Evidence for Deficiency of Low Density Lipoprotein Receptor on Human Colonic Carcinoma Cell Lines

Michele Fabricant and Selwyn A. Broitman

Departments of Microbiology [M. F., S. A. B.] and Pathology [S. A. B.], Boston University School of Medicine, Boston, Massachusetts 02118

ABSTRACT

Cells from six human colonic adenocarcinoma lines (CaCo-2, HT29, LS174T, SW480, SW403, and SW1417) and a normal skin fibroblast cell line (AG1519) were assayed in vitro for their ability to use low density lipoprotein (LDL). All tumor cell lines grew well in lipoprotein-deficient serum, implying that LDL in culture medium was not critical for cell growth. When cell growth was inhibited with mevinolin, a cholesteryl synthesis inhibitor, the addition of LDL to the medium had no effect on the growth of cells from five of six tumor cell lines. CaCo-2 cells showed a moderate reversal while the fibroblast control showed total reversal of inhibition. A monoclonal antibody to bovine/human LDL receptor, used in an enzyme-linked immunosorbent assay, indicated that only CaCo-2 cells and human skin fibroblast cells consistently demonstrated the presence of LDL receptors. Thus, five of six colon tumor cell lines were unable to overcome a mevinolin block in cholesterol metabolism indicating that these cells were deficient in LDL receptors.

INTRODUCTION

Cellular requirements for cholesterol are met either by de novo synthesis from acetate or by uptake of exogenous cholesterol from the extracellular environment. In the human, the source of exogenous cholesterol is LDL, which is distributed by the systemic circulation to cells with LDL receptors and internalized by receptor-mediated endocytosis. Brown and Goldstein (1, 2) first identified this process in cultured human fibroblasts.

Receptor-mediated uptake of LDL is the major source of cholesterol for mammalian cells grown in culture (2). While cholesterol synthesis occurs in cultured cells, it is generally at a low rate, since cholesterol is preferentially derived from the LDL present in the serum of the culture media (2, 3). Once internalized, LDL is degraded in the lysosome. Derived cholesterol (or an oxy derivative) serves as a negative regulator of cholesterol synthesis by suppressing the activity of HMG CoA reductase, the rate-limiting step in cholesterol synthesis (4, 5). Conversely, cells cultured in serum devoid of LDL also maintain their growth; in the absence of negative suppression, the activity of HMG CoA reductase, cholesterol synthesis, and LDL receptor synthesis simultaneously increase.

Cell growth can be made dependent on LDL uptake by utilizing inhibitors of cholesterol synthesis. Compactin and its structural analogue, mevinolin, bind to HMG CoA reductase and act as reversible competitive inhibitors of the enzyme (6, 7). When culture medium is devoid of LDL and mevinolin is added to cultures, cell growth is inhibited and can be restored only by addition of LDL to the medium (8). Therefore, by using inhibitors of cholesterol synthesis, cells can be assayed for the ability to use LDL as a source of cholesterol.

LDL receptors have been shown to be present on a number of human tumor cells in culture (8-14). In addition, several reports (15-19) have suggested that LDL metabolism is altered in tumor cells. Data from studies in rats conducted previously in our laboratory had indicated that: (a) colon tumors (N-methyl-N-nitrosourea induced) took up cholesterol from the serum much more avidly than adjacent colon or normal colon, and (b) colon tumors synthesized cholesterol at a slower rate than adjacent colon or normal bowel (20). However, these studies had not been designed to measure specifically the uptake of LDL-cholesterol and were conducted in rodents in which the mode of cholesterol delivery to tissues is different than in humans (21).

Nevertheless, the possibility that human colon tumor cell lines similarly utilized external cholesterol for growth was explored in this report. To ascertain if this was mediated via LDL receptors, cultured human tumor cell lines were grown in the presence of mevinolin with and without LDL. Results of these experiments indicate that colon tumor cells from all but one of the cell lines tested were unable to use LDL as a source of cholesterol. A monoclonal antibody to the human LDL receptor was used to determine if these cells lacked LDL receptors.

