylsilyl ethers and methyl estertrimethylsilyl ether derivatives, respectively.

| No. | Steroid name | Structurea | Retention indexb | Molecular and significant ionsm/z |
|-----|--------------|------------|-----------------|---------------------------------|
| 1   | 7α-Hydroxycholesterol | C5,3α,7α-dl | 3115 | 546, 456 |
| 2   | 7α-Hydroxy-4-cholesten-3-one | C5,3α,7α-dl-3-one | 3210 | 472, 457, 382, 269 |
| 3   | 7β-Hydroxycholesterol | C5,3β,7β-dl | 3235 | 546, 456 |
| 4   | 7-Oxocholesterol | C5,3α-dl-7-one | 3375 | 472, 382, 367, 129 |
| 5   | 24-Hydroxycholesterol | C5,3α,24(R/S)-dl | 3385 | 546, 413, 145, 249 |
| 6   | 25-Hydroxycholesterol | C5,3α,25-dl | 3405 | 546, 456, 131 |
| 7   | 7α,25-Dihydroxycholesterol | C5,3α,7α,25-dl | 3390 | 634, 544, 131 |
| 8   | 7α,25-Dihydroxy-4-cholesten-3-one | C5,3α,7α,25-dl-3-one | 3490 | 560, 545, 412, 131 |
| 9   | 27-Hydroxycholesterol | C5,3α,27-dl | 3455 | 546, 456, 417, 129 |
| 10  | 7α,27-Dihydroxycholesterol | C5,3α,7α,27-dl | 3445 | 634, 544 |
| 11  | 7α,27-Dihydroxy-4-cholesten-3-one | C5,3α,7α,27-dl-3-one | 3545 | 560, 545, 470, 269 |
| 12  | 7β,27-Dihydroxycholesterol | C5,3β,7α,27-dl | 3555 | 634, 544 |
| 13  | 27-Hydroxy-7-oxocholesterol | C5,3β,27-ol-7-one | 3710 | 560, 545, 470, 129 |
| 14  | 3β-Hydroxy-5-cholestenoate | CA5,3β-dl | 3425 | 502, 412, 373, 129 |
| 15  | 3β,7α-Hydroxy-5-cholestenoate | CA5,3α,7α-dl | 3415 | 590, 500 |
| 16  | 7α-Hydroxy-3-oxo-4-cholestenoate | CA5,3α,7α-dl-3-one | 3415 | 516, 501, 426, 269 |
| 17  | 3β,7β-Hydroxy-5-cholestanoate | CA5,3β,7β-dl | 3530 | 590, 500 |
| 18  | 3β-Hydroxy-7-oxocholestenoate | CA5,3β,7β-dl-7-one | 3680 | 516, 426, 411, 129 |

a C, cholestane; CA, cholestanoate; superscript indicates position of double bond; greek letters denote configuration of hydroxyl groups.
b Kovats, on a fused silica capillary column coated with cross-linked methyl silicone.
c Intensities of fragment ions with m/z values above 200–300 were enhanced relative to those of lighter fragments; base peak is shown in italics; m/z, mass/charge.
d Tentative identification, reference compound not available.

Oxysterols in medium after incubating normal fibroblasts with lipoproteins. The metabolism of 7α-hydroxycholesterol and 7-oxocholesterol was studied by incubating these sterols (5 nmol) with normal human fibroblasts (Table II). The metabolism of oxysterols in transformed fibroblasts (Table II) which may be related to a decrease of the amount of its potential precursor 7α-hydroxycholesterol. These results indicated that 27-hydroxylation of sterols may be obstructed in transformed fibroblasts (see below).

The oxysterols were also studied with regard to the time course of their cellular production. Incubation of normal human fibroblasts for different lengths of time showed that the formation of 27-hydroxylated sterols had started 3–8 h after exposure to lipoproteins (Table III). No production of 25-hydroxylated sterols was observed during the first 24 h. A decrease of the amounts of 7α-hydroxycholesterol was noted after 8 h of incubation. Since the oxysterols having oxy groups both in the 7- and 27-positions could be derived from either 27-hydroxylated LDL cholesterol or 7-oxygenated cholesterol derivatives (autooxidation products present in the medium), their origin was investigated.

Metabolism of LDL Cholesterol and 7-Oxygenated Sterols in Normal Fibroblasts—The metabolism of 7α-hydroxycholesterol, 7β-hydroxycholesterol, and 7-oxocholesterol was studied by incubating these sterols (5 nmol) with normal human fibroblasts (protein content about 0.5 mg, dish size 57 cm2) for 24 or 48 h in media (10 ml) containing 10% LDS. The values given in percent represent the distribution of observed metabolites.

When 7α-hydroxycholesterol was incubated (24 h), the major metabolites found were 7α-hydroxy-4-cholesten-3-one (57%) and 7α,27-hydroxy-4-cholesten-3-one (43%) (acids were not analyzed). Only a small amount of 7α,27-dihydroxycholesterol (0.5%) was noted suggesting that oxidation/imerization of 7α-hydroxycholesterol to 7α,27-dihydroxycholesterol-3-one preceded 27-hydroxylation. The corresponding enzyme activities have been found previously in fibroblasts (20). When 7α-hydroxycholesterol can be converted to 7α-hydroxy-4-cholesten-3-one, 7α,27-dihydroxy-4-cholesten-3-one, and 7α-hy-
Regulation of Cholesterol Biosynthesis

Fig. 1. Gas chromatographic-mass spectrometric analysis of neutral oxysterols isolated from the medium after incubating normal fibroblasts with lipoproteins. Normal human fibroblasts (protein content: 1.1 mg, dish size: 143 cm²) were incubated with 10 ml of medium containing 10% FCS (cholesterol concentration: 1.2 mM) for 24 h, and the medium was then taken for analysis by GC/MS. Fragment ion current chromatograms characteristic of the trimethylsilyl ethers of oxysterols were constructed by the computer from mass spectra taken every 2 s during the analysis and for the purpose of illustration the intensities of the ions (m/z) were multiplied by appropriate factors. The principal sterols indicated by the numbers are listed in Table I. The equivalent of about 0.2 ml of medium was injected onto a Finnigan 55Q 710 instrument housing a 23-m fused-silica column coated with methyl silicone, and the oven temperature was programmed from 185 to 280 °C at a rate of 5 °C × min⁻¹.

droxy-3-oxo-4-cholestenic acid by normal fibroblasts and this could explain the appearance of these metabolites and the disappearance of 7α-hydroxycholesterol in media during the incubations with FCS (Table I).

