Kinetic and Morphometric Responses of Heterogeneous Populations of Experimental Breast Cancer Cells in Vivo

Scott Lancaster, Hugh F. English, Laurence M. Demers, and Andrea Manni

ABSTRACT

Although the hormone responsiveness of some breast cancers is well known, the differential sensitivity of tumor cell subpopulations to hormonal effects is not well established. These experiments were designed to address this issue using the hormone-responsive N-nitrosomethylurea-induced rat mammary tumor. Rats bearing these tumors were randomly assigned to no treatment, 7-day castration, and 7-day castration followed by 1-, 3-, 7-, and 10-day treatment with estradiol benzoate (5 µg) and perphenazine (1 mg) to stimulate prolactin release. Under these conditions, the proportion of different cell populations was estimated with morphometric analysis, while their replicative activity was assessed using [3H]thymidine autoradiography. In tumors of intact rats the fractions of glandular epithelial, myoepithelial, and nonepithelial cells were 88.2%, 3.8%, and 8.0%, respectively. All cell types manifested a similar kinetic response to our hormonal treatments characterized by a drastic decline in labeling indices after castration followed by a progressive increase with hormone repletion which peaked on Day 7 of treatment. The magnitude of the response was, however, greater in the epithelial components of the tumor (glandular and myoepithelial cells), whereas the peak labeling indices significantly exceeded those observed in the tumors of control intact rats. Castration reduced the proportion of glandular cells while increasing the fractions of myoepithelial and nonepithelial cells. Furthermore, castration reduced the volume of the glandular-epithelial cells by 35%, which accounted for approximately half of the overall tumor volume reduction induced by ovariectomy. These alterations in tumor morphology were partially reversed by hormone repletion. These results underscore the exquisite hormonal sensitivity of different cellular counterparts of this experimental breast cancer with regard to both kinetic and morphological characteristics. They also provide support for stromal-epithelial interaction in the hormonal modulation of breast cancer growth.

INTRODUCTION

Regimens involving sequential induction of hormone depletion and repletion have recently been proposed to optimize synchronous growth of endocrine-dependent neoplasms, such as breast and prostate cancer, in the attempt to enhance the efficacy of cytotoxic chemotherapy (1–4). The rationale for such therapeutic approach is based on the assumption supported by some experimental evidence (5) that cytotoxic drugs are more effective on rapidly dividing cells. The possibility of successful implementation of these treatment strategies can be significantly enhanced by improved understanding of the proliferative responses of hormone-responsive populations of cancer cells to modifications of the endocrine milieu of the host. Consequently, these experiments were undertaken to evaluate, in detail, the effects of sequential hormone depletion and repletion on cell kinetic characteristics of experimental breast cancer. The experimental system used was the NMU2-induced rat mammary tumor, a well-established model of hormone-responsive breast cancer (6, 7), the growth of which is primarily affected by estradiol and prolactin (8, 9). Since heterogeneity is a salient feature of human and experimental tumors, including breast cancer, we were particularly interested in individually analyzing the growth characteristics of heterogeneous subpopulations of cells within each tumor. The technique of tritiated thymidine autoradiography provided us with a powerful tool to address this important biological question.

In addition to examining the kinetic activity of the tumor, changes in tissue morphology were also investigated. The technique of quantitative morphometry allowed us to critically evaluate the relative contribution of changes in cell size versus number to the alterations in tumor volume induced by our hormonal manipulations.

MATERIALS AND METHODS

Tumor Induction. Fifty-day-old female Sprague-Dawley rats (Charles River) were given injections of N-nitrosomethylurea (Sigma) via the jugular vein. The dose given was 5 mg/100 g of body weight in a vehicle of 0.3% acetic acid.

The rats were given food and water ad libitum and were housed undisturbed in light- and temperature-controlled rooms (12 h of light and 21°C) in the Department of Comparative Medicine, The Milton S. Hershey Medical Center. Mammary tumors developed 5 to 15 wk after NMU administration.

Measurement of Tumor Growth. Tumor growth was monitored by weekly measurements of tumor volume. The length (l), width (w), and height (h) of the tumor were measured with a Jamison caliper. From these dimensions the geometric volume was calculated using the formula \( V = \frac{1}{6} \times l \times w \times h \), which assumes the shape of a hemielipsoid (10).

Experimental Protocol. Tumor-bearing rats were randomly assigned to one of five ovariectomy groups or to one of 2 control groups. Ovariectomy was performed on each rat when one of the individual tumors reached a volume of 1600 mm³. One wk after castration, one group was sacrificed, while the remaining four were started on hormone replacement for 1, 3, 7, and 10 days, respectively. Hormone replacement consisted of daily s.c. injections of 5 µg of 17β-estradiol benzoate (Steraloids) and 1 mg of perphenazine. Perphenazine was given to stimulate endogenous prolactin secretion. Tumors that did not regress more than 30% 7 days after castration were considered hormone insensitive and were excluded from the study. Of the 37 animals entered, 2 were rejected by this criterion. One of the intact groups was sacrificed when the tumors had grown to a volume of 1600 mm³, while the other was sacrificed when the tumors had only reached a volume of 600 mm³. The latter group was selected on the basis of the estimated effect of 7-day castration on tumor growth based on our previous experience (6, 8). Inclusion of this group was deemed necessary in order to take into account Gompertzian kinetics (11) when comparing data obtained in the intact versus treatment groups. Specifically, we wanted to exclude that the observed alterations in tumor cell kinetics following ovariectomy were simply due to the smaller volume of the tumors rather than to the effect of the treatment.

Tissue Processing and Autoradiography. One h following the i.v. injection of 1 µCi/g body weight [3H]thymidine (New England Nuclear; specific activity, 20 Ci/mmol) the animals were killed by cervical dislocation, and the tumors were rapidly removed. The tissues were fixed and embedded in plastic following procedures described earlier.
Sections 0.8 μm in thickness, were cut on a Porter Blum MT2 ultramicrotome, dried onto a glass slide, and dipped into Kodak NTB-2 emulsion diluted 1:1 with distilled water. The slides were then placed in dessicant-containing boxes impervious to light and allowed to expose for 4 wk at 4°C. After exposure, the slides were developed in Kodak D-19 and stained with Azure II.

For every animal, three blocks were randomly selected, sectioned, and processed for light microscope autoradiography as described above. The labeling index was determined by direct counting of the nuclei in sections viewed with a × 100 oil objective lens on a Leitz Dialux light microscope. Quantitations were performed on defined areas optically superimposed on the section by inserting a grid of measured dimensions into one eye piece. The grid areas chosen for quantitation were selected by the systemic random sampling technique (13, 14). Sampling was accomplished by scoring all nuclei enclosed within the grid area into the appropriate category (see below).

Grain count analysis was performed on representative autoradiographs in order to establish a reliable limit of defining a nucleus as being labeled (15). The number of nonnuclear grains was exceedingly small so that nuclei displaying greater than five grains were considered to be well within the range of being truly labeled. For every animal in each treatment group, the numbers of labeled and unlabeled cells in each cell population defined below were tabulated separately and recorded as the percentage of labeled cells. In addition, the figures for each category were combined in order to obtain the total percentage of labeled cells.

Categorization of Cell Types. All nuclei counted were categorized into one of three groups: glandular epithelial; myoepithelial; and nonepithelial (Fig. 1). The criteria for differentiation were those used by Tseng (16) in which cell location, nuclear shape, and cytoplasmic density are the primary indications of cell type. Specifically, glandular epithelial cells were aggregated into clusