Cholesterol-induced Growth Stimulation, Cell Aggregation, and Membrane Properties of Ascites Tumor Cells in Culture

E. W. Haeffner, C. J. K. Hoffmann, M. Stoehr, and H. Scherf

Institut für Zell- und Tumorbiologie [E. W. H., C. J. K. H.]; Institut für experimentelle Pathologie [M. S.]; und Institut für Toxikologie und Chemotherapie [H. S.], Deutsches Krebsforschungszentrum, D-6900 Heidelberg, Federal Republic of Germany

ABSTRACT

Ascites tumor cells can be cultivated at a reduced serum concentration if cholesterol (2.50 mg per 100 ml of medium) is added to the culture medium. At serum concentrations of 3%, optimal growth properties are obtained; below 3%, cell cultures usually perish after a few days. Cells grown in the presence of added cholesterol have an elevated content of this molecule per cell as well as in the plasma membrane, and they also show a cholesterol concentration-dependent rate of proliferation. Precursors of the cholesterol-biosynthetic pathway like mevalonic acid, added in mM amounts, or squalene and lanosterol cannot be substituted for cholesterol itself. This is due to the observation that the biosynthetic pathway is blocked at the stage of lanosterol conversion to cholesterol. Cholesterol de novo synthesis from acetate is regulated by the cholesterol content of the cells, which also affects the production of ubiquinone and dolichol. Growth factors such as insulin, prostaglandin F2α, and transferrin added to the medium do not mimic the cholesterol-induced effect. Distribution of DNA during cell cycle and the cell density-dependent reduction in macromolecule synthesis is very similar to the control cells. In contrast, cells without added cholesterol show reduced growth properties accompanied by the accumulation of cells in the mitotic and G2 phase.

The cholesterol/phospholipid ratio of the plasma membranes of cholesterol-rich cells is about 15% lower than of the control cells and 40% higher compared to the cholesterol-poor cells, which, however, does not significantly alter the membrane fluidity between the cholesterol-rich and -poor cells as revealed by fluorescence polarization measurements. The most dramatic behavior of the cholesterol-rich cells is their tendency to form aggregates, which is demonstrated either by concanavalin A-induced agglutination or by cell density-dependent aggregation shown by interference microscopy in vivo.

INTRODUCTION

The requirement of some cell types for preformed lipids, especially cholesterol, normally supplied by the serum has recently been established (27, 29, 32, 33). Brennemann et al. (9) have shown that ascites cells take up major amounts of cholesterol from the ascites fluid and, in the same study, it has further been found that the cholesterol de novo synthesis is rather low. In another paper (26), we have reported that ascites tumor cells do not grow when cultivated in delipidated serum medium. Attempts to induce cell proliferation in delipidated serum medium to which cholesterol has been added were only partially successful.3 In this study, we have investigated the effect of a reduced serum concentration with and without the addition of cholesterol upon ascites cell growth, lipid de novo synthesis, and surface properties. We present evidence for permanent cultures of these cells at drastically reduced serum concentrations, and we also show data revealing changes at the surface which may be responsible for inducing a highly increased cell-cell interaction. Preliminary results of these experiments have been reported elsewhere (25).

MATERIALS AND METHODS

Materials. Concanavalin A, linoleic acid, palmitoleic acid, linolenic acid, prostaglandins E and F2α, phenylmethylsulfonyl fluoride, 4,6-diamidino-2-phenylindol, and 1,6-diphenyl-1,3,5-hexatrien were purchased from Serva, Heidelberg, Federal Republic of Germany, and insulin plus transferrin was from Sigma, Munich, Federal Republic of Germany. The radioisotopes [methyl-3H]thymidine, 2-[14C]acetic acid, and N-[acetyl-3H]methionine A were purchased from New England Nuclear, Dreieichenhain, Federal Republic of Germany.