The metabolism of 7β-hydroxycholesterol differed from that of 7α-hydroxycholesterol. When this sterol was incubated for 48 h with normal fibroblasts, the major metabolites were 7β,27-dihydroxycholesterol (1%) and 3β,7β-dihydroxy-5-cholestenic acid (62%). In addition, a large portion (about one-third) of 7β-hydroxycholesterol was converted to 7-oxocholesterol (20%), 27-hydroxy-7-oxocholesterol (1%), and 3β-hydroxy-7-oxo-5-cholestenic acid (14%). Oxidation of the 7β-hydroxy group also occurred when 7β,27-dihydroxycholesterol was incubated. No conversion of 7β-hydroxycholesterol to the corresponding 3-oxo-Δ⁴ derivative was observed, which was consistent with the absence of 7β-hydroxylated 3-oxo-Δ⁴ sterols in media after incubating fibroblasts with FCS (Table I).

Incubation of 7-oxocholesterol with normal fibroblasts resulted in the formation of 27-hydroxy-7-oxocholesterol (55%) and the corresponding C27-acid (35%). A small amount was converted to 7β-hydroxycholesterol (10%), but a conversion to 7α-hydroxylated products was not observed. Thus, the formation of C27-steroids having oxygen groups in both the 7- and 27-positions by normal fibroblasts could be due to the presence of autooxidation products of cholesterol in the medium. However, this did not exclude the possibility that 7α,27-dihydroxy-4-cholesten-3-one and 7α-hydroxy-3-oxo-4-cholestenic acid could also be derived from 27-hydroxycholesterol. 7α-Hydroxylation of 27-hydroxycholesterol in human fibroblasts was first noted by us (15) and was later found to be occurring generally in these cells (21). The product 7α,27-dihydroxycholesterol is extensively converted to 7α,27-dihydroxy-4-cholesten-3-one and the corresponding acid in the cells (15, 21).

In order to determine whether LDL cholesterol (via 27-hydroxycholesterol) could be converted to 7α,27-dihydroxy-4-cholesten-3-one and the acid, the contribution from 7α-hydroxycholesterol (which is always present when the medium contains lipoproteins) had to be accounted for. This was made possible by the following experiment. LDL and other lipoproteins in FCS were first labeled with [3H]cholesteryl oleate and then incubated with normal fibroblasts in the presence and absence of cyclosporin A (CsA), a selective inhibitor of the sterol 27-hydroxylase (6, 22, 23). When lipoproteins are labeled in this way, the cellular uptake of [3H]cholesteryl oleate is due solely to a LDL receptor-dependent process (i.e. a physiological uptake of LDL) (6). After the incubations, radioactive 27-hydroxycholesterol, 7α,27-dihydroxy-4-cholesten-3-one, and 7α-hydroxy-3-oxo-4-cholestenic acid were analyzed by HPLC. 3H-Labeled 7α-hydroxy-4-cholesten-3-one, the direct metabolite of 7α-hydroxycholesterol, was also determined. If 3H-labeled 7α-hydroxycholesterol (free or esterified) was present in the medium, its 3-oxidized metabolite was expected to accumulate in the presence of CsA, since the drug prevented further metabolism (see above). The amount of the metabolite would then reflect the contribution to 7α,27-dihydroxy-4-cholesten-3-one and the acid from 7α-hydroxycholesterol in the absence of CsA. Obviously, essentially no formation of 27-hydroxylated compounds was expected in the presence of CsA (see also Fig. 5). For comparison, fibroblasts were also incubated with lipoproteins labeled with [3H]cholesterol, whose cellular uptake is not entirely dependent on LDL receptors.

The results of these incubations are summarized in Table IV. In addition to 27-hydroxycholesterol, both 7α,27-dihydroxy-4-cholesten-3-one and 7α-hydroxy-3-oxo-4-cholestenoic acid were found as [3H]cholesteryl oleate. The HPLC analyses of these metabolites are shown in Fig. 2. Since no accumulation of radioactive 7α-hydroxy-4-cholesten-3-one was observed in the corresponding incubation with CsA (Table IV), autooxidation (7α-hydroxylation) of [3H]cholesteryl oleate had not occurred during the incubations. In the incubations with [3H]cholesterol, much larger amounts of radioactive 7α,27-dihydroxy-4-cholesten-3-one and the corresponding acid were found, although the amount of 27-hydroxycholesterol was less than with [3H]cholesterol oleate. However, the incubation with [3H]cholesterol and CsA resulted in a significant accumulation of 3H-labeled 7α-hydroxy-4-cholesten-3-one suggesting that most of the 27-oxygenated metabolites had been produced from autooxidized [3H]cholesterol (7α-hydroxycholesterol). The difference in chemical stability toward oxygen between [3H]cholesteryl oleate and the 5-cholesten-3-one.
de and [3H]cholesterol was surprising, but was confirmed by exposing them to air and heat in an aqueous/methanolic environment for 24 h. No autooxidation products (<0.1% of [3H]cholesterol de) could be detected, whereas about 2% of [3H]cholesterol were autooxidized. Thus, the results demonstrate that LDL cholesterol esters are hydrolyzed and are converted to 27-hydroxycholesterol, which is then 7α-hydroxylated and oxidized to 7α,27-dihydroxy-4-cholesten-3-one and 7α-hydroxy-3-oxo-4-cholesten-3-one. The latter seems to be the major metabolic end product of this extended LDL pathway in fibroblasts.

Metabolism of LDL Cholesterol and Side-chain Hydroxylated

Sterols in Transformed Fibroblasts—In contrast to normal fibroblasts, only small amounts of 27-oxygenated steroids were detected in media after incubating transformed human fibroblasts with lipoproteins (Table II). Although the increased amounts of 7α-hydroxy-4-cholesten-3-one could indicate that 27-hydroxylation of steroids was obstructed by cyclasepoin A (CSA, 10 μM), the accumulation of [3H]-labeled 7α-hydroxy-4-cholesten-3-one indicated the presence of autooxidized [3H]cholesterol or [3H]cholesterol de (i.e. free or esterified 7α-hydroxycholesterol) in the media during the incubation.

