Culture of Cloned Cells from the Mouse Preputial Gland Tumor

ESR 586

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SUMMARY

Cells from the mouse preputial gland tumor ESR 586 have been cultured and cloned. Trypsin-ethylenediaminetetraacetate was used to obtain single cells. The cells are grown in a modified CMRL-1415 medium supplemented with 10% fetal calf serum. Clones tend to fall into two classes: Class 1, those that undergo morphological differentiation in liquid medium to form round bodies filled with lipid vacuoles; and Class 2, those that do not differentiate. Preliminary studies on the control of differentiation in Class 1 clones suggest that a minimum cell density is required before differentiation takes place. Analysis of the lipids from differentiating and nondifferentiating clones reveals the presence of sebaceous-type lipids in differentiating clones only. No requirement for testosterone was found for these cells.

INTRODUCTION

The preputial gland is a holocrine gland in which the peripheral acinar cells undergo a lethal differentiation to form the final secretion of the gland. Thus, in theory at least, it should not be possible to maintain such cells in culture under conditions in which they differentiate, and to date there has been no report of successful culture of these or other types of sebaceous cells. The mouse preputial gland tumor, on the other hand, is highly differentiated but grows rapidly, thereby indicating that its cells are able to both proliferate and differentiate. Therefore, the possibility of maintaining such cells in culture was explored, and the characteristics of such cells are described.

MATERIALS AND METHODS

Establishment of Cell Lines. An 8-week-old tumor was grown as previously described (7). It was approximately 2 cm in diameter and was removed from the mouse and placed in a sterile 100-ml Falcon 3003 culture dish (Falcon Plastics, Los Angeles, Calif.) containing 10 ml F12 medium (Grand Island Biological Co., Grand Island, N. Y.). Gentamycin (50 μg/ml; Schering Corp., Port Reading, N. J.) and amphotericin B (Fungizone, 2 μg/ml; E. R. Squibb & Sons, Inc., New York, N. Y.) were included in all media. The tumor was minced into pieces small enough to allow pipetting with a 5-ml plastic disposable pipet. The tumor was minced into pieces small enough to allow pipetting with a 5-ml plastic disposable pipet. The medium was decanted. 5 ml trypsin-EDTA (Grand Island Biological Co.) were added, and the minced tumor was transferred to a trypsinizing flask. An additional 10 ml trypsin-EDTA were added, and the mince was stirred in a magnetic stirrer at moderate speed at room temperature for 20 min. Ten ml of supernatant were decanted and discarded, and 10 ml Earle’s balanced salt solution (Grand Island Biological Co.) were added to the flask. The flask was stirred for 20 min. 10 ml of supernatant were decanted and saved, and 10 ml of Earle’s balanced salt solution were added to the flask. This step was repeated 5 times, resulting in 5 supernatants, which were then centrifuged in an International Model CL clinical centrifuge and examined.

Supernatants 1 and 2 contained mostly RBC’s and were discarded. Supernatants 3, 4, and 5 were cultured by seeding 10^6 cells in 35-mm Falcon 3001 culture dishes in duplicate in the following media: F12 medium (Grand Island Biological Co.) containing 10% fetal calf serum; Dulbecco’s modified Eagle’s medium (Grand Island Biological Co.) containing 10% fetal calf serum; a modified CMRL-1415 medium (5) made in our laboratory, in which the cystine was omitted and the cysteine concentration reduced to 35 μg/ml (Dr. R. G. Ham, personal communication), to which 10% fetal calf serum was also added. Cultures were grown at 37° in an atmosphere of 5% CO₂ in air.

Lipid Analysis. The extraction and analysis of lipids was performed as previously described (7).

RESULTS

Description of Clones

Within 10 days, most cells in Dulbecco’s medium had died and were discarded. The cells in F12 or CMRL-1415 were passed when confluent by removing cells with trypsin-EDTA and replating first in 60-mm Falcon 3002 culture dishes and then in 250-ml Falcon 3024 culture flasks. No differences were seen between the cells resulting from the different supernatants seeded.

The cells were cloned by seeding 500 cells in a 60-mm dish, incubating for 1 to 2 weeks, and removing the well-isolated clones with cloning rings. More than 40 clones were obtained in this manner and frozen in liquid nitrogen.
in medium containing 10% dimethyl sulfoxide. All clones were subsequently grown in the modified CMRL-1415 supplemented with 10% fetal calf serum. This medium was found to support a faster growth rate and a higher cloning efficiency than F12.

Cells appeared in many shapes, from flat, epithelial types to very elongated fibroblastoid types. Each clone was recloned at least once, but morphological variants almost always appeared. However, all clones could be divided into 2 classes on the basis of their ability to differentiate in liquid medium:

**Class 1.** This class consists of clones that can undergo a morphological differentiation in culture. For example, clone 34 (Fig. 1) tends to have a flat or wedge-shaped morphology as it grows, but, occasionally, cells will be seen that contain many vacuoles that seem to increase in number and coalesce. Clone 38 (Fig. 2) is less flat and more elongated than clone 34. Over a monolayer of these cells appear large roundish cells with a “honeycombed” appearance. Fig. 3 shows clone 38 cells after 2 weeks in culture. A monolayer of flat cells persists, giving rise to many differentiated cells which become detached and accumulate in the medium.

**Class 2.** This consists of clones that do not undergo the type of morphological differentiation described above. Clone 20 (Fig. 4) is typical of this class. The cells tend to be very flat at first, but later some take on a dendritic appearance.

In order to be certain that both cell types from Class 1 clones result from the same precursor cell, single-cell cloning was carried out with the use of a Falcon 3034 Micro Test plate. An average of 1 cell/well was seeded in 0.3 ml medium, using clones 17 and 26 (both of Class 1). Microscopic examination of the wells was performed, and all wells containing one cell were marked. One single-cell subclone of each was isolated and grown to confluence. In both cases, morphological differentiation typical of the parent clone was observed.

**Factors Leading to Morphological Differentiation**

These were studied, using Clone 38 (Fig. 2). When these cells were grown in clonal culture (500 cells seeded per 60-mm culture dish), approximately 100 clones resulted. The morphology of the cells was like those of the background of Fig. 2. Differentiated cells did not appear until the clones were large. Medium conditioned by cultures containing differentiated cells failed to induce differentiation in younger cultures. It seemed as if a minimal cell density was required before differentiation occurred. To test this hypothesis, cells from Clone 38 were seeded at 10⁴, 10⁵, and 10⁶ cells/60-mm culture dish. Cultures were examined on the 3rd day after seeding. Differentiated cells appeared in cultures seeded with 10⁵ and 10⁶ cells but not in those cultures seeded with 10⁴ cells. Thus it appears that, at least for this clone, morphological differentiation depends on cell density. In another experiment, 10⁶ cells were seeded and examined on the following day. Differentiated cells were seen in the culture, indicating that once the proper cell density is obtained, morphological differentiation can occur within 24 hr.

As the cultures age, many differentiated cells become detached and accumulate in the medium. If these "floaters" are replated, most can attach to the dish but only a few can spread and divide. Table 1 compares the cloning efficiency of cells from: (a) a young, rapidly growing, undifferentiated culture; (b) the monolayer (washed to remove loosely adhering cells) from an age-differentiated culture (Fig. 3); and (c) the supernatant plus wash from the differentiating culture. The cloning efficiency of undifferentiated cells is about 20%. The differentiated cells can no longer give rise to clones. The very low cloning efficiency of cells that have accumulated in the medium suggests that many of these cells (viable by the criterion of trypan blue exclusion) may be committed to differentiate, even though only 47% show morphological changes when examined by light microscopy. Floaters were also allowed to accumulate in a culture and were then collected on a Millipore filter for examination by electron microscopy. Preliminary results revealed the presence of lipid droplets in these floaters.

**Lipid Changes on Differentiation**

The lipids were extracted from cells of clones of both Class 1 and Class 2 and analyzed. In Table 2, a representative analysis of a clone from each class is presented. The nondifferentiating cells contain predominantly polar lipids and free sterols, while the differentiating cells show the presence of sebaceous-type lipids (glyceryl ether diesters, esters, and free sterols, while the differentiating cells show the presence of sebaceous-type lipids (glyceryl ether diesters,

<table>
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<tr>
<th>Total viable cells</th>
<th>% morphologically differentiated cells</th>
<th>Cloning efficiency (%)</th>
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<tbody>
<tr>
<td>Undifferentiated monolayer (10⁶ cells seeded, 2 days growth)</td>
<td>5.6 x 10⁴</td>
<td>0</td>
</tr>
<tr>
<td>Differentiated washed (10⁶ cells seeded, 2 wk growth)</td>
<td>2.4 x 10⁴</td>
<td>4</td>
</tr>
<tr>
<td>Differentiated supernatant and wash (10⁶ cells seeded, 2 wk growth, 2-day accumulation)</td>
<td>3.6 x 10⁴</td>
<td>47</td>
</tr>
</tbody>
</table>

* Trypan blue exclusion, hemocytometer count.

Table 2

<table>
<thead>
<tr>
<th>% composition of lipids</th>
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<tbody>
<tr>
<td>Non-differentiatiating cells (clone 23)</td>
</tr>
<tr>
<td>Polar lipids</td>
</tr>
<tr>
<td>Free sterols</td>
</tr>
<tr>
<td>Free fatty acids</td>
</tr>
<tr>
<td>Triglycerides</td>
</tr>
<tr>
<td>Glyceryl ether diesters</td>
</tr>
<tr>
<td>Sterol esters</td>
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<td>Wax esters</td>
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sterol and wax esters) which are absent from the former cells.

**Hormone Effects**

The mouse preputial gland is controlled by a very complex endocrine interaction. Testosterone is known to play a central role in the control of mitosis in all sebaceous glands (3). Some primary cultures were grown in the presence of 1 \( \mu \text{M} \) testosterone. We found, however, that this addition was not necessary for growth and it was discontinued. It is possible that the cells are receiving steroid hormones from the fetal calf serum. To test this hypothesis, the serum was treated with Norit A and dextran to remove steroids (2). The treated serum was still able to support growth of the preputial cells.

**DISCUSSION**

The successful culture of sebaceous-like cells represents the addition of an important cell type to the list of established cell lines. Not only does this permit detailed studies to be made of sebaceous cell biology, but it provides a cell type which not only differentiates in culture but differentiates to form lipids rather than proteins. In these cells, the initiation of differentiation can be detected early by the formation of lipid droplets, as seen by electron microscopy, or by changes in the chemical composition of the cell lipids. Furthermore, the cells we have cultured were obtained from a rapidly growing tumor which, one must presume, resulted from some form of transformation. From such a tumor we have isolated 2 distinct classes of cells, 1 still capable of undergoing differentiation, the 2nd unable to differentiate but capable of continual growth. Careful comparison of the behavior and properties of the 2 cell lines should, it is hoped, provide some clue to the tumorigenesis of this tumor. We are currently investigating the differences in tumorigenicity of the 2 types of cell.

The sebaceous secretion, sebum, serves to lubricate and protect the skin, hair, or feathers of animals and birds. With the exception of man, the predominant lipid components are mono- and diester waxes and sterol esters, where the sterol moiety can be lathosterol, lanosterol, etc., as well as cholesterol (6). Mouse sebum is predominantly alkane-diol diester waxes with the remainder almost entirely lathosterol esters (9). The preputial gland secretion of the mouse serves a more specialized role as a territorial marker and sex attractant (1) and contains glyceryl ether diesters and alkyl acetates (8) not present in other sebaceous secretions. Triglycerides are formed by the differentiating preputial cells but do not form part of the final glandular secretion (V. R. Wheatley, unpublished observations). In our experiments, glyceryl ether diesters, sterol, and wax esters were found only in the differentiating cultured cells; such lipids are typical of the differentiating preputial cell. The nondifferentiating cultured cell lipids are predominantly polar lipids and free sterols (i.e., membrane lipids). Free fatty acids and a little triglyceride are also present, but these lipids are probably mainly derived from the culture medium (4).

The preputial gland, in common with all sebaceous glands, is controlled by complex endocrine interactions (3). We were unable to demonstrate a specific requirement of testosterone by these cells for growth, and hence this was not incorporated in any of the media used. However, other hormones should affect the growth of these cells. This and other aspects of hormonal action, e.g., cell receptors, could conveniently be studied *in vitro* with these cultured cells. Hence, cell types now become available for the study of both normal and aberrant differentiation as well as for *in vitro* studies of hormonal effects.

**ACKNOWLEDGMENTS**

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**REFERENCES**


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