Growth Rates of Normal and Abnormal Human Mammary Epithelia in Cell Culture

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SUMMARY

The in vitro growth rates of different classifications of human mammary epithelium were compared. Samples included 4 established breast cell lines and excised tissue or breast fluid cells originating from 40 different women and comprising 3 classifications: normal, nonmalignant atypical, and malignant. Growth was quantitated in situ and expressed as population-doubling time. Principal findings were: (a) malignant cells divided at a slower mean rate than normal cells; (b) population-doubling time values of malignant cells were more heterogenous than those of normal cells; (c) cultures from nonmalignant atypias showed population-doubling time means and standard deviations between those of normal and malignant cells; and (d) long-term mammary tumor cell lines divided more slowly than did normal cells. Discussion includes implications of data for the preneoplastic state and cell culture of mammary epithelium.

INTRODUCTION

Knowledge of the growth of tumors is important to understand the genesis and outcome of human cancer. Necessary for tumor growth studies are an appropriate biological system and an accurate method for growth quantitation. Because in vivo experimentation with humans is difficult, models must suffice as biological systems. Accurate quantitation of growth has been achieved in biological systems by methods such as measurement of tumor size or quantitation of cells, nuclei, total protein, mitoses, and DNA synthesis. We describe here monolayer area measurement as a growth quantitation method and its application to a model system of primary cultures of human mammary epithelium, to compare, in vitro, growth rates of cells from normal, nonmalignant atypical, and malignant human breast tissue. Hopefully, such values will serve as baselines against which to measure the effects of biological, chemical, and physical agents on the growth of human mammary epithelial cells in culture. Because primary cultures are the in vitro growth of cells taken directly from the human host, study of their growth may enhance our understanding of some aspects of human mammary tumor growth in vivo.

MATERIALS AND METHODS

Source and Preparation of Specimens. Breast fluids contain mammary epithelial cells which are viable and can be grown in culture (6, 15). About 50% of women (Caucasian) have fluid in their breasts, whether or not they are pregnant or lactating (31). Breast fluid specimens giving rise to proliferating epithelial cells were obtained from 10 nonlactating women and 1 weaning woman by either manual expression or nipple aspiration with a Sartorius aspirator (Diagnostic, Inc., Indianapolis, Ind.) (33). Fluid was collected into isotonic solution and centrifuged at low speed to pellet cells, which were then inoculated into a single well of either 32-sq mm microtest plates (Falcon No. 3040, Oxnard, Calif.) or 182-sq mm microtest plates (Linbro No. FB-16-24-TC, Van Nuys, Calif.)

Mammary epithelial cells were also obtained from excised tissue (biopsies, mastectomies, reduction mammoplasties, and metastatic tumors) of 35 different women by either spilling or collagenase treatment as described by Lasfargues (21). Each specimen was trimmed of fat, sliced thinly in a large Petri dish containing 5 to 10 ml isotonic solution, and agitated gently through the solution to spill epithelial cells from their supporting framework. The solution was then centrifuged at low speed to pellet the cells, which were then inoculated into microtest plates (Linbro No. FB-16-24-7C) or 25-sq cm plastic flasks (Falcon No. 3013).

The remaining tissue slices were treated for 2 to 4 days with a 0.1% solution of collagenase ( Worthington Biochemical Corp., Freehold, N. J.) in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.). Treatment was terminated by vigorous shaking to break up clumps and centrifuging at low speed to pellet cells, which were then inoculated into microtest plates (Linbro No. FB-16-24-7C) or 25-sq cm plastic flasks.

Four permanent mammary epithelial cell lines, BT-20, MDA-MB-157, MCF-7, and SKBR-3, were also used in this experiment. Cultures were obtained from the Cell Culture Laboratory, School of Public Health, University of California, Berkeley, which received the lines from E. Y. Lasfargues (BT-20), R. Cailleau (MDA-MB-157), C. M. McGrath (MCF-7), and J. Fogg (SKBR-3). The development of these lines and their identity evaluation have been described (7, 13, 22, 27, 37, 39).

Cell Cultures. We could not standarize our seeding densities of primary cultures because so few cells were available,
but inocula usually ranged from $2 \times 10^3$ to $2 \times 10^4$ cells/ml. Cells of all types were maintained in Dulbecco's modified Eagle's medium (Grand Island Biological Co.) supplemented with 10% fetal calf serum (Grand Island Biological Co.), insulin (Calbiochem, San Diego, Calif.) (10 $\mu$g/ml), penicillin (Eli Lilly and Co., Indianapolis, Ind.) (250 units/ml), streptomycin (Lilly) (0.1 mg/ml), polymixin (Calbiochem) (50 units/ml), and amphotericin B (Fungizone) (E. R. Squibb & Sons, New York, N. Y.) (5 $\mu$g/ml). All cells were maintained in an atmosphere of 5% CO$_2$.

**Specimen Classification.** We determined cytological classifications of breast fluid cells by standard Papanicolaou smears (29). Because some nonmalignant atypias (fibrocystic disease) cannot be cytologically distinguished from normal, we used, as a source of normal breast fluid cells, only donors without palpable lumps or a history of fibrocystic disease. Cells from excised tissues were classified by examination of histological preparations. Trypsinized cells from monolayers of the permanent cell lines were examined by Papanicolaou smear technique to confirm their identity as neoplastic (16). All specimens for cytological examination were coded to eliminate bias.

**Determination of Growth Rate.** Growth curves of the permanent mammary tumor cell lines were plotted from successive cell counts of trypsinized replicate cultures grown in 25-sq mm plastic flasks. These counts were performed using a hemocytometer and diluted trypan blue exclusion as a test of cell viability.

Because cell number in primary cultures of breast fluids and excised tissue is too small to seed replicate flasks for trypsinized cell counts, growth curves were constructed from successive in situ cell counts on the same epithelial patch using phase-contrast microscopy at $\times 100$ magnification to distinguish cell nuclei (Fig. 1) or from successive in situ area estimations of individual epithelial patches at $\times 40$ magnification, using an ocular grid (0.5 sq cm in 1-sq mm divisions) inserted into the microscope eyepiece (Fig. 2).

**PDT's** were calculated from the maximum slope of the logarithmic portion of the growth curves of each culture (Chart 1). Patches chosen for measurement were always larger than 0.5 sq mm, were well isolated from other patches or fibroblasts, and were without curled borders, loose cells, or debris. Area measurements were begun only after 3-dimensional pieces of explanted tissue had spread to a 2-dimensional monolayer and were free of tissue fragments. Only growth curves with enough points to include the entire logarithmic phase were included in the data. The PDT value for each specimen was the average of values of several individual patches.

**RESULTS**

When inoculated into culture, healthy mammary epithelial cells attached to the substrate and formed isolated epithelial patches that increased in area but usually failed to reach confluency. Mitotic figures (Fig. 3) were regularly present in patches derived from all specimen types. Necrotic cells released from some of the malignant specimens failed to attach to the substrate and therefore did not contribute to monolayer area measurements. Epithelial cells exfoliated into breast fluids or spilled from excised tissue grew as fibroblast-free cultures, whereas epithelial cells released by collagenase treatment were usually contaminated with fibroblasts.

Growth curves were completed for 46 of the 52 cultures with epithelialoid patches. The principal reason for incomplete growth curves was failure to plot at least 3 successive measurements during the logarithmic phase of the growth curve. Our data do not include PDT's on cells from metastatic mammary tumors, because we were unable to complete growth curves on cells from our only 2 specimens, bone and lymph node.

In order to evaluate whether area estimations reflected actual growth of cells or merely spreading of the cells after attaching to the substrate, parallel determinations of cell count and area were done in 2 ways. Cell line MCF-7, which grows as distinct epithelialoid patches like the primary cultures, was monitored by both dye exclusion counts of trypsinized cells in replicate flasks and in situ area measurements in the 3 vessel types used in this study. The results (Table 1) indicate that PDT's based on in situ measurements correspond closely to those based on counts of trypsinized cells and that the type of culture vessel used did not significantly affect the growth rate.

Second, growth curves plotted from area measurements were compared with growth curves plotted from in situ total cell counts for the same patch of epithelialoid cells in primary culture. This was done for 1 specimen of each classification: normal, nonmalignant atypical, malignant, and malignant cell line (MCF-7). The results, illustrated in Chart 2,
PDT's of MCF-7 determined by different methods

<table>
<thead>
<tr>
<th>Method</th>
<th>PDT (hr)</th>
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<tbody>
<tr>
<td>Cell counts of trypsinized replicate flasks (25-sq cm)</td>
<td>38</td>
</tr>
<tr>
<td>In situ quantitation in 25-sq cm flasks</td>
<td>38</td>
</tr>
<tr>
<td>In situ quantitation in 182-sq mm microtest plates</td>
<td>38</td>
</tr>
<tr>
<td>In situ quantitation in 32-sq mm microtest plates</td>
<td>40</td>
</tr>
</tbody>
</table>

Table 1

indicate little difference in PDT's calculated from growth curves determined by each method. In each case, PDT determined by cell count was higher than PDT determined by area measurement, indicating that, during logarithmic growth phase, cell number was increasing more rapidly than total cell area.

PDT's calculated from completed growth curves and plotted against specimen classification resulted in the spot distribution chart, Chart 3. PDT's of normal cells were very homogeneous. Seven of the 11 normal cultures had PDT's of 23 to 25 hr. The cells of 5 breast fluid donors, sampled on multiple occasions, over 1- to 2-year periods, repeatedly showed the same PDT. PDT values of atypical cells were more heterogeneous than those of normal cells. The means of PDT values of cultures classed as nonmalignant atypical and malignant each differed significantly from the mean PDT value for normal cells and increased with increasing dysplasia. For the cell lines, standard deviation and significance of the difference of the mean from normal cells were not calculated because the sample size was only 4.

The method described to determine PDT from growth curves plotted from successive in situ monolayer area measurements permits growth studies on populations of cells too small for cell enumeration by standard hemocytometer and Coulter Counter methods and preserves the viability of the cells for use in other experiments. In situ methods utilizing an ocular grid have been used by others to enumerate cell nuclei (23) and to measure volume of excised tissue (9). Our experiments have established that increase of patch area reflects an increase in cell number rather than spreading ability of the cells.

Hosick (17) found that, unlike typical fibroblasts in culture, mouse mammary epithelial cells exhibited a growth rate and saturation density dependent on concentration of cells in the initial inoculum. We found a similar density-dependent effect of PDT for patches smaller than 0.5 sq mm, but this effect disappeared for patches over 0.5 sq mm. Therefore, only patches greater than 0.5 sq mm were included in these data.

We considered the possibility that our data reflect a differential growth effect of medium additives on normal and atypical cells, rather than intrinsic differences in growth rate. Normal cells in culture are more sensitive to high concentrations of Fungizone (amphotericin B) than are transformed cells (1), yet normal cells in this experiment grew faster than transformed cells. Moreover, antimicrobial...
agents at concentrations we used have little effect on growth of cultured cells (1). The benefits of insulin for the growth of mouse mammary epithelium is well documented (8, 18), but differential effects on cultures of normal and malignant cells at the glucose concentrations present in Dulbecco's modified Eagle's medium have not been reported. Although fetal calf serum contains estrogens and progesterones, of which binding to mammary cells may vary with cellular concentration (25), amounts are at pg levels (11), whereas levels experimentally effective for human mammary epithelial cells in vitro are in the μg range (2). Removal of serum steroids with dextran-coated charcoal did not alter doubling time of primary cultures of human mammary epithelium (2). Our data may reflect a differential growth effect of a medium additive other than those considered above. However, intrinsic differences in growth rate, either in vitro or in vivo, may ultimately be due to differential responses of normal and atypical cells to their nutrient environment.

Our finding that cells from primary cultures of malignant breast tumors doubled, on the average, more slowly than did normal controls is not surprising, because a number of other tumor cell types in both humans and animals were found to grow more slowly than their normal counterparts (5, 24). In our system, this cannot be explained solely on the basis of morbid or necrotic cells often present in malignant tumors, because healthy, established mammary tumor cell lines divided on the average, even more slowly than did the primary cultures from malignant tumors.

The heterogeneity of PDT values among malignant cells from different patients may indicate a high degree of tumor individuality. Alternatively, it may reflect a mixture of cell types within each tumor. There was no way to distinguish morphologically between normal and atypical cells in culture.

Interestingly, cells derived from nonmalignant atypias doubled at mean rates and exhibited standard deviations between those of normal and malignant cells. The concept that nonmalignant atypias represent a preneoplastic stage in the progression from normal to neoplastic was proposed many years ago (14) and has been supported by the mouse mammary tumor model (26), subgross and histological examination of human breasts (19, 30), and epidemiological studies indicating that women with nonmalignant breast lesions are 4 to 5 times more likely to develop mammary carcinomas than are those without such a history (3). It is impossible to deduce from our data whether an increase in PDT preceded or followed a progression from normal to preneoplastic.

Replication rate is a stable property of cells in culture and is included in the characterization of cell lines of the American Type Culture Collection (34). Our finding that malignant breast cells in long-term culture replicate more slowly than normal cells suggests that the capacity to become a permanent mammary cell line depends on a trait other than fast growth rate. The frustrations of establishing cell lines from primary explants of breast tissue are common, and defining baseline PDT values for primary cultures of each cellular classification is fundamental to the proper testing of potential growth-promoting substances.

Several other investigators have studied the growth kinetics of human mammary cells (4, 10, 12, 20, 35, 36, 38). Our data, showing that atypical mammary cells divided at a slower average rate than their normal counterparts, agree with another study in which normal and atypical breast tissue were compared after tritiated thymidine labeling (20). For malignant cells, our PDT range of 10 to 86 hr approximates the value (29 to 120 hr) obtained by another group (35), estimating potential doubling times of malignant breast cells in vitro from tritiated thymidine labeling indices, and our mean PDT (41 hr) compares well with the estimated median intermitotic time (2 days) of human metastatic mammary cells in vivo (36).

Extrapolation of our in vitro results to the in vivo situation is complicated because tumor growth in vivo may be influenced by the immune system, the extent of tumor vascularization, and the physiology and endocrinology of the host (4). Further growth kinetics studies are needed to assess the relative contributions of cell replication time, longevity, adherence, and state of cell differentiation to the observed slower in vitro growth rate of populations of malignant cells (5, 28, 32) and to determine whether these in vitro growth kinetics relate to the unlimited growth of human mammary carcinomas in vivo.

ACKNOWLEDGMENTS


REFERENCES

G. C. Buehring and R. R. Williams


Fig. 1. A patch of mammary epithelium shown by phase-contrast microscopy with superimposed ocular grid image for estimating cell number. × 150.

Fig. 2. A patch of mammary epithelium with superimposed ocular grid image for estimating area of monolayer. × 60.

Fig. 3. A mitotic figure within an epithelioid patch in a primary culture derived from a nonmalignant breast atypia. × 300.
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