Table II

Production of oxysterols in normal and virus-transformed human fibroblasts when incubated with lipoproteins

The amounts of neutral and acidic oxygenated cholesterol derivatives were determined in the media (10 ml) containing 10% FCS (cholesterol concentration: 1.2 mM) after incubation for 0.25–24 h with normal human fibroblasts, only small amounts of 27-oxygenated sterols were detected in media after incubating transformed human fibroblasts, only small amounts of 27-oxygenated sterols were detected in media after incubating transformed human fibroblasts. Cell cultures incubated with lipoproteins in media containing 10% LDS were preincubated for 24 h in media containing 10% LDS.

| No. | Steroid structure | Normal fibroblasts | Virus-transformed fibroblasts |
|-----|------------------|--------------------|-----------------------------|
|     | Amount of oxysterol found in media | Amount of oxysterol found in media |
|     | 0.25 h; n = 2d | 48 h; n = 4d | 0.25 h; n = 2d | 48 h; n = 4d |
| 1   | C5-3,7α-3,4-7α | 171:117–225 | 54:39–81 | 156:81–228 | 60:54–123 |
| 2   | C5-3,7α-3,4-7α | <6:3–6 | 12:6–18 | <3:6–6 | 69:51–159 |
| 3   | C5-3,7α-3,4-7α | 138:108–165 | 114:99–156 | 144:81–207 | 129:75–216 |
| 4   | C5-3,7α-3,4-7α | 879:840–915 | 816:609–1140 | 993:633–1350 | 867:657–1881 |
| 5   | C5-3,7α-3,4-7α | 6:6–6 | 6:6–6 | 3:3–3 | 9:6–18 |
| 6   | C5-3,7α-3,4-7α | 6:3–12 | 45:25–150 | 9:3–18 | 27:15–30 |
| 7   | C5-3,7α-3,4-7α | <3:3–3 | <6:3–9 | <3:3–3 | <3:3–3 |
| 8   | C5-3,7α-3,4-7α | <6:3–<6 | 21:9–27 | <3:3–<6 | <3:3–<6 |
| 9   | C5-3,7α-3,4-7α | 12:6–18 | 219:153–267 | 12:9–12 | 21:15–33 |
| 10  | C5-3,7α-3,4-7α | <3:3–<3 | <3:3–<3 | <3:3–<3 | <3:3–<3 |
| 11  | C5-3,7α-3,4-7α | <12:12–12 | 270:144–321 | <9:3–12 | <9:3–<15 |
| 12  | C5-3,7α-3,4-7α | <3:3–3 | <3:3–3 | <3:3–3 | <3:3–<3 |
| 13  | C5-3,7α-3,4-7α | <9:6–12 | 81:33–108 | <12:6–18 | <9:6–15 |
| 14  | C5-3,7α-3,4-7α | 21:12–27 | 21:18–27 | 15:12–15 | 12:18–15 |
| 15  | C5-3,7α-3,4-7α | 9:6–9 | 6:3–6 | 6:6–6 | 6:3–6 |
| 16  | C5-3,7α-3,4-7α | 75:63–117 | 18:5–18 | 9:6–15 | 6:5–15 |
| 17  | C5-3,7α-3,4-7α | 12:9–12 | 51:45–72 | 3:3–3 | <3:3–<3 |
| 18  | C5-3,7α-3,4-7α | <3:3–<3 | 27:18–30 | <3:3–<3 | <3:3–<3 |

Table III

Time-response for the production of oxysterols in normal fibroblasts when incubated with lipoproteins

Table IV

Formation of radioactive metabolites from LDL [3H]cholesterol in normal fibroblasts

The amounts of [3H]labeled 27-hydroxycholesterol, 7α,27-dihydroxycholesterol, 7α,27-dihydroxycholesterol, 7α,27-dihydroxycholesterol, and 7α,27-dihydroxycholesterol were determined in media containing 10% FCS (cholesterol concentration: 1.2 mM) after incubation for 0.25–24 h with normal human fibroblasts (cholesterol content 1.7 mg, size of dishes 143 cm²). All cells had been preincubated for 24 h in media containing 10% LDS.
cholesteryl esters after incubating normal and transformed fibroblasts with lipoproteins labeled with radioactive cholesteryl or cholesteryl oleate for 48 h. Results on the oxysterol production from the same incubations are those shown in Table II. As seen in Table V, the cellular uptake and retention (cell content) of \(^{3}H\)cholesteryl oleate in normal and transformed cells were about 16 and 22%, respectively. About 4% had been esterified by both cell types. After the incubations with \(^{3}H\)cholesteryl oleate, the retention of the compound was about 11% in the normal and 15% in the transformed cells, although a major portion had been hydrolyzed in the cells. About 9% and 35% of hydrolyzed \(^{3}H\)cholesterol were also present in media of the normal and transformed fibroblasts, respectively, due to an efflux of hydrolyzed LDL cholesterol from the cells (6, 24).

When the cellular content and the efflux of cholesterol in the incubations with \(^{3}H\)cholesteryl oleate were added together, the total uptake of \(^{3}H\)cholesteryl oleate in the normal and the transformed cells was 19% (32%/mg of protein) and 50% (36%/mg of protein), respectively. These results show that the uptake of LDL (reflected by that of \(^{3}H\)cholesteryl oleate) was not decreased but possibly increased in the transformed cells also when the protein content of the cells was taken into account. Thus, a possible reduced formation of 27-hydroxylated sterols by transformed fibroblasts (Table II) was not due to a decreased uptake of LDL.

In order to determine whether the shortage of 27-hydroxysterols could be due to an increased metabolism (other than formation of C\(_{27}\) acids) or conjugation, the metabolism of 25-hydroxycholesterol was studied. The major reason for selecting this sterol instead of 27-hydroxycholesterol was that 25-hydroxycholesterol was available in a \(^{3}H\)-labeled form, so that major metabolites or conjugates could be traced and would not escape detection. Because of their similarities in structure (both having a \(3\beta\)-hydroxy-\(\Delta^{4}\) structure and one hydroxyl group in the side chain), the cellular handling of the two sterols was expected to be similar (except that the 25-hydroxy group could not be oxidized to a carboxyl group). Table VI shows the distribution of radioactivity when \(^{3}H\)-labeled 25-hydroxycholesterol (plus unlabeled, 1.2 nmol) had been incubated with normal and transformed fibroblasts for 48 h in 10% LDS. In addition to 25-[\(^{3}H\)]hydroxycholesterol, two major radioactive metabolites, one polar and one nonpolar, were found by HPLC. Other metabolites constituted less than 1% of the recovered radioactivity. No radioactivity (\(<0.1\%\)) corresponding to oxidized 25-[\(^{3}H\)]hydroxycholesterol without a \(7\)-hydroxy group (i.e. 25-hydroxy-4-cholesten-3-one, see below) was found. Neither did we find any radioactivity (\(<1\%\)) in fractions containing weak acids (e.g. having a free carboxyl group) or stronger acids (e.g. glucuronides or mono- or disulfates) which were isolated from the extracts by anion exchange chromatography. After collecting a fraction of the HPLC effluent containing the polar metabolite and derivatization, it was identified by GC/MS as 7\(\alpha\),25-dihydroxy-4-cholesten-3-one (21). The nonpolar metabolite(s) had a retention time (3.7 min), which was similar to that of the 3-acetate derivative of 25-hydroxycholesterol (retention time 4.9 min) by straight-phase HPLC (retention time of free 25-hydroxycholesterol was 11.8 min). It was therefore tentatively characterized as being fatty acid esters of 25-[\(^{3}H\)]hydroxycholesterol. This was supported further by the recovery of free 25-[\(^{3}H\)]hydroxycholesterol after treating the nonpolar metabolite(s) with mild alkali in a methanolic solution. Table VI clearly reveals large differences in the handling of 25-[\(^{3}H\)]hydroxycholesterol between the two cell lines. Intact 25-[\(^{3}H\)]hydroxycholesterol was found mainly in the cells (32% in the normal and 50% in the transformed cells). A large portion (about 43% 71%/mg of protein) of 25-[\(^{3}H\)]hydroxycholesterol had been converted to 7\(\alpha\),25-dihydroxy-4-cholesten-3-one by normal cells (21) but much less so (about 3% 2%/mg of protein) by the transformed cells. This sterol was recovered mainly in the media. On the other hand, esterification of 25-[\(^{3}H\)]hydroxycholesterol was noted only in the transformed cells, although the amount of esters was relatively small (about 7%). These results show that 25-hydroxycholesterol is readily taken up by both normal and transformed cells, but whereas the sterol is extensively 7\(\alpha\)-hydroxylated in normal cells, this reaction is obstructed in transformed cells. The results also suggested that the apparent shortage of 27-hydroxycholesterol in transformed cells was due to a decreased formation rather than an increased conjugation, since only a minor amount of the analogous sterol 25-hydroxycholesterol was esterified and since no other conjugates were found.

After these observations, the rates of oxidation/somerization of \(3\beta\),7\(\alpha\)-dihydroxy-3-oxo-\(\Delta^{4}\) steroids to 3-oxo-\(\Delta^{4}\) steroids in normal and transformed cells were also investigated. 7\(\alpha\),27-Dihydroxycholesterol (1.2 nmol) was therefore incubated with normal and transformed fibroblasts (protein contents 0.4 and 1.1 mg, respectively, size of dishes 57 cm\(^2\)) for 48 h in media (10 ml) containing 10% LDS, and the metabolites were then analyzed by GC/MS. Incubations for 15 min served as controls. The analyses showed that this sterol was readily taken up by the cells, since only about 1% remained in the media. In media from normal cells, about 26% and 39% were recovered as 7\(\alpha\),27-
TABLE V
Uptake and handling of free and esterified [3H]cholesterol in normal and transformed fibroblasts
Distribution of radioactivity after incubating normal and virus-transformed human fibroblasts for 48 h with media containing lipoproteins (10% FCS) labeled with [3H]cholesterol or [3H]cholesteryl olate. The total concentration of unlabeled cholesterol in FCS was 1.2 mM. Incubations for 0.25 h served as controls.

| Time (h) | Additions to incubation medium | Distribution of free and esterified [3H]cholesterol |
|---------|--------------------------------|---------------------------------------------------|
|         | Medium                          | Free (%)  | Ester (%) | Total (%) | Cells                          | Free (%)  | Ester (%) | Total (%) |
| 0.25    | [3H]Cholesterol in FCS          | 95        | 4         | 99        | Normal cells                           | 1         | <1        | 1        |
| 48      | [3H]Cholesterol in FCS          | 82        | 3         | 85        | Normal cells                           | 11        | 4         | 15       |
| 48      | [3H]Cholesterol in FCS          | 80        | 4         | 84        | Normal cells                           | 11        | 4         | 16       |
| 0.25    | [3H]Cholesteryl olate in FCS    | 2         | 98        | 100       | Normal cells                           | <1        | <1        | <1       |
| 48      | [3H]Cholesteryl olate in FCS    | 10        | 81        | 91        | Normal cells                           | 6         | 4         | 9        |
| 48      | [3H]Cholesteryl olate in FCS    | 11        | 77        | 88        | Normal cells                           | 9         | 3         | 12       |
| 0.25    | [3H]Cholesterol in FCS          | 95        | 4         | 99        | Transformed cells                       | 1         | <1        | 1        |
| 48      | [3H]Cholesterol in FCS          | 66        | 12        | 79        | Transformed cells                       | 17        | 4         | 21       |
| 48      | [3H]Cholesterol in FCS          | 73        | 5         | 78        | Transformed cells                       | 18        | 4         | 22       |
| 0.25    | [3H]Cholesteryl olate in FCS    | 3         | 99        | 100       | Transformed cells                       | <1        | <1        | <1       |
| 48      | [3H]Cholesteryl olate in FCS    | 38        | 49        | 86        | Transformed cells                       | 11        | 3         | 14       |
| 48      | [3H]Cholesteryl olate in FCS    | 34        | 51        | 84        | Transformed cells                       | 12        | 3         | 16       |

a Protein contents of normal and virus-transformed fibroblasts were 0.6 mg and 1.4 mg, respectively. The size of the incubation dishes was 57 cm². All cells had been preincubated for 24 h in media containing 10% LDS. Results from these incubations are also shown in Table II.

b The amounts of [3H]cholesterol and [3H]cholesteryl olate added to the medium were 22.8 × 10⁶ dpm and 18.9 × 10⁶ dpm, respectively. The sterols were preincubated with FCS for 16 h at 20°C.

c The total recovery was >90% of the radioactivity added.

TABLE VI
Metabolism of 25-[3H]hydroxycholesterol in normal and transformed fibroblasts
Distribution of recovered radioactivity after incubating normal and virus-transformed human fibroblasts for 48 h with media containing [3H]-labeled and unlabeled 25-hydroxycholesterol and 10% LDS (cholesterol concentration, 0.1 mM). Incubations for 0.25 h served as controls.

| Radioactive compound     | Normal fibroblasts | Transformed fibroblasts |
|--------------------------|--------------------|-------------------------|
|                          | 0.25 h             | 48 h                    | 48 h |
|                          | 0.25 h             | 48 h                    | 48 h |
| % recovered radioactivity|                    |                        |

a The amounts of [3H]-labeled and unlabeled 25-hydroxycholesterol added to the medium (10 ml, containing 10% LDS) were 1.4 × 10⁶ dpm and 1.2 nmol, respectively. Protein contents of normal and virus-transformed fibroblasts were 0.6 mg and 1.4 mg, respectively. The size of the incubation dishes was 57 cm². All cells had been preincubated for 24 h in media containing 10% LDS.

b The total recovery was about 90% of the radioactivity added.

c Retention time on reversed-phase HPLC was 3.5-4.0 min using hexane/propyl alcohol (97:3) as the mobile phase and a flow-rate of 1 ml × min⁻¹. It was tentatively identified as 25-hydroxycholesterol esterified with a fatty acid.

d Retention time on reversed-phase HPLC was 3.0-4.5 min using methanol/ethanol/water (80:20:10) as the mobile phase and a flow-rate of 1 ml × min⁻¹. In this fraction, 7α,25-dihydroxy-4-cholesten-3-one was identified by gas chromatography-mass spectrometry, and the amounts were similar to those calculated from the radioactivity.

e Also includes other radioactive compounds, each accounting for less than 1% of the total radioactivity.

Regulation of Cholesterol Biosynthesis

dihydroxy-4-cholesten-3-one and 7α-hydroxy-3-oxo-4-cholestenic acid, respectively. The corresponding values for the transformed cells were 28% and 19%. Trace amounts (about 1%) were converted to 3β,7α-dihydroxy-5-cholestenic acid in the transformed cells. No 7-oxo-, 7β-hydroxy-, or other metabolites were found. Thus, almost the same amounts of 7α,27-dihydroxycholesterol were oxidized by the normal and transformed fibroblasts. However, when the number of incubated cells (cell protein content) were taken into account, the oxidation rate in transformed cells was calculated to be about 25% of that of normal cells. These studies show that the apparent activities of 27- and 7α-hydroxylation enzymes are much lower in transformed than in normal fibroblasts (estimated to be <2% of the normal activity when corrected for the cellular protein content or total uptake of LDL), whereas the enzyme catalyzing oxidation of 3β-hydroxy-Δ⁵ sterols is affected to a lesser extent.

Suppression of HMG-CoA Reductase by LDL and Oxysterols in Fibroblasts—The suppression of HMG-CoA reductase by LDL in normal and transformed fibroblasts was also studied and was related to the production of different oxysterols. Fig. 3 shows that the response to LDL was defective in the transformed cells when compared with normal fibroblasts. In fact, almost 10 times higher concentrations of FCS (4% versus 0.4%) were required to cause a 50% suppression of the activity of HMG-CoA reductase in the transformed cells.
The effects of the different metabolites of LDL cholesterol and other oxysterols on HMG-CoA reductase in normal and transformed cells were also tested. A low oxysterol concentration (0.12 μM) was selected, so that a partial conversion into a more potent metabolite might be discovered, at least when the reaction was obstructed. Also, a 0.06 μM concentration of the most potent sterols could induce >50% suppression of HMG-CoA reductase in normal cells, but 0.03 μM had only a weak suppressive effect (<20%).

Table VII shows that oxysterols which were normally 27-hydroxylated or 7α-hydroxylated in fibroblasts, had much less suppressive effects in transformed than in normal cells (mean: 32% versus 68% suppression). Also, sterols that were normally 3-oxidized/isomerized had less suppressive effects in transformed than in normal cells (mean: 46% versus 74% suppression). In contrast, the sterol metabolites with a 3-oxo-Δ5 structure and a hydroxyl group in the side-chain (7α,27-dihydroxy-4-cholesten-3-one and 7α,25-dihydroxy-4-cholesten-3-one) were potent suppressors also in the transformed cells, and their effects were essentially the same as in normal cells. This result was surprising. It implied that 27-hydroxycholesterol and 25-hydroxycholesterol had to be metabolized in order to be active, since the transformed fibroblasts could respond normally to their metabolites. Furthermore, the regulatory defect in the transformed cells was evidently not beyond the formation of these metabolites. We have previously reported that 27-hydroxylated 3-oxo-Δ5 sterols are potent suppressors in normal fibroblasts (15), and, more recently, we have found that one of the mechanisms by which they act is by inhibiting the synthesis of HMG-CoA reductase. In agreement with our previous results (15), these compounds were active also when they lacked a 7α-hydroxy group (Table VII). Oxidation of the 27-hydroxy group to an acid seemed to decrease their biological activity (15) at least in the transformed fibroblasts. In addition to this group of sterols, 27-hydroxy-7-oxo-cholesterol was also found to be a potent suppressor in the transformed fibroblasts, although it seemed to have a stronger effect in normal cells (Table VII).

Since the production of 7α,27-dihydroxy-4-cholesten-3-one and 27-hydroxy-7-oxocholesterol had started at the time when a significant suppression (73%) of the activity of HMG-CoA reductase occurred (3–8 h after exposing normal fibroblasts to lipoproteins, Fig. 4), there was a possibility that the two events were related. However, if a hindered conversion of LDL cholesterol and autooxidation products to side-chain hydroxylated 3-oxo-Δ5 sterols and 27-hydroxy-7-oxocholesterol was causing the definitive response to the former sterols in the transformed cells, then the same was expected to be seen in normal fibroblasts when treated with inhibitors of the sterol metabolizing enzymes. Table VIII shows the effects of treating normal human fibroblasts with CsA and thereby preventing 27-hydroxylase in the cells. Consistent with the behavior of transformed fibroblasts, the suppressive effects on HMG-CoA reductase of LDL cholesterol or oxysterols, which were 27-hydroxylated in untreated cells, decreased or were abolished when CsA was present in the medium. Table VIII also shows that 3β-hydroxy-5α-cholest-8(14)-en-15-one, a potent suppressor of HMG-CoA reductase with an oxo group in the D-ring, most likely had to be 27-hydroxylated before being active (25). As expected, CsA did not interfere with the activity of 27-hydroxylated sterols. Since the metabolism of 7α-hydroxy-4-cholesten-3-one was limited to 27-hydroxylation in normal fibroblasts (see above), its metabolite 7α,27-dihydroxy-4-cholesten-3-one (or possibly the acid) was likely to be a true suppressor of HMG-CoA reductase.

Attempts to prevent 7α-hydroxylation in normal cells using ketoconazole, a general cytochrome P-450 inhibitor (26), were unsuccessful. This drug had a significant effect on the 27-hydroxylase when added to the medium 30 min before the sterols, but the effects on the 7α-hydroxylase were inconsistent. No further attempts were made to find an inhibitor of the sterol 7α-hydroxylase.

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Regulation of Cholesterol Biosynthesis

TABLE VII

| No. | Steroid structure<sup>a</sup> | Observed metabolism<sup>b</sup>                                                                 | Activity of HMG-CoA reductase<sup>c</sup> | ∆ Suppression<sup>d</sup> |
|-----|-----------------------------|------------------------------------------------------------------------------------------------|-----------------------------------------|-------------------------|
|     |                             |                                                                                                 | Normal cells                           | Transformed cells       |                        |
| 2   | LDL cholesterol<sup>e</sup> | 27-Hydroxylation                                                                                 | 15–31                                   | 40–88                   | 32–57                  |
| 3   | C<sub>3</sub>·7α-ol-3-one    | 27-Hydroxylation                                                                                 | 26                                      | 50                      | 24                     |
| 4   | C<sub>3</sub>·3β,7β-ol       | 7-Oxidation or 27-Hydroxylation                                                                   | 39                                      | 60                      | 21                     |
| 5   | C<sub>3</sub>·3β,7β-ol-7-one | 27-Hydroxylation                                                                                 | 38                                      | 100                     | 62                     |
| 6   | C<sub>3</sub>·3β,7α-ol-15-one<sup>e</sup> | 27-Hydroxylation<sup>e</sup>                           | 22                                      | 41                      | 19                     |
| 7   | C<sub>3</sub>·3β,7α,25-ol    | 7α-Hydroxylation                                                                                 | 37                                      | 77                      | 40                     |
| 8   | C<sub>3</sub>·3β,7α,25-ol-3-one<sup>e</sup> | 3-Oxidation/isomerization                        | 33                                      | 79                      | 46                     |
| 9   | C<sub>3</sub>·3β,7α,25-ol-7-one<sup>e</sup> | 7α-Hydroxylation<sup>e</sup>                           | 23                                      | 49                      | 26                     |
| 10  | C<sub>3</sub>·3β,7α,27-ol    | 3-Oxidation/isomerization                                                                         | 30                                      | 60                      | 30                     |
| 11  | C<sub>3</sub>·3β,7α,27-ol-3-one<sup>e</sup> | 7α-Hydroxylation<sup>e</sup>                           | 31                                      | 38                      | 7                      |
| 12  | C<sub>3</sub>·3β,7α,27-ol-7-one<sup>e</sup> | 7α-Hydroxylation<sup>e</sup>                           | 33                                      | 34                      | 1                      |
| 13  | C<sub>3</sub>·3β,7α,29-ol-3-one<sup>e</sup> | 7α-Hydroxylation<sup>e</sup>                           | 7                                       | 33                      | 26                     |
| 14  | C<sub>3</sub>·25β-ol-3-one   | None                                                                                             | 33                                      | 37                      | 4                      |
| 15  | CA<sub>3</sub>·7α-ol-3-one  | None                                                                                             | 26                                      | 31                      | 5                      |
| 16  | CA<sub>3</sub>·7α-ol-3-one  | None                                                                                             | 30                                      | 47                      | 17                     |

<sup>a</sup> For abbreviations and steroid names, see Table I.

<sup>b</sup> Metabolism observed in normal human fibroblasts.

<sup>c</sup> Difference in degree of suppression of HMG-CoA reductase in normal and transformed fibroblasts induced by LDL or the oxysterol.

<sup>d</sup> The activities of HMG-CoA reductase in normal and transformed fibroblasts were 58 and 96 pmol/min/mg of protein, respectively.

<sup>e</sup> Results also shown in Fig. 3.

<sup>f</sup> Oxysterol not normally detected in medium or cells (fibroblasts) after incubation with FCS.

<sup>g</sup> Not studied, but concluded from the results in Table VIII.

Fig. 4. Time-response curves for the LDL-induced production of 7α,27-dihydroxy-4-cholesten-3-one (●), 27-hydroxy-7-oxocholesterol (●●), and 7α,25-dihydroxy-4-cholesten-3-one (⊡), and suppression of HMG-CoA reductase (○) in normal human fibroblasts. The cells (protein content: 1.7 mg; dish size: 143 cm<sup>2</sup>) were incubated with medium (15 ml) containing 10% FCS and were harvested at the indicated times. The concentration of cholesterol in FCS was 1.2 mM. All cells were preincubated for 24 h in media containing 10% LDS (see also Table III). For comparison, the production of 27-hydroxycholesterol (△) is also shown.

In cultured human fibroblasts, cholesterol is metabolized in breast and colonic carcinoma cells, a short fall of LDL receptors and an increased sulfation rate in the latter could contribute to a low production of potent 27-hydroxylated metabolites.

