Lipid metabolism in cultured cells XVI. Lipoprotein binding and HMG CoA reductase levels in normal and tumor virus-transformed human fibroblasts

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Abstract The loss in feedback control of cholesterol biosynthesis in tumor cells was examined in tissue culture. Human fibroblasts from normal subjects, SV40 tumor virus-transformed cell lines, and homozygous familial hypercholesterolemic cells as reference, were grown in tissue culture. Experiments were conducted to relate the regulatory enzyme for cholesterol biosynthesis, HMG CoA reductase, and the membrane-located binding receptors for low density lipoproteins (LDL) that mediate feedback control in normal cells. Monolayers of virus-transformed tumor cells exhibited specific ^{125}I-labeled LDL binding of 152 ± 21 ng/mg cell protein, which was essentially the same as that of normal fibroblasts (135 ± 20 ng/mg). Binding of LDL by familial hypercholesterolemic cells used as controls was only 8 ± 3 ng/mg under the same test conditions. Basal levels of HMG CoA reductase in tumor cells of 45.2 ± 6.5 units/mg cell protein were about twice those of normal cells. However, in contrast to the lack of feedback control of this enzyme observed with tumors in vivo, in both the normal and the transformed cells in vitro, activity of the enzyme decreased about fourfold when serum lipids were added. These findings demonstrate that tumor cells growing in vitro contain a normal complement of the membrane-located binding receptors for low density lipoproteins and, although the basal levels are higher than normal, an effective feedback regulation of the enzyme HMG CoA reductase is retained.

Supplementary key words lipoproteins · tissue culture · tumor cells · HMG CoA reductase · feedback control · cholesterol synthesis

It has been shown by Siperstein, Fagan, and Morris (1) that hepatomas and leukemic cells in vivo have a defect in the regulatory mechanisms for cholesterol biosynthesis. It was shown that this defect was the loss of effective feedback control of the enzyme HMG CoA reductase, which converts HMG CoA to mevalonic acid and is the principal regulatory point in the biosynthetic pathway for cholesterol (2). Previous studies have shown that the loss of feedback control of cholesterol biosynthesis, which can be demonstrated in hypercholesterolemic cells in vitro, is due to a deficiency in a cell surface receptor for low density lipoproteins (LDL) (3).

These observations, plus the well-documented alterations in various membrane components that accompany cellular transformation, suggested that the relationship between defective control of cholesterol synthesis and membrane receptor sites for LDL in tumor cells should be examined in tissue culture.

This paper describes studies in which human fibroblast cell lines from normal and hypercholesterolemic subjects as well as tumor cell lines derived from normal fibroblasts by transformation with the oncogenic SV40 virus were grown in tissue culture. These cells were then studied for the responses of HMG CoA reductase levels to added serum lipoproteins. Furthermore, the interaction of ^{125}I-labeled LDL with normal fibroblast cells was compared with those of their virus-transformed counterparts to investigate the membrane-bound LDL receptor sites in the various types of cells.

MATERIALS AND METHODS

3-Hydroxy-3-methyl[3-^{14}C]glutaryl coenzyme A (11.7 μCi/μmol), DL-[5-^{3}H]mevalonic acid (DBED salt), 6.74 Ci/mmol, and Na^{125}I were purchased from New England Nuclear Corp., Boston, MA. DL-[2-^{14}C]Mevalonic acid lactone, 10.3 mCi/mmol was obtained from Amersham/Searle, Arlington Heights, IL. Glucose-6-phosphate (Na salt), glucose-6-phosphate dehydrogenase (300 units/mg), dithioerythritol,

Abbreviations: FH, familial hypercholesterolemia; LDL, low density lipoprotein; HMG CoA, hydroxy-methylglutaryl coenzyme A; MEM, Eagle's minimal essential medium.

and NADP were from Sigma Chemical Co., St. Louis, MO. Precoated plates for thin-layer chromatography (Silica gel 60, 0.25 mm thickness, 5 × 20 cm) were purchased from Brinkman Instruments Co., Westbury, N.Y.