MATERIALS AND METHODS

Cell Culture. Cells of six human colon cancer lines were grown in monolayer cultures: CaCo-2, HT29, LS174T, SW480, SW403, and SW1417. CaCo-2 and SW1417 cells were obtained from the American Type Culture Collection (Rockville, MD). The others were provided by Dr. Herbert Kupchik (Boston University School of Medicine, Boston, MA). Stock cultures were grown in plastic 75-cm² flasks and incubated at 37°C in a 5% CO₂/95% air atmosphere. Culture medium consisted of Eagle’s MEM containing antibiotics, glutamine, and nonessential amino acids and supplemented with 10% FBS (Hazelton, St. Lenexa, KS). Cultures were divided weekly using trypsin/EDTA. Cells used in each experiment were grown in plastic 35-mm dishes and 96-well microtiter plates. Normal human skin fibroblast cells AG1519 (obtained from the Institute for Medical Research, Camden, NJ) were grown as monolayers in MEM supplemented with antibiotics, glutamine, nonessential amino acids and 10% FBS. C7 hybridoma cells from the American Type Culture Collection were grown as suspension cultures in Dulbecco’s high glucose medium supplemented with antibiotics, glutamine, and 15% FBS. Chinese hamster fibroblast cells (V-79) were provided by Dr. I. N. Chou (Boston University School of Medicine). Cell cultures were assayed for the presence of Mycoplasma using the Gen-Probe Mycoplasma T.C.11 Rapid Detection System (San Diego, CA).

Preparation of LPDS. Lipoproteins were removed from FBS by density gradient centrifugation. FBS was adjusted to a density of 1.21 g/ml with solid potassium bromide (64.4 g KBr per 200 ml FBS) and centrifuged for 48 h at 5°C at 200,000 × g (45,000 rpm in a 70 Ti rotor...
in a Beckman L8-70 ultracentrifuge. The top lipoprotein fraction (density <1.21 g/ml) was removed. The bottom lipoprotein-deficient fraction (LPDS) was dialyzed extensively against 0.01% EDTA/double-distilled water (pH 8) and then PBS. The LPDS was sterilized by passage through a 0.45-μm filter.

Mevinolin. Mevinolin, in the lactone form, was kindly provided by A. Alberts of Merck Sharp & Dohme (Rahway, NJ). Saponification was carried out by adding 0.1 ml ethyl alcohol and 0.1 ml 0.1 N NaOH to 4 mg of mevinolin and heating at 50°C for 2 h. The mevinolin was neutralized with 5% HCl to pH 7.3. Stock solutions (4 mg/ml) were prepared by bringing the volume to 1 ml with DMSO and were stored at −20°C. Mevinolin was diluted in MEM to the concentrations used in experiments.

Inhibition of Cell Growth with Mevinolin. Cells were grown in 35-mm dishes in duplicate in MEM + 10% LPDS. Mevinolin, at varying concentrations, was added to some of the cultures at day 0. The final volume per dish was 1 ml. Cells received fresh medium every 2 days. Cell growth was measured by counting the number of viable cells per plate at the times indicated in Figs. 1 and 2. Cells were removed from dishes with trypsin/EDTA and resuspended in medium containing serum. An aliquot was stained with trypan blue and cells were counted in a hemacytometer.

Since solutions of mevinolin were prepared in DMSO, the effect of DMSO alone on cell growth was determined. Cells were grown in MEM + 10% LPDS + DMSO, a concentration of DMSO equivalent to a 10-μM solution of mevinolin. DMSO alone had no effect on cell growth.

LDL Preparations of human LDL were purchased from Sigma (St. Louis, MO). LDL was stored at 4°C and used within 1 month. Appropriate dilutions were made in PBS or medium immediately before use in experiments.

Uptake of LDL by Cells in Culture. Cells were grown in 35-mm dishes in duplicate in MEM + 10% LPDS. Mevinolin was added to cultures at day 0 at a previously determined concentration that inhibited cell growth by approximately 50% (see “Results”). At day 2 some of the cultures received LDL. The final volume per dish was 1 ml. Cells received fresh medium and reagents every 2 days. Cell growth was measured by counting the number of viable cells per dish at the times indicated in Figs. 1 and 2. Cells were counted in a hemacytometer after trypan blue staining, as described above. Reversal of the mevinolin-induced inhibition of cell growth in the presence of LDL is interpreted as the ability of cells to take up and use LDL.

Monoclonal Antibody to Human LDL Receptor. C7 hybridoma cells, which secrete IgG-C7, a mouse monoclonal antibody against the human LDL receptor, were grown in suspension cultures. The cells were resuspended in serum-free medium and injected into the peritoneal cavities of pristane-primed female BALB/c mice (1 × 10⁶ cells per mouse) for the collection of antibody-containing ascites fluid several weeks later. Ascites fluid was centrifuged to remove cells and diluted for use in the ELISAs described below. Aliquots of ascites fluid were stored at −20°C. Cloning of the C7 hybridoma cells and characterization of the monoclonal antibody had been done previously (22). IgG-C7 reacts with human and bovine LDL receptors, but not with receptors from the mouse, rat, Chinese hamster, rabbit, or dog (22).

ELISA for Binding of IgG-C7 to Cell Monolayers. Cells were plated in microtiter wells in 0.1 ml MEM + 10% FBS at densities that resulted in confluent monolayers by the time the cells were fixed for the assay. Plating densities ranged from 1000 to 4000 cells per well, depending on the cell line. At day 2 of culture the medium was changed to MEM + 10% LPDS. Mevinolin also was added at this time in some experiments. After 48 h in LPDS, the number of cells per well for each cell line was determined. For each line, cells in several wells were trypsinized, resuspended in medium containing serum, stained with trypan blue, and counted in a hemacytometer. Cells in all other wells were fixed with 0.25% glutaraldehyde in PBS (100 μl per well) for 10 min at room temperature. Cells then were washed three times with PBS, and stored overnight at 4°C in PBS if the ELISA could not be done the same day. After fixation, cells were incubated in 1% BSA in PBS (100 μl per well) for 1 h at 37°C to reduce nonspecific background staining. BSA then was aspirated from the wells. For each cell line assayed, 100 μl per well of the primary antibody was added: IgG-C7 to one set of three wells and IgG-31-64, a nonrelevant mouse ascites used as a control, to another set of three wells. IgG-31-64 was provided by Dr. Ann Marshak-Rothstein (Boston University School of Medicine). IgG-C7 and IgG-31-64 were diluted 1:2000 in 1% BSA for use in these assays. Preliminary assays had been done to determine optimal antibody dilutions. Wells were incubated with primary antibody for 90 min at 37°C, and then washed three times with 1% BSA. All wells next received 100 μl of a peroxidase-conjugated secondary antibody: goat anti-mouse IgG/IgM (Boehringer, Indianapolis, IN), diluted 1:2000 in 1% BSA. After a 90-min incubation at 37°C, wells were washed three times with 1% BSA. Substrate solution (100 μl per well) was added, and wells were incubated for 30 min at 37°C. Substrate solution was prepared immediately before use by adding 12.5 ml substrate buffer (0.1 M sodium citrate, 0.1 M NaH₂PO₄, pH 5) and 42 μl 3% H₂O₂ to 0.005 g orthophenylene diamine. The reaction was stopped by adding 100 μl 2.5 M H₂SO₄ to the wells. Absorbance was read using a Microelisa Reader (Dynatech Laboratories Inc., Alexandria, VA) with a 488-nm filter on high gain. Wells of uninfected cells were used to calibrate to zero. Absorbance per well was calculated by subtracting the average background absorbance from the sample absorbance. Background absorbance was determined from wells incubated with the control primary antibody and sample absorbance from wells incubated with IgG-C7. Results are expressed as absorbance per number of cells.

RESULTS

The six cell lines used in experiments are listed in Table 1. All the lines were established from primary colon adenocarcinomas. Three cell lines were derived from differentiated tumors and three from undifferentiated tumors. In culture, all cells appeared epithelial-like, and each line retained morphological characteristics of its tumor of origin. In addition, two of the three differentiated lines produced mucin, a glycoprotein secreted by normal colonic mucosal cells. All of the cell lines, except SW1417, have been shown by their originators to be tumorigenic in nude mice.

All six cell lines were grown in MEM + 10% LPDS in the presence of mevinolin at varying concentrations to ascertain if mevinolin would inhibit cell growth. In this report growth inhibition is defined as a decrease in cell density (number of cells per dish). The results of these preliminary experiments were used to determine concentrations of mevinolin appropriate for the inhibition studies. When HMG CoA reductase is inhibited completely, LDL alone cannot restore growth. A small amount of mevalonate, the product of the HMG CoA reductase reaction, in addition to LDL, is necessary for cell proliferation (23). Because the cholesterol synthesis pathway is branched, some mevalonate must be available for the synthesis of several nonsterol products. For the experiments to follow, it was important to inhibit cell growth only partially, so that it would be possible to overcome this inhibition with LDL. For each cell

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tumor origin</th>
<th>Gland formation* in culture</th>
<th>Mucin*</th>
<th>t₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCo-2</td>
<td>MWD</td>
<td>+</td>
<td>+</td>
<td>43.8</td>
</tr>
<tr>
<td>HT29</td>
<td>MWD</td>
<td>-</td>
<td>-</td>
<td>23.7</td>
</tr>
<tr>
<td>LS174T</td>
<td>D*</td>
<td>+</td>
<td>-</td>
<td>22.2</td>
</tr>
<tr>
<td>SW480</td>
<td>U</td>
<td>-</td>
<td>-</td>
<td>18.1</td>
</tr>
<tr>
<td>SW403</td>
<td>U</td>
<td>-</td>
<td>-</td>
<td>25.8</td>
</tr>
<tr>
<td>SW1417</td>
<td>U*</td>
<td>-</td>
<td>-</td>
<td>40.1</td>
</tr>
</tbody>
</table>

* MWD, moderately well-differentiated; D, differentiated; U, undifferentiated.

Tumor had metastasized to surrounding lymph node and to small intestine.
line, a concentration of mevinolin was chosen that inhibited cell density (number of cells per dish) by approximately 50%. Different cell lines varied somewhat in their susceptibilities to the same concentrations of mevinolin. The growth of CaCo-2, LS174T, SW403, and SW1417 cells were inhibited by about 50% with 1 μM mevinolin, but HT29 and SW480 cells required 2 μM mevinolin (data not shown).

Colon tumor cells treated with mevinolin were evaluated for their ability to use LDL as a source of cholesterol. Human LDL was added to cultures of mevinolin-treated cells growing in LPDS-supplemented medium. If tumor cells were able to bind, internalize and utilize LDL, it would be expected that mevinolin-induced growth inhibition would be reversed. The protocol for these studies was derived from available information about LDL metabolism in human fibroblasts, which have been the only cells studied extensively in this regard (3, 24-28). Experiments were done with exponentially growing colon tumor cells, with varying concentrations of LDL added to cells at day 2 of culture. Concentrations of LDL initially were determined on the basis of data from fibroblast studies, in which 10–50 μg/ml LDL were used (3). Human fibroblast cells, AG1519, known to have LDL receptors (1, 2), served as positive controls. The effect of mevinolin on AG1519 cells was reversed in the presence of 50 μg/ml LDL (Fig. 1). LDL concentrations of 50 and 100 μg/ml were used in subsequent experiments. Results of experiments on the effect of LDL on the growth of mevinolin-treated cells are shown in Fig. 2, A–F. Inhibition of cell growth by mevinolin was not reversed by the presence of LDL, except in one tumor cell line. Studies carried out with CaCo-2 cells (Fig. 2F) showed a modest but significant reversal of mevinolin inhibition of growth with the addition of 50 μg/ml LDL to the culture medium, but no additional reversal was obtained with 100 μg/ml LDL. Studies with HT-29 (Fig. 2E) illustrate a problem utilizing mevinolin induction of LDL receptor in this tumor cell line. At 2 μM mevinolin (which is greater than required or adequate to inhibit growth about 50% in all the other cell lines) inhibition of the growth of HT-29 was 38.1% compared to control at day 4 and 20.6% at Day 6. While both these differences were statistically significant (paired t test, P < 0.025) the biological difference at day 6 was marginal. Increasing the concentration of mevinolin above this level depicted for HT-29 increased the toxicity beyond the range required for the detection of LDL uptake.

An ELISA that measured the binding of IgG-C7, a monoclonal antibody to the human LDL receptor, to cell monolayers was used to determine if the inability to use LDL was due to a
lack of receptors for LDL. The ELISA was evaluated first with receptor-positive AG1519 human fibroblast cells; these also served as positive controls each time the assay was performed. The protocol for the assay was based on standard procedures for the maximum induction of LDL receptor activity in fibroblasts (3). V-79 Chinese hamster fibroblasts served as negative controls since IgG-C7 does not recognize hamster LDL receptors (8). The validity of the ELISA was evaluated by including fibroblasts treated with mevinolin. Since cells with LDL receptors increase the number of receptors in response to inhibition of cholesterol synthesis (1), such cells would be expected to show an increase in IgG-C7 binding in the presence of mevinolin. Data on the binding of IgG-C7 to AG1519 cells, presented in Table 2, indicate that these cells have receptors for LDL, which are increased in number approximately threefold after mevinolin treatment.

Binding of IgG-C7 to cells from the six colonie tumor lines is shown in Table 3, which includes data for both untreated and mevinolin-treated cells. Compared to the positive control AG1519 cells, only the CaCo-2 cells demonstrate the presence of LDL receptors. AG1519 and V-79 cells were included as positive and negative controls, respectively. As expected, IgG-C7 binding to AG1519 cells increased threefold after treatment with mevinolin. Among the tumor cells studied only CaCo-2 cells provided evidence of antibody binding. Treatment with mevinolin had no effect on IgG-C7 binding to HT29, LS174T, SW480, SW403, or SW1417 cells. The data support the conclusion that these five types of tumor cells are deficient in LDL receptors.

**DISCUSSION**

Results from cell growth experiments presented here indicate that six tumor cell lines grew well in delipidized media and that the addition of LDL had no appreciable influence upon cell growth. When cells were treated with mevinolin to impair cell growth approximately 50%, it was possible to totally restore the growth of AG1519 fibroblast cells by the addition of LDL. The addition of LDL in concentrations twofold greater than that used with fibroblasts failed to reverse mevinolin inhibition of growth of five of six tumor cell lines. In the sixth tumor cell line CaCo-2 growth was partially reversed by the addition of LDL. These findings suggest the possibility that all but one of the tumor cell lines tested lacked LDL receptors.

Cells were screened for the presence or absence of LDL receptors by using an ELISA that measured the binding of monoclonal antibody IgG-C7 to cell monolayers. Binding of antibody was readily detected on the positive control fibroblasts. All but one of the human colon tumor cell lines exhibited values that indicated the antibody did not bind. Thus, five of the six tumor cell lines appear to be deficient in LDL receptors. Why these cells are deficient in receptors is unclear.

CaCo-2 cells were the only tumor cells that were able to use LDL in the culture medium after treatment with mevinolin and that consistently demonstrated the presence of LDL receptors by ELISA. It is not apparent from the available data why CaCo-2 differs from the other tumor cells. Neither doubling time nor degree of differentiation seemed to be correlated with the presence of LDL receptors. It is not known whether a deficiency in LDL receptors is a particular characteristic of certain colon tumors or a general characteristic of both normal colon and colon tumor tissue. Evidence of whether LDL receptors are present on normal human colon cells is not available. Attempts to study normal colonic cells in culture are hampered by the difficulty in growing gut epithelial cells in vitro. However, studies are in progress using tissue sections of normal colon and colon tumors to determine the presence or absence of LDL receptors. Studies of the binding of IgG-C7 to frozen tissue sections currently are being done in our laboratory.

Even in the absence of information about LDL receptors on normal colon cells, the results of the experiments presented here are significant in the context of the pathology of human colon cancer. If many, or a few, colon tumors are found devoid of LDL receptors at surgery, a strategy for inhibiting growth of hepatic metastases via cholesterol deprivation is apparent. Colorectal tumors are readily removed surgically, but approximately one third of patients have clinical evidence of hematogenous metastases at that time (29). Occasionally metastases occur in the lung, but far more frequently they occur in the liver (30). The liver is the major site of clearance of LDL from the plasma (1), and normal human hepatocytes are known to have a great affinity for the uptake of LDL (31, 32). Therefore, treatment with mevinolin, which is concentrated in hepatic cells during clearance by the liver, would be expected to inhibit the growth of metastatic colonic tumor cells devoid of LDL receptors in the liver, but not the growth of normal hepatic cells. Mevinolin (Lovastatin) already has been approved for clinical use, as a hypocholesterolemic agent for patients at high risk for heart disease (33, 34).

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REFERENCES


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