In contrast, the rates of metabolism of LDL cholesterol and oxysterols were increased in malignant melanoma cells (1.0 mg of cell protein) when incubated with lipoproteins (10% FCS) for 48 h. Yet the responses to LDL and oxysterols were similar to those seen in the other tumor cells (Table IX). However, like in liver cells (19, 27, 28), 27-hydroxylated metabolites were extensively converted to their corresponding acids, the major ones being 7α-hydroxy-3-oxo-4-cholestenolic acid and 3β-hydroxy-7-oxo-5-cholestenoic acid (2.6 and 2.0 nmol/mg of cell protein/48 h, respectively). Consequently, the accumulation of the 27-hydroxy suppressors were low also in these cells, as reflected by their concentrations in media. The amounts of 7α,27-dihydroxy-4-cholesten-3-one and 27-hydroxy-7-oxocholesterol were only about 0.2 and 0.06 nmol/mg of cell protein/48 h, respectively. These studies show that common to all these tumor cell lines were a defective response to LDL and a low intracellular concentration (accumulation) of 7α,27-dihydroxy-4-cholesten-3-one and 27-hydroxy-7-oxocholesterol, which was probably due to either a decreased formation or an increased metabolism.

DISCUSSION

Cultured human fibroblasts have been widely used in studies of regulatory mechanisms of cholesterol homeostasis (1–6, 29–33). Although oxysterols have documented suppressive effects on HMG-CoA reductase (34–38), their formation in human fibroblasts has not been studied until we recently showed that fibroblasts converted LDL cholesterol to 27-hydroxycholesterol (6). We also showed that this oxysterol is an important mediator between LDL and the suppression of HMG-CoA reductase in the cells (6). The involvement of oxysterols in the regulation of cholesterol homeostasis, their formation, and biological activity in human fibroblasts have now been investigated further.

In the absence of LDL, a production of oxysterols in normal fibroblasts was not observed. In the presence of LDL, normal fibroblasts produced several oxysterols and C<sub>27</sub>-acids in addition to 27-hydroxycholesterol. These oxygenated C<sub>27</sub>-steroids were all formed from LDL cholesterol or autooxidation products of cholesterol by a relatively extensive metabolism. Fig. 5 shows a simplified scheme of their metabolic pathways and their conversion into potent HMG-CoA reductase suppressors in normal human fibroblasts. Briefly, LDL is internalized by LDL receptors. Following hydrolysis of cholesteryl esters, a portion of LDL cholesterol is converted to 27-hydroxycholesterol, which is metabolized further to 7α,27-dihydroxy-4-cholesten-3-one and the corresponding C<sub>27</sub>-acid. However, the lat-
ter two sterols can also be formed from the autooxidation product 7α-hydroxycholesterol (Fig. 5), and their origin will therefore depend on the presence and cellular uptake of LDL as well as 7α-hydroxycholesterol. It is interesting that the metabolic pathways of these sterols in fibroblasts show so many similarities to those in human liver cells (19, 27, 28). This suggests that many of the reactions of sterols seen in fibroblasts may be occurring generally in human cells (see also below) although the activities of the catalyzing enzymes may vary in different tissues.

The two other major autooxidation products, 7-oxocholesterol and 7β-hydroxycholesterol, were both 27-hydroxylated and then oxidized to the corresponding C27-acids (Fig. 5). A large portion of 7β-hydroxycholesterol was also converted to 7β-cholesten-3-one and its side-chain oxygenated metabolites. 25-Hydroxycholesterol is only a minor autoxidation product of cholesterol, and it was found to be metabolized analogously to that of 27-hydroxysterol, except that it will not form an acid. Interestingly, 25-hydroxylation of sterols and the formation of 7α,25-dihydroxy-4-cholesten-3-one was by far the major oxysterol formed (0.9–1.1 nmol/mg of cell protein) after incubation with media containing 10% FCS for 48 h, and this could explain how these cells can respond to LDL (see below) (6). Thus, 25-hydroxylation of sterols may serve as an extra hydroxylating system in cells, being active when the capacity of the 27-hydroxylase is exceeded by substrate.

Exposure of lipoproteins to oxygen (air) prior to or during the incubations will result in autoxidation of cholesterol, and the amounts of the products can be considerable, especially when LDL-particles have been isolated from serum. The concentrations of autoxidation products are usually low in the circulation of man (39), but their presence has to be considered when the biological effects of LDL on cultured cells are being studied. Most of these sterols will contaminate the LDL particles (40), and, thus, the biological effects of LDL cholesterol are difficult to distinguish from those of the autoxidation products. In order to limit the amounts of autoxidation products present in our incubations, we have deliberately used intact serum instead of purified LDL.

As expected, LDL cholesterol and the autoxidation products 7α-hydroxy-, 7β-hydroxy-, and 25-hydroxycholesterol and 7-oxocholesterol all had strong suppressive effects on HMG-CoA reductase in normal fibroblasts (29, 37). However, none of these was particularly active in transformed fibroblasts. When the effects of their metabolites were determined in the latter cells,
only 7α,27-dihydroxy-4-cholesten-3-one, 7α,25-dihydroxy-4-cholesten-3-one, and 7α,27-dihydroxycholesterol were potent suppressors of HMG-CoA reductase. The apparently normal response to these metabolites suggested that the regulatory defect in transformed cells was localized prior to and not beyond their formation. In transformed fibroblasts, their formation was shown to be hindered by reduced activities of enzymes catalyzing 27-hydroxylation, 7α-hydroxylation, and 3-oxidation with isomerization of the 5-double bond; and IV, oxidation to a 27-carboxy group. Hydrolyzed LDL cholesterol was shown to be metabolized via these reactions (filled arrows). Oxidation of a 7β-hydroxy group (V) to a ketone was also observed. Minor reactions noted are shown by broken arrows. The formation of 7α,25-dihydroxy-4-cholesten-3-one by 25-hydroxylation of sterols (not shown) was observed under specific conditions. Reactions I, II, and III were obstructed in virus-transformed fibroblasts displaying a defective suppression of HMG-CoA reductase by LDL cholesterol and autoxidation products of cholesterol. Sterol metabolites with an apparently normal suppressive effect also in transformed cells are indicated in-frame.