Cell Cultures. Hyperdiploid ascites tumor cells, type Karzel, were cultivated in minimum essential medium with Earle’s salt, 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, pH 7.4, and horse serum from Boehringer-Mannheim, Federal Republic of Germany. The serum concentration in the medium varied between 0.5 and 10% as indicated in the chart legends. Cell cultures were seeded at an initial cell density of about 10⁴ cells/ml using sterile 75-sq cm Corning tissue culture flasks. The medium was changed every second day by centrifugation of cell cultures and 40% higher compared to the cholesterol-poor cells, which, however, does not significantly alter the membrane fluidity between the cholesterol-rich and -poor cells as revealed by fluorescence polarization measurements. The most dramatic behavior of the cholesterol-rich cells is their tendency to form aggregates, which is demonstrated either by concanavalin A-induced agglutination or by cell density-dependent aggregation shown by interference microscopy in vivo.

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of Marnarli et al. (31). A nuclear-free 12,000 × g pellet was prepared from the cell homogenate by differential centrifugation which was then used as starting material for the purification of the plasma membranes by gel filtration on Sephacryl S-1000 superfine (Pharmacia Fine Chemicals, Uppsala, Sweden). The membranes were eluted with 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, at a flow rate of 50 ml/hr. After collection of the plasma membrane-containing fractions, the material was dialyzed against 10 mM Tris-HCl, pH 7.5, containing 0.2 mM MgCl₂ and phenylmethylsulfonfyl fluoride as protease inhibitor, was freeze-dried, and the residual material was taken up in Tris-buffered isotonic sucrose solution for further experiments. Details of the enzymatic assays have been described elsewhere (20).

Labeling Experiments. Thymidine incorporation rates were determined by using [methyl-³H]thymidine (specific activity, 20 Ci/mmol) at concentrations of 0.25 to 0.75 μCi to 0.5-mi aliquots of the cell suspensions (2.5 × 10⁶ to 7 × 10⁶ cells) and incubated at 37° for 30 min. Determination of labeled cholesterol derived from [¹⁴C]acetate was as follows. Amounts of 5 × 10⁶ cells were taken from the culture flasks; centrifuged; washed once with phosphate-buffered saline, pH 7.4, consisting of 0.138 M NaCl, 2.7 mM KCl, 0.49 mM MgCl₂, 0.90 mM CaCl₂, 1.47 mM KH₂PO₄, and 8.1 mM Na₂HPO₄; and suspended in 2 ml of the same buffer, to which 62.5 μCi of [2-¹⁴C]acetate (specific activity, 51 μCi/μmol) were added. After incubation of the reaction mixtures at 37° for the time indicated in the chart, 0.6-ml aliquots were taken and centrifuged, and the pellets were washed 3 times with phosphate-buffered saline, pH 7.4. The cell lipids were then extracted by the Folch procedure (18) and analyzed by TLC using SiG-covered plastic sheets (Macherey-Nagel, Düren, Federal Republic of Germany) and petroleum ether:diethyl ether:acetic acid (80:20:2) as solvent system. After visualization with iodine vapor and disappearance of the color, the spots were cut off the plates and quantitated by liquid scintillation counting.

For the determination of cholesterol precursors and polyprenoids, the Folch extracts were saponified with 1 ml of 5% NaOH in methanol for 1 hr at 100° in a sealed ampul. The unsaponifiable material was extracted 3 times with petroleum ether (b.p. 40-60°) and then analyzed by TLC with chloroform as solvent system. Radioactivity distribution in the various spots was performed as described above.

Quantitation of cholesterol was done by gas-liquid chromatography (21), and for comparison by an enzymatic procedure according to the method of Röschlau (35). P₃ was determined by the method of Eibl and Lands (16). The membrane polar lipids were quantified by phosphorus analysis after TLC separation using SiG-covered plastic sheets. Microscopic examinations of the cell suspensions were made by carefully putting the cells on glass plates and analyzing them by interference microscopy.

