Effect of Cell Density and Confluency on Cholesterol Metabolism in Cancer Cells in Monolayer Culture

David Gal, Paul C. MacDonald, John C. Porter, Jesse W. Smith, and Evan R. Simpson

ABSTRACT

Cholesterol metabolism in four gynecological cancer cell lines in monolayer culture was evaluated as a function of cell density. The rate of uptake and degradation of $[^{125}I]$-iodinated low-density lipoprotein increased during the first 24 to 48 hr of culture, but decreased thereafter. Once the cells became confluent, the rate of metabolism of $[^{125}I]$-iodinated low-density lipoprotein was only one-tenth that in cells which were in the preconfluent state. The specific activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase increased during the first 24 to 48 hr of culture and subsequently declined, reaching a nadir after confluency was attained. The rate of incorporation of $[^{14}C]$-oleate into cholesteryl esters was low when the cells were in the log-exponential phase of replication but increased gradually as cell density increased. The highest specific activity of acylcoenzyme A: cholesterol acyltransferase was attained after the cells became confluent. Generally speaking, there was an inverse relationship between the specific activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase, on the one hand, and the rate of $[^{125}I]$-iodinated low-density lipoprotein metabolism and cholesteryl ester synthesis, on the other. It is concluded that cholesterol metabolism in cancer cells in monolayer culture is regulated, in part, by the rate of cell division. In the cancer cells utilized in this study, it is apparent that cholesterol metabolism was subject to the same regulatory mechanisms as are present in nonneoplastic cells.

INTRODUCTION

Most studies designed to evaluate lipoprotein metabolism and the intracellular metabolism of cholesterol by cells in monolayer culture have been conducted after the cells were confluent (1-6, 9-11, 15-17, 19, 21-24, 26, 27). The confluent state of cells in monolayer culture was chosen by most investigators because it provided a condition in which cell replication was limited; thus, growth-dependent factors of replication might have influenced the results of various experiments were reduced or eliminated.

In the present investigation, we evaluated cholesterol metabolism in 4 different human gynecological cancer cell lines in order to establish whether or not normal control mechanisms which regulate cholesterol metabolism in nonneoplastic cells were operative also in cancer cells. Initially, the experiments were conducted with cells in the confluent state as has been described for human fibroblasts (4, 5, 9). In those initial studies, we found low rates of metabolism (uptake and degradation) of LDL and low rates of de novo synthesis of cholesterol by such cells. Since cholesterol is essential for cell membrane synthesis, it seemed unreasonable that cancer cells, which generally exhibit a high rate of replication in vivo, should have such limited capacity to metabolize LDL or to synthesize cholesterol. For this reason, the present study was conducted to evaluate the effect of the density of cells in monolayer culture on various components of cholesterol metabolism in 4 different cancer cell lines.

MATERIALS AND METHODS

Cells. Poorly differentiated epidermoid cervical cancer cells EC-50 (18), epidermoid vaginal cancer cells EC-82 (18), endometrial adenocarcinoma cells AC-258, and well-differentiated endometrial adenocarcinoma cells HEC-B-296 (13) were utilized in the present investigation (Table 1). The cells were derived from primary cultures of minced neoplastic tissues and were passed whenever they became confluent. The histological nature of these cells was confirmed by periodical evaluation of tumors grown after inoculation in nude mice. The histological nature of these cells was confirmed by periodical evaluation of tumors grown after inoculation in nude mice. The cells were maintained in T-75 flasks in the presence of Waymouth’s enriched medium containing 10% FCS at 37°C in a humidified atmosphere of 95% air:5% CO2.

Lipoproteins. Human LDL (density, 1.019 to 1.063 g/ml) and LPPS, (density, >1.215 g/ml) were prepared by differential ultracentrifugation of plasma obtained from healthy subjects according to the method of Bilheimer et al. (3). $[^{125}I]$-Iodo LDL was prepared by iodination of LDL according to the method of Bilheimer et al. (3) as modified by Goldstein and Brown (9). Effect of Cell Density of LDL Metabolism. Cells (10^6) from each cancer cell line were transferred to 60-mm Petri dishes and maintained in medium containing 10% FCS. The cells in 2 other dishes of each cell line were maintained from the beginning of culture in medium containing 10% LPPS. The medium in 2 dishes of each cell line originally maintained in medium containing FCS was changed to one containing 10% LPPS at 24-hr intervals. After 24 hr in medium containing LPPS, $[^{125}I]$-Iodo LDL (50 μg/ml) was added to the medium of each dish. Six hr after the addition of $[^{125}I]$-Iodo LDL, the medium was removed, and an aliquot was taken for the measurement of trichloroacetic acid-soluble degradation products of $[^{125}I]$-Iodo LDL according to the method described by Goldstein and Brown (9). The cells were washed 5 times with 50 mM Tris

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buffer (pH 7.4) containing 0.15 M NaCl and bovine serum albumin (2 mg/ml) and then once with buffer that did not contain bovine serum albumin. After washing, the cells were removed from the dishes with 1 ml of 0.5 N NaOH. One aliquot of the solubilized cell suspension (0.5 ml) was assayed to determine the amount of radioactivity that remained bound to the cells; another aliquot (50 μl) was used for the measurement of cellular protein according to the method of Lowry et al. (14).

129I]iodo LDL uptake and degradation are expressed as ng LDL per mg cell protein per 6 hr. Each value is the mean of results obtained utilizing cells from duplicate dishes.

**Effect of Cell Density on HMG CoA Reductase Activity.**

Cells (10^6) from each cell line were placed in Petri dishes as described for the previous experiments. After 24 hr in medium containing 10% LPPS, the medium was removed, and the cells were washed twice with the Tris:NaCl buffer. The cells were scraped from the dishes into 1 ml Tris:NaCl buffer using a rubber policeman. The cells were pelleted by centrifugation at 1000 rpm for 5 min; the supernatant fraction was removed, and the cells were stored in liquid N2. Two dishes of cells from each line were processed in this manner every 24 hr.

HMG-CoA reductase activity was assayed in detergent-solubilized extracts of the cells according to the method of Brown and Goldstein (5), except that 1.25% Brij 96 detergent was used rather than 0.25% Kyro EOB. The HMG-CoA reductase activity is expressed as pmol of [14C]mevalonate formed per mg microsomal protein per min. Each value presented is the average of results obtained utilizing cells from duplicate dishes.

**Effect of Cell Density on ACAT Activity.**

Cells (10^6) from each cancer cell line were placed in Petri dishes and maintained in Waymouth's enriched medium containing 10% FCS. The cells in 2 other dishes of each cell line were maintained in medium containing LDL (400 μg protein per ml) for 24 hr. The medium in 2 dishes of each cell line originally maintained in medium containing FCS was replaced with one containing 10% LPPS and LDL (400 μg/ml) at 24-hr intervals, after which [14C]oleic acid (150 μM) was added for 6 hr. After 6 hr, the medium was removed, and the cells were washed 2 times with Tris:NaCl buffer. The cells were harvested and stored in liquid N2 until homogenized and extracted with chloroform:methanol (2:1). Cholesteryl [14C]oleate was isolated by thin-layer chromatography and quantified according to the method described by Brown et al. (4). The rate of [14C]oleate incorporation into cholesteryl esters proceeded at a linear rate for up to 24 hr and is expressed as nmol/mg cell protein per 6 h. Each value is the average of results obtained utilizing cells from duplicate dishes.

**Effect of Cell Density on Regulation of HMG-CoA Reductase Activity by LDL and Progesterone.**

Epidermoid cervical cancer cells (EC-50) were studied at 2 time periods after initially placing the cells in culture. After 24 hr, the medium of the cells of Group 1 was replaced with one containing 10% LPPS; the cells had not yet become confluent. The cells of Group 2 were allowed to reach confluency prior to replacing the medium with one containing 10% LPPS. After maintaining the cells in medium containing LPPS for 48 hr, the medium was replaced with medium containing 10% LPPS and LDL in various amounts. Twelve hr later, progesterone, dissolved in acetone, was added to some dishes to achieve a final concentration of 40 μM. Acetone alone was added to the other dishes. After an additional 12 hr of incubation, the cells were removed from the medium, washed, harvested, homogenized, and assayed for HMG-CoA reductase activity according to the method described above. Each value presented represents the results obtained utilizing cells from duplicate dishes.

**Protein Measurement.**

Aliquots for protein measurement were taken from either cell or microsomal extracts in all experiments and assayed according to the method described by Lowry et al. (14). The results are expressed as mg protein per dish.

**Materials.**

Culture media and FCS were obtained from Grand Island Biological Co. (Grand Island, N. Y.). All culture flasks were purchased from Corning Glass Works (Corning, N. Y.). The following isotope-labeled compounds were purchased from New England Nuclear (Boston, Mass.): [1,2-3H]mevalonic acid (3 Ci/mmol); [1,2-3H]mevalonic acid DBED salt (2 Ci/mmol); [1-14C]oleic acid (40 mCi/mmol); and [7-3H]cholesterol (20 Ci/mmol). Polyoxyethylene 10-oleylether (Brij 96), NADP*, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Co. Sodium [125I]iodide (13 to 15 Ci/mg) was purchased from Amersham/Searle Corp. (Arlington Heights, Ill.).

All experiments were performed at least 3 times, and the results were qualitatively and quantitatively similar on each occasion. In all cases, the values for determination of mean rates for each time and concentration point were within 5% of the mean.

**RESULTS**

The rate of metabolism of [125I]iodo LDL in all cell lines increased during the first 24 to 48 hr of culture and, in the case of EC-50 and HEC-B-296 cells, increased throughout the first 72 hr of culture (Chart 1). Thereafter, the rate of metabolism decreased as cell density increased. After the cells became confluent, the rate of [125I]iodo LDL metabolism in one cell line (AC-258) was only one-tenth that found in cells in the preconfluent state.

After commencement of culture, there was an increase in the specific activity of HMG-CoA reductase in all cell lines. However, the specific activity of HMG-CoA reductase decreased significantly as cell density increased and reached a nadir after confluency was attained (Chart 2).

The rate of incorporation of [14C]oleate into cholesteryl esters (ACAT activity) was low when the cells were in the log-expo-
Cholesterol Metabolism in Cancer Cells

Chart 1. The effect of time in culture on metabolism in LDL by cancer cells in monolayer culture. Cells (10^6) from each cell line were plated in 60-mm Petri dishes. All cells were maintained in medium containing 10% LPPS for 24 hr prior to harvesting. After 6 hr, the uptake and degradation of [125I]iodo LDL were determined in cells from 2 dishes from each line as described in "Materials and Methods." Each point is the average rate of LDL metabolism by cells in duplicate dishes. Cell confluency was determined by microscopic examination. EC-50, cervical cancer; EC-82, vaginal cancer; HEC-B-296, well-differentiated endometrial cancer; AC-258, poorly differentiated endometrial cancer.

Chart 2. The effect of time in culture on HMG-CoA reductase activity in cancer cells in monolayer culture. Cells (10^6) from each cell line were plated in 60-mm Petri dishes. All cells were maintained in medium containing 10% LPPS for 24 hr prior to harvesting. At the indicated times, cells from 2 dishes of each cell line were harvested and stored in liquid N2 until assayed as described in "Materials and Methods." Each point is the average specific activity of HMG-CoA reductase obtained utilizing cells from duplicate dishes. Cell confluency was determined by microscopic examination. EC-50, cervical cancer; EC-82, vaginal cancer; HEC-B-296, well-differentiated endometrial cancer; AC-258, poorly differentiated endometrial cancer.

Chart 3. The effect of time in culture on ACAT activity in cancer cells. Cells were incubated with LDL (400 μg/ml) for 24 hr and [14C]oleic acid (150 μM) for 6 hr prior to harvesting. At each time interval, cells from duplicate dishes of each cell line were harvested and stored in liquid N2. ACAT activity was determined as described in "Materials and Methods." Each point is the average of the results obtained utilizing cells from duplicate dishes. Cell confluency was determined by microscopic examination. EC-50, poorly differentiated epidermoid cervical cancer; HEC-B-296, well-differentiated adenocarcinoma of endometrium; EC-82, poorly differentiated epidermoid vaginal cancer; AC-258, poorly differentiated adenocarcinoma of endometrium.

DISCUSSION

Increasing cell density and the attainment of confluency resulted in marked changes in the rates of several key reactions involved in cholesterol metabolism in 4 cancer cell lines maintained in monolayer culture. Specifically, the activity of HMG-CoA reductase and the rate of LDL metabolism declined, whereas the rate of cholesteryl ester synthesis increased. These results are consistent with the concept that, in these 4 cancer cell lines, cholesterol metabolism is regulated by the requirements of the cells for cholesterol for cellular membrane biosynthesis, in accordance with the model of cholesterol metabolism regulation proposed by Brown et al. (4), Brown and Goldstein (5), and Goldstein and Brown (9).

Vlodavsky et al. (25) concluded that contact inhibition was of major importance in the regulation of LDL uptake by bovine vascular endothelial cells maintained in monolayer culture. These investigators also proposed that LDL metabolism was

It has been shown that progesterone stimulates HMG-CoA reductase activity and attenuates the inhibitory effect of LDL on this enzyme activity (7). In the present study, it was found that the progesterone effect on cholesterol metabolism was greater in cells in the preconfluent state than in cells that were confluent (Chart 5). In cells that were confluent, progesterone did not stimulate HMG-CoA reductase activity but still attenuated LDL-induced inhibition of HMG-CoA reductase activity.

The amount of protein in each dish was proportional to cell number. After confluency was attained, there was no further increase in cell protein (Chart 4).

Chart 4. Growth curves and total cell protein in dishes containing cancer cells from 4 different lines (EC-50, EC-82, HEC-B-296, and AC-258). The media were changed as they turned acid, which occurred no earlier than 6 days of culture. Each point is the average cell protein, mg/dish, at the indicated times of culture. The photomicrographs were taken of a representative cell line (EC-82) at the times indicated. The progression of cell density as a function of time in culture is shown.

Chart 5. The effect of cell density on regulation of HMG-CoA reductase activity by LDL and progesterone. Epidermoid cervical cancer cells (EC-50) were studied as described in "Materials and Methods." Each point is the average specific activity of HMG-CoA reductase in cells from 2 dishes.

not affected by contact inhibition in subendothelial smooth muscle cells which therefore would accumulate cholesteryl esters after injury to the endothelial cells, and, through such a process, atherosclerotic lesions were formed. Kruth et al. (12), using immunofluorescent methods, investigated the effect of cell density and cell confluency on lipid accumulation within human fibroblasts. They found that noncycling (confluent) cells contained fewer functional LDL receptors.

It has been suggested previously that neoplastic cells may have defective biochemical functions and that normal extracellular control of intracellular processes may be lacking (20). However, in the cell lines utilized in the present investigation, control mechanisms involved in regulation of cholesterol metabolism appeared to be normal (7, 8). It was of interest to note that the maximum rate of a particular reaction which could be achieved appeared to be an intrinsic property of a particular cell line. Thus, the specific activity of HMG-CoA reductase was low in cells with high rates of LDL metabolism (EC-50 and HEC-B-296) and high rates of ACAT activity. On the other hand, in cells in which there was a low rate of LDL metabolism (AC-258), the specific activity of HMG-CoA reductase was high, and ACAT activity was low.

Previously, we investigated the effect of progesterone on the regulation of HMG-CoA reductase activity by LDL in 8 different cancer cell lines (7). Progesterone stimulated HMG-CoA reductase activity in each of the 8 cell lines studied, and progesterone prevented the LDL-induced inhibition of HMG-CoA reductase activity. In the present study, this effect of progesterone on HMG-CoA reductase activity was found to be greater in replicating cells than in cells which were confluent.

It is obvious that cancer cells require large amounts of cholesterol for cell membrane synthesis when cell replication is rapid and unbridled. However, on the basis of the results reported here, we would conclude that loss of control mechanisms regulating cholesterol metabolism is not a general feature of cancer cells in monolayer culture. Rather normal control mechanisms are utilized by such cells to optimize cholesterol supply to meet the demands of cell growth and division.

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