Normal human skin fibroblasts (Bo Mat and T.J.) and normal human fetal lung fibroblasts (WI-26 and WI-38) were from the Wistar Institute and American Type Cell Collection respectively. VA-4 (SV40 viral-transformed product of WI-38) were gifts from the Wistar Institute. Homozygous familial hypercholesterolemic skin fibroblasts (V.C. and GM-361) were obtained from the National Heart and Lung Institute and The Institute for Medical Research, N.J., respectively. Cell cultures were grown in a CO_2 incubator at 37°C in Eagle's minimum essential medium (MEM), (Grand Island Biological Co., Grand Island, N.Y.) supplemented with $NaHCO_3$ (2.2 g/l), 10% fetal bovine serum, 1% essential vitamin mixture (100X), and antibiotics (50 units/ml of penicillin and 50 μg/ml of streptomycin). Plastic T flasks containing normal and VA-4 cells took 5–6 days and 3–4 days, respectively, to reach confluency as determined by microscopic examination. Flasks of confluent cells were then treated with 0.25% trypsin and seeded into four 25 cm^2 T flasks. Cells were grown an additional 3 days to reach confluency. The protein content of normal and VA-4 cells was found to be 160–200 μg and 550–750 μg per flask, respectively, by the method of Lowry, et al. (4).

Preparation and iodination of human low density lipoproteins

Human low density lipoproteins (d 1.019–1.063 g/ml) and lipoprotein-free serum (LPFS), (d > 1.215 g/ml), were isolated (5) after centrifuging fresh human serum for 30 min at 10,000 rpm to remove chylomicrons. The infranatant was removed and centrifuged in a Beckman L3-50 ultracentrifuge using a Type 65 rotor at 50,000 rpm for 16 hr. The very low density lipoproteins (VLDL) floated and were removed; the density of the infranatant was adjusted to 1.019 g/ml by adding 0.8 ml of salt solution of d 1.085 g/ml to each 4 ml. The d 1.019 mixture was then centrifuged for 20 hr at 50,000 rpm and LDL class 1 (LDL1) was isolated. The infranatant was adjusted to a density of 1.063 g/ml by adding 1.5 ml of d 1.21 salt solution to each 4.5 ml, and was again centrifuged for 20 hr at 50,000 rpm. This floated the low density class 2 (LDL2). The infranatant was adjusted to a density of 1.21 g/ml by adding 1.117 g of KBr to each 4.5 ml, made up to 5 ml with H_2O, and was centrifuged at 50,000 rpm for 27 hr to remove the HDL. The infranatant from the final solution was saved as the source of lipoprotein-free serum. Each lipoprotein fraction was further purified by a second ultracentrifugation in solutions of the appropriate buoyant density. Removal of KBr and buffer exchange were accomplished by concentration dialysis of the lipoprotein fractions through a PM-10 Diaflo membrane with buffer containing 20 mM Tris-HCl (pH 7.4), 0.15 M NaCl, and 0.3 mM EDTA (buffer A). The purity of lipoprotein fractions was confirmed by polyacrylamide gel electrophoresis and agarose gel immuno-electrophoresis. The LDL 2 fraction (d 1.019–1.063) was concentrated and iodinated by a modification of the iodine monochloride method of McFarlane (6). A solution of LDL (0.4 ml, 16 mg protein) was diluted with glycine buffer (0.75 ml, pH 10) at 0°C. Stock $Na^{125}I$ solution (10 mCi in 0.1 ml of 0.05 N NaOH diluted to 0.5 ml with glycine buffer) and freshly prepared iodine monochloride stock solution (15 μl, 4.2 mg/ml) were mixed with LDL and incubated for 5 min in an ice bath. The reactants were diluted with an excess of buffer A, filtered through an XM-100 Diaflo membrane, and washed repeatedly with buffer A to remove non-protein-bound ^{125}I. The final preparation was sterilized by filtration through a 0.45 μm Millipore filter using a Swinnex syringe adapter. The ^{125}I-labeled LDL contained 0.024 mol of iodine per mol of LDL. Only 5.4% was extractable by lipid solvents and more than 90% of the radioactivity was precipitable by trichloroacetic acid. The specific activity of ^{125}I-labeled LDL was 192 dpm/ng protein. In addition, ^{125}I-labeled LDL was shown to retain its antigenic integrity as judged by immunodiffusion.

HMG CoA reductase assay

Bo Mat, WI-38, V.C., and VA-4 cells were incubated in MEM with and without serum lipids for 24 hr. At the end of the incubation, the cell monolayers were scraped from the flasks with 1 ml of 0.5 M Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl. Cell pellets were collected by centrifugation at 900 *g* for 3 min. The pellets were dissolved in 1 mM K_2HPO_4 buffer (0.2 ml, pH 7.4) containing dithioerythritol (5 mM), EDTA (1 mM), and Triton X-100 (0.25%), and the mixture was incubated for 10 min at 37°C. HMG CoA reductase was assayed by a minor modification of the method described by Goldstein and Brown (7). Fifty-μl samples of cell extracts, as prepared above, were incubated for 10 min at 37°C in a total volume of 0.2 ml of reaction mixture (pH 7.4) that contained K_2HPO_4 (0.1 M), glucose-6-phosphate (20 mM), glucose-6-phosphate dehydrogenase (0.74 units), NADP (2.5 mM), and dithioerythritol (5 mM).

The reaction was started by adding DL-3-hydroxy-3-methy[3-^{14}C]glutaryl coenzyme A to a final concentration of 62.4 μM. Reaction vessels were incubated for 120 min at 37°C and the reaction was terminated by adding 20 μl of 5N HCl. Approximately 40,000 dpm of DL-[5-^{3}H]mevalonic acid were added as an internal standard, and 10 μl (1 mg/ml) of unlabeled mevalonolactone was added as a carrier. Samples were allowed to incubate for an additional 15 min period to ensure lactonization of the mevalonic acid formed. A blank, in which 20 μl of 5 N HCl was added before the substrate, was also run for each sample. The samples were extracted with two volumes of ether (10 ml). The ether extracts were evaporated to dryness, dissolved in 200 μl of acetone, and applied to a silica gel G thin-layer chromatographic plate which was then developed in acetone–benzene 1:1. Standards of [^{14}C]mevalonolactone were also spotted and chromatographed by the same procedure. Mevalonolactone product that was formed was identified by chromatography with [^{14}C]mevalonolactone standard. The appropriate TLC fractions were then scraped into the scintillation vials and counted. HMG CoA reductase was calculated using the double labeling method of Goldfarb and Pitot (8). HMG CoA reductase activity of each cell was expressed as pmol of [3-^{14}C]mevalonate formed/min of incubation per mg cell protein.

^{125}I-Labeled LDL binding assay

The interaction of ^{125}I-labeled LDL with cell monolayers was determined according to a modification of the methods of Brown and Goldstein (7). Confluent cultures of T.J., WI-38, GM-361, and VA-4 cells were incubated 24 hr in MEM (serum-free) supplemented with lipoprotein-free serum (2.5 mg protein/ml). The following day, the medium was removed and replaced with MEM (2 ml) that contained lipoprotein-free serum (5 mg) and 10 μg of ^{125}I-labeled LDL. Cultures were incubated in triplicate; excess unlabeled LDL (1 mg) was added to one control culture of each set as an aid in measuring nonspecific binding of ^{125}I-labeled LDL. After 3 hr of incubation at 37°C, the cell monolayer was washed twice with 50 μM Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl, and bovine serum albumin (2 mg/ml), and then washed seven times with the same buffer without albumin. The washed cell monolayers were then extracted with ether–ethanol 1:1 to remove the lipid and the cell residue was digested in 1 N NaOH (0.75 ml) overnight and diluted with H_2O (0.75 ml). Aliquots were used for protein determination and for scintillation counting. The specific binding of each cell line was determined from the difference between total binding and that in control cultures and expressed as ng of LDL protein bound/mg cell protein. The binding reported for VA-4 cells is the average of five determinations that ranged from 131 to 170 ng per mg cell protein in separate experiments. T.J., WI-38, and GM-361 are averages of duplicate determinations.

RESULTS

HMG CoA reductase levels in hypercholesterolemic skin fibroblasts grown in the absence of serum lipoproteins averaged 39 units (pmol mevalonate synthesized/minute per mg protein), and were about twice those of the corresponding normal cells (**Table 1**). When serum lipoproteins were added, there was a striking decrease to only 2 units in normal cells whereas enzyme levels in the defective cells increased to 45 units. Similarly high levels of HMG CoA reductase (45.0 units) were found in tumor fibroblasts grown in the absence of serum lipoproteins. These contained about twice as much enzyme as normal fibroblasts grown under the same conditions. When serum lipoproteins were added however, the tumor cells exhibited an effective feedback control of this enzyme, with HMG CoA reductase levels decreasing by about 75%, a decrease of similar magnitude to that observed in normal lung fibroblasts. Despite this effective feedback control, however, tumor cells still had about twice the enzyme activity of normal cells when grown in the presence of similar concentrations of serum lipoproteins.

Unlike normal diploid fibroblasts, which displayed contact inhibition, the transformed cells grew to multilayered densities. Binding of ^{125}I-labeled LDL

TABLE 1. HMG CoA reductase levels in normal, hypercholesterolemic Type II, and SV40 virus-transformed human fibroblasts grown in the presence and absence of serum lipids

Cell Type	HMG CoA Reductase Units (pmol mevalonate/min/mg protein)[a]	
	Control	Plus Serum
Bo Mat (normal skin)	13.5 ± 4.5	2.3 ± 0.3
WI-38 (normal lung)	21.5[b]	6.6 ± 2.4
V.C. (hypercholesterolemic skin)	39.5 ± 3.5	44.7 ± 14.1
VA-4 (tumor-lung)	45.2 ± 6.5	11.8 ± 3.7

[a] ±SEM.
[b] Single determination on pooled cultures.
Cultures of the indicated cell types were grown to confluence in MEM supplemented with 10% serum, washed, and then incubated in MEM either with the addition of solvent-delipidized serum protein (controls), or 10% fetal calf serum, for 24 hr. Cells were harvested by scraping, and HMG CoA reductase levels were measured as described in Methods.

to normal and tumor cells was therefore studied both under conditions of confluence and at greater population densities for the tumor cells. Monolayers of normal skin fibroblasts took up ^{125}I-labeled LDL, fairly rapidly reaching equilibrium levels between 2 and 3 hr. A similar time course was shown by virus-transformed tumor cells (**Fig. 1**) whereas, under similar conditions of incubation and time periods, little or no uptake by FH fibroblasts occurred.

Quantitative comparisons of specific LDL binding were made at the 3 hr time interval after correction for nonspecific binding, which was measured after addition of a 100-fold excess of unlabeled LDL to the monolayers (**Table 2**). At steady-state, normal diploid skin fibroblasts displayed specific binding that averaged 120 ng of ^{125}I-labeled LDL per mg of cell protein. Hypercholesterolemic skin fibroblasts showed essentially no binding activity, averaging less than 8 ng bound per mg protein (Fig. 1). Normal human lung fibroblasts behaved essentially like skin fibroblasts, averaging 135 ng of LDL bound per mg of protein. VA-4 tumor cells grown to the monolayer stage bound 152 ng/mg, which was not significantly different than that of the normal diploid cells.

DISCUSSION

The original observations of Siperstein et al. (1) on the failure of feedback inhibition of cholesterol biosynthesis were made by feeding cholesterol to tumor-bearing animals and examining the metabolic activity of freshly excised tissue slices. Whereas feeding 5% cholesterol decreased [^{14}C]acetate incorporation into normal liver slices up to 60-fold, incorporation into slices of hepatoma tissue from the same animal continued unchanged (2). Similar loss of the feedback system was observed in other types of tumors, such as leukemic cells, and also in livers of rats and fish treated for only short periods of time with the carcinogen aflatoxin (9).

Further investigation showed that the loss of feedback control occurred at the level of HMG CoA reductase that was reduced approximately 10-fold in activity in normal liver from cholesterol-fed animals. Basal levels of this enzyme activity in hepatoma tissue were some fourfold higher than in normal liver, and cholesterol feeding produced no decrease in activity. It was concluded from these studies that the defect in feedback control in malignant tissue was at the level of the HMG CoA reductase although the extract mechanism whereby the feedback control was lost was not established.

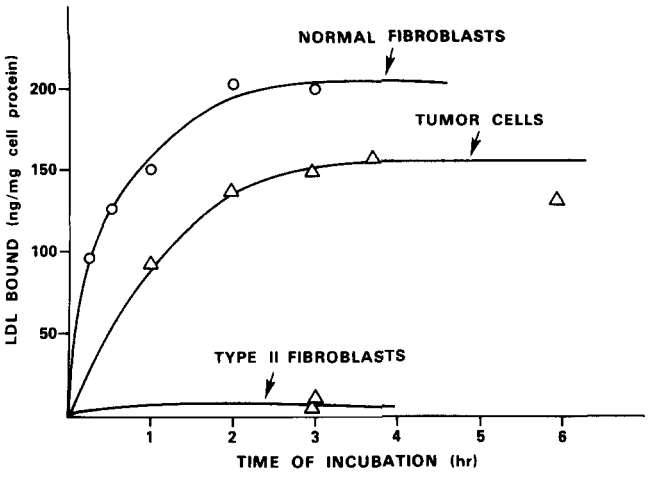

Fig. 1. Time-course of ^{125}I-labeled LDL binding to normal, tumor virus-transformed and Type II hypercholesterolemic human fibroblasts. Type II hypercholesterolemic human skin fibroblasts and WI-38-VA-13A tumor cells were grown to confluence in MEM supplemented with fetal bovine serum (10%). The medium was then replaced for a further 24 hr with MEM supplemented with lipoprotein-free serum (2.5 mg protein/ml). At zero time the medium was replaced with MEM containing lipoprotein-free serum and ^{125}I-labeled LDL (5 mg/ml). Cultures were harvested in duplicate at the indicated intervals and the total LDL binding was determined as described in Materials and Methods.

Concurrently with these later studies, it was reported by Rothblat, Boyd, and Deal (10) that cholesterol synthesis was markedly enhanced in human diploid fibroblasts in tissue culture following transformation with the oncogenic SV40 virus. In contrast to hepatomas in vivo, however, cholesterol synthesis in the transformed cells displayed effective feedback control when cholesterol was added to the medium.

TABLE 2. Binding of ^{125}I-labeled LDL by normal, hypercholesterolemic, and tumor virus-transformed human fibroblasts

Cell Type	^{125}I-Labeled LDL bound (ng LDL/mg cell protein)		
	Total	Nonspecific	Specific
Normal			
T.J. (skin fibroblast)	181 ± 6	61 ± 6	120 ± 12
WI-38 (lung fibroblast)	158 ± 19	23 ± 1	135 ± 20
Tumor			
VA-4 (transformed lung fibroblast)	207 ± 19	55 ± 2	152 ± 21
Hypercholesterolemic			
GM-361 (skin fibroblast)	16 ± 2	8 ± 1	8 ± 3

Cells were grown for 4 days until confluent in MEM supplemented with 10% fetal calf serum and for a further 24 hr in MEM supplemented with lipoprotein-free serum. Total binding (column 1 above) was measured in cultures supplemented with ^{125}I-labeled LDL at a level of 5 μg/ml, and nonspecific binding was measured in similar cultures to which a 100-fold excess of unlabeled LDL was added as described by Goldstein and Brown (7). After 3 hr, cultures were washed, the radioactivity in lipids was extracted, and the protein-bound activity remaining was determined as described in Methods.

We found that cholesterol synthesis from both acetate and mevalonate was elevated in a number of transformed cells and that synthesis from acetate, but not mevalonate, was effectively repressed when serum lipids were added (11, 13). Watson (12) also showed that cholesterol synthesis in the cultured minimal deviation hepatoma 7288C was under effective feedback control.

The present observations demonstrate that there is no significant loss of the membrane-located binding receptors for low density lipoproteins following transformation with the oncogenic SV40 virus. That these measurements were detecting the specific receptors involved in feedback control of HMG CoA reductase is shown by the almost complete lack of binding of ^{125}I-labeled LDL by a receptor negative mutant cell line incubated under the same conditions.

The enhanced ability of the transformed cells to incorporate ^{14}C-labeled acetate into cholesterol is consistent with the approximately twofold increase in the activity of HMG CoA reductase in these cells. However, when serum lipids were added, the transformed cells exhibited a similar degree of inhibition of this enzyme, as did normal diploid cells.

There is thus no evidence in transformed fibroblasts in tissue culture of a lesion in the LDL receptor–HMG CoA reductase feedback loop. These findings suggest therefore that fundamentally different mechanisms underlie the enhanced cholesterol biosynthesis observed for hepatoma cells in vivo and tumor virus-transformed cells growing in tissue culture.

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