Although the three most potent suppressors of HMG-CoA reductase had different structures, there were apparent similarities between them as illustrated in Fig. 6. Common to the sterols were an oxo group with a conjugated double bond in the steroid nucleus and a distal hydroxyl group in the side chain. Additional hydroxyl groups in the steroid nucleus did not seem to affect their biological activity (15). Interestingly, 3β-hydroxy-5α-cholestan-8(14)-en-15-one (25) also belongs to this group after 27-hydroxylation. This was in agreement with the results obtained for this compound (see Tables VII–IX). These sterols did not seem to be metabolized further in order to be active. For example, saturation of the double bond resulted in an almost complete loss of their activity (15), reduction of the oxo group to a hydroxyl group also decreased the activity as seen when the activity of 27-hydroxy-7-oxo-cholesterol was compared with that of 7α,27-dihydroxycholesterol. Further oxidation to an acid was apparently not required, since the active 25-hydroxylated sterols did not form acids. However, the corresponding steroids with a 27-carboxy group may also be active, when present in a nonionized form. Furthermore, we did not find any evidence that additional oxygen groups were required in the steroid nucleus or in the side chain for these sterols to be active. Due to the flexibility of the side chain, its hydroxyl group and the oxo group (with double bond) in the steroid nucleus may not have to be situated at certain positions (Fig. 6). Thus, in addi-
Regulation of Cholesterol Biosynthesis

While the actions of oxysterols are able to respond to the esterification of sterols (41). A binding protein for this group of oxysterols have been characterized previously (42-45), but we do not know if this protein can be involved in the actions of acyl-CoA:cholesterol acyltransferase.

The formation of 7α,27-dihydroxy-4-cholesten-3-one from LDL cholesterol was obstructed by low activities of sterol-metabolizing enzymes in the transformed fibroblasts. The mechanisms underlying these multiple enzyme deficiencies are not known. However, when normal fibroblasts were incubated for 48 h with conditioned media containing 10% LPS, transformed fibroblasts and then were exposed to lipoproteins for 24 h, the uptake of LDL increased by 40%, while the formation of 27-oxygenated sterols decreased by 20%. This may suggest that a substance (or substances) could have been released from the transformed cells into the medium, which affected the regulatory response to LDL also in normal cells. In relation to this, the observation that interleukin 1α and tumor necrosis factor α stimulate the activity of the 7α-hydroxylating enzyme in rat ovaries is interesting (46). According to our results and in contrast to the speculations of the authors and others (47), this would be expected to increase the suppression of HMG-CoA reductase by side-chain hydroxylated oxysterols (and possibly by LDL) if rat cells behave in the same way as human fibroblasts. Thus, our observation that LDL cholesterol and autooxidation products seem to be metabolized prior to being biologically active implies that cholesterol homeostasis in cells can be regulated by factors that determine the activities of the sterol-metabolizing enzymes.

Common to all the tumor cell lines studied was a low intracellular accumulation of active HMG-CoA suppressors due to a decreased formation or an increased metabolism. Although an altered sterol metabolism may be a common cause of a defective feedback control in tumor cells, it cannot be excluded that other defects may also exist in some cancer cells. Nonetheless, a reduced response to LDL seems to be characteristic of malignant cells (11), and it has been speculated that this phenomenon may be essential for the growth of tumor cells by increasing the cellular supply of cholesterol and intermediates in the mevalonate pathway (11). This hypothesis can now be tested, since the enzyme deficiencies in tumor cells can be by-passed and their cholesterol homeostasis normalized by the described HMG-CoA reductase suppressors. Preliminary results show that the growth of transformed fibroblasts is stopped in a cell cycle specific way by these suppressors.

In conclusion, the results of this study provide information about how LDL and various oxysterols suppress HMG-CoA reductase in human cells, about structural requirements of oxysterols for being suppressors of HMG-CoA reductase, and about mechanisms underlying a defective regulatory response to LDL in neoplastic cells. The results may stimulate the interest in finding a receptor protein which can bind this new group of oxysterols, in finding factors regulating cholesterol homeostasis in cells by affecting the activities of sterol-metabolizing enzymes and in elucidating the biological significance of malignant cells having a defective response to LDL.

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Fig. 6. Structures of the three naturally occurring cholesterol derivatives that were potent suppressors of HMG-CoA reductase also in transformed fibroblasts. The sterols 7α,27-dihydroxy-4-cholesten-3-one (I), 7α,25-dihydroxy-4-cholesten-3-one (II), and 27-hydroxy-7α-cholesten-3-one (III) did not seem to require further metabolism in order to suppress HMG-CoA reductase in human fibroblasts. Common to these sterols is the presence of an oxo group with a conjugated double bond in the steroid nucleus and a distal hydroxyl group in the side chain. The sterols are drawn in such a way that their apparent structural similarities are illustrated. For comparison, 27-hydroxylated 3β-hydroxy-5α-cholestan-8(14)-ene-15-one (IV) is also shown.

In a separate study, we have found that 27-hydroxylated 3-oxo-Δ5 steroids, the corresponding 24-hydroxylated steroid was found to be a highly potent suppressor of HMG-CoA reductase. These results suggest that the oxysterols may bind to a common receptor protein, which could be involved in the suppression of HMG-CoA reductase. To our knowledge, none of the oxysterols shown in Fig. 6 has been used in the search for such a receptor. The relatively extensive metabolism of sterols in fibroblasts (Fig. 5) and the apparently few structural requirements for being a suppressor of HMG-CoA reductase (Fig. 6) may explain how such a wide range of different oxysterols can be biologically active in the cells. On the other hand, not until an oxysterol receptor has been found, can the true nature of its ligands be established with certainty.

Out of the three suppressors formed, 7α,27-dihydroxy-4-cholesten-3-one seems to be biologically most important under normal conditions (see above). It was quantitatively the major suppressor, it was rapidly formed and it was derived from LDL cholesterol. Although suppressors may also be formed from autooxidation products of cholesterol, the latter sterols were evidently not required for fibroblasts to maintain their cholesterol homeostasis. For simplicity we are now calling 7α,27-dihydroxy-4-cholesten-3-one “cytosterone” in analogy with the names of potent steroid hormones and since the sterol can be formed in several different human tissues.

In a separate study, we have found that 27-hydroxylated 3-oxo-Δ5 steroids decrease the syntheses of HMG-CoA reductase and LDL receptors in fibroblasts. These effects were expected of a suppressor derived from LDL (4). However, the formation of 7α,27-dihydroxy-4-cholesten-3-one or the other suppressors did not seem to be required for stimulating esterification of sterols in the cells. In fact, this reaction may be triggered by 3β-hydroxy-Δ5 steroids with a hydroxyl group in the side chain, since 25-hydroxycholesterol was more esterified in the transformed than the normal fibroblasts (Table V1). This is in agreement with the previous finding that mutant cells

2 M. Axelsson and S. Vitols, manuscript in preparation.
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