DNA Histograms. Cells were washed with 0.85% NaCl solution (isotonic salt solution), fixed with 70% ethanol, and collected at this stage at 4°. After removal of ethanol, the above buffer containing the fluorochrome 4,6-diamidino-2-phenylindol (3 μg/ml) was then added. Flow cytometric analysis was carried out with a computerized FACS II cell sorter (Becton Dickinson) which was equipped with a UV laser beam. The laser was tuned at 363 nm; cellular fluorescence was collected above 390 nm.

Fluorescence Polarization Measurements. Fluorescence polarization was measured at 90° angle relative to the exciting beam, using a Perkin-Elmer MFP 4 fluorescence spectrometer. Two commercial polarizers (Zeiss, Oberkochen, Federal Republic of Germany) were mounted at the excitation (360 nm) and emission (426 nm) site of the cuvet. The samples were excited with vertically polarized light, and the intensity of the polarized fluorescence emission was measured both at vertical and horizontal orientation of the emission polarizer. The labeling conditions of the membrane preparations are described in Fig. 1. The 1,8-diphenyl-
Chart 1. Growth curves of ascites cells in culture. A, curves at 0.5% and B, at 3% serum concentration in the medium. Control serum (---), serum plus 2.50 mg of cholesterol and 250 μg of C₁₈₅ per 100 ml of medium (-----), the same as before but with 25 μg C₁₈₅ (-----), serum minus cholesterol (O-O), serum plus growth factors such as insulin, transferrin, or prostaglandin (V-V) at concentrations between 1 and 4 μg/10⁶ cells.

Chart 2. Growth curves of ascites cells. A, in control medium with 10% serum (D-G), in medium with 3% serum supplemented with 38.7 μmol (0.39 mM) mevalonic acid (x-x), and 6.5 μmol each of squalene (∆-∆) and lanosterol (-----) per 100 ml of medium; B, in medium with 3% serum supplemented with increasing concentrations of cholesterol, 0.4 mg (x-x), 1.25 mg (∆-∆), and 2.5 mg/100 ml of medium (-----).
Control of Growth and Surface Properties of Ascites Cells

the growth-stimulating effect of cholesterol (Chart 2A), and it has also been shown that the rate of proliferation is dependent upon the cholesterol concentration in the medium (Chart 2B). The feedback control of cholesterol synthesis has generally been accepted. To test this also in case of ascites tumor cells, we have measured the rate of cholesterol de novo synthesis from labeled acetate of cells grown in 3% serum medium either with or without cholesterol added to the medium. The results (Chart 3) indicate that the rate of acetate incorporation is depressed by a factor of about 6 in the cholesterol-supplemented cells. Similar results are obtained for acetate incorporation into the entire sterol fraction and the radioactivity distribution between sterol precursors and the products of the branched pathways, ubiquinone and dolidol, is shown in Chart 4. Unlike in liver, the major labeled products were found to be lanosterol, followed by squalene, and the lanosterol-labeling was about 7-fold higher in the cholesterol-depleted compared to the cholesterol-rich cells. From these data, it becomes clear that a block exists in the sterol synthesis pathway at the stage of lanosterol conversion to cholesterol. This also explains why neither mevalonic acid nor squalene nor lanosterol added to the culture medium had a growth-stimulating effect. The data in Chart 4 further show that the main compound of the isoprenoid pathway does not seem to be cholesterol but ubiquinone.

For a further characterization of the growth properties of these
cells kept at a reduced serum concentration, we have measured the thymidine incorporation rate and also determined the amount of DNA within the cell cycle which is shown in Chart 5. A comparison between the control cells (A) and the cells grown at 3% serum plus cholesterol in the medium (C) reveals very similar patterns not only in the cell density-dependent macromolecule synthesis but also in the distribution of DNA between the G1, S, and G2 phases. On the other hand, cells grown at 3% serum without cholesterol in the medium show a reduced growth rate resulting in the accumulation of cells in the mitotic and G2 phase. From these results, we can postulate that cholesterol must play a significant role in the expression of the growth properties of these ascites cells.

In the following, we have measured the cholesterol content, and the C/P ratio of the plasma membranes of control, cholesterol-enriched, and -depleted cells. As shown in Table 1, the cells grown at 3% serum plus cholesterol contain about 30% more cholesterol, and the cells grown at 3% serum concentration without cholesterol contain about 54% less cholesterol than the control cells. Also, differences were observed in the amount of phospholipids of these cell membranes, but their relative composition was mainly unchanged (Table 2). Determination of the C/P ratio as a measure of cell surface alterations gave values of 0.29 for the control, 0.17 for the cholesterol-depleted, and 0.25 for the cholesterol-rich cells. In order to correlate these data with membrane fluidity changes within the lipid bilayer, we have performed fluorescence polarization measurements by using 1,6-diphenyl-1,3,5-hexatriene as membrane probe. The results of these experiments (Chart 6) indicate some small differences in the temperature-dependent fluorescence depolarization between the cholesterol-rich and -poor cells in the higher temperature range only.

To further characterize the surface properties, we have tested the capacity of these cells to agglutinate in the presence of concanavalin A. The results of these experiments are shown in Chart 7. Under the conditions applied, the agglutination occurred during the first 3 to 8 min. However, the extent of agglutination was most dramatic with the cholesterol-enriched cells. Almost 90% of all cells were agglutinated after 2 min of reaction time, whereas the control showed only about 50%, and the cholesterol-poor cells, 30% agglutination. Since, in all these cases, some agglutinates were present before the experiments were started, we have counted only the single cells from the beginning of the tests. Binding studies with labeled concanavalin A (Chart 8) revealed some differences in the binding capacity of these ascites tumor cells.

### Table 1

<table>
<thead>
<tr>
<th>Cholesterol and phospholipid content, and the C/P molar ratio of the plasma membranes of control, cholesterol-enriched, and -depleted cells grown under normal and reduced serum concentrations (3%) without and with cholesterol supplementation</th>
<th>Cholesterol (µg/mg protein)</th>
<th>Phospholipid (µg/mg protein)</th>
<th>C/P (x)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum essential medium</td>
<td>+10% serum</td>
<td>19.6</td>
<td>151.8</td>
</tr>
<tr>
<td></td>
<td>+3% serum</td>
<td>8.0</td>
<td>106.0</td>
</tr>
<tr>
<td></td>
<td>+3% serum and cholesterol (2.50 mg/100 ml)</td>
<td>22.2</td>
<td>256.0</td>
</tr>
</tbody>
</table>

Data represent the average of at least 2 experiments with duplicate analyses.

### Table 2

| Phospholipid composition of horse serum and the plasma membranes of cholesterol-depleted and -enriched ascites tumor cells |
|---|---|---|
| Serum | Cholesterol-depleted cells | Cholesterol-enriched cells |
| ng of phosphorus per 0.1 ml of serum (at 1 ml of medium) | ng phosphorus/mg of membrane protein | ng phosphorus/mg of membrane protein |
| Lipid | % | % |
| Lyso phosphatidylcholine | 766.8 ± 228 | 15.2 | 885.9 ± 9.1 | 20.4 |
| Sphingomyelin | 480.8 ± 208 | 9.5 | 813.5 ± 12.0 | 50.2 |
| Phosphatidylcholine | 3135.9 ± 996 | 62.1 | 1798.9 ± 72.8 | 41.4 |
| Phosphatidylethanolamine | 226.3 ± 55 | 4.5 | 302.5 ± 81.9 | 7.0 |
| Cardiolipin | 436.8 ± 199 | 8.7 | 1251.3 ± 113.3 | 28.8 |
| Cardiolipin | 0.0 | 0 | 1073.3 ± 28.3 | 2.5 |

*Mean ± S.D.*

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CANCER RESEARCH VOL. 44
cells mainly at the low-affinity binding site. From the Scatchard plots, the high-affinity binding capacity was calculated to be $9.5 \times 10^4$ for the cholesterol-depleted and $14 \times 10^4$ for the cholesterol-enriched cells with about the same association constant of $2.4 \times 10^7 \times M^{-1}$. The low-affinity binding capacities were determined with 1.3 and $2.5 \times 10^4$, respectively, with association constants of $4.2$ and $1.1 \times 10^4 \times M^{-1}$.

For further substantiation of the increased agglutinability of the cells at 3% serum plus cholesterol in the medium, we have cultivated the cells until they reached the plateau phase, and then analyzed by interference microscopy under \textit{in vivo} conditions. The results of these experiments are shown in Fig. 1. In contrast to control cells and the cells kept without cholesterol, the cholesterol-enriched cells showed numerous aggregates in various configurations. These aggregations seem to be formed by the cell plasma or microvilli building strong rod-like bridges between cells.

**DISCUSSION**

It has been suggested that the primary role of serum is to provide hormones for cell growth (23). Although the importance of hormones with respect to the cultivation of cells seems to be undisputed, there have been found a number of low-molecular-weight nutrients such as lipids and others which are equally necessary for cell proliferation [see review by Barnes and Sato (6)]. Some of these substances, like phosphoethanolamine and selenic acid, have been tested by us in addition to the compounds described before, but without any effect. Our results clearly demonstrate that ascites tumor cells need preformed cholesterol for growth which normally is supplied by the serum. The observation that cells at 0.5% serum plus exogenous cholesterol in the medium grow only to some extent indicates that other growth-promoting factors become limiting. These factors may be different from those growth factors which we have added to the medium and which have been shown to induce proliferation in ascites cells (30), in a human mammary tumor cell line (5), and in others (8). The block at the G2 phase of the cholesterol-depleted cells has not been observed previously. However, it has been known that the physical state of the lipid-protein matrix of the plasma membrane undergoes profound changes after stimulation of cell division and that the changes are correlated in one way or another to the presence of cholesterol (11). The exact function of cholesterol in this respect is still open to debate (1, 14). We are presently investigating the question in which way cell proliferation, surface function, and morphology may be regulated by the presence or absence of lipids.5

The regulation of cholesterol biosynthesis and the homeostatic control of the cholesterol level are very complex mechanisms, still far from being resolved. For liver and also some hepatomas (3), it is known that the $\beta$-hydroxymethylglutaryl coenzyme A reductase is the key enzyme in the sterol synthesis pathway which is controlled through a multivalent feedback mechanism, involving cholesterol itself and possibly also a nonsterol compound (10). With respect to the involvement of cholesterol, there are at least 3 independently operating negative-feedback mechanisms, which have been described by Sabine (37). One of these mechanisms, namely, the dietary feedback regulation, may be

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5E. W. Haeffner, N. Pawletz, and C. J. K. Hoffmann, manuscript in preparation.
functioning in our ascites cells, since acetate incorporation into cholesterol can be suppressed by a factor of about 7 at a high cholesterol level in the medium. Thus far, these ascites cells differ from a number of extrahepatic tissues including rodent 


Fig. 1. Interference phase contrast microscopy of cholesterol-rich and -poor cells. Cells were kept in minimum essential medium to which 3% horse serum and cholesterol (2.50 mg/100 ml) were added. Cell cultures were kept for 4 days without diluting the cell number but with one medium change. At the time of the analysis, the cell density was $8.6 \times 10^5$ cells/ml. The cells were carefully placed on glass plates and observed immediately under the microscope; a and b, cholesterol-depleted cells; c and d, cholesterol-enriched cells; magnification: a and c, bar equals 50 $\mu$m; b and d, bar equals 12.5 $\mu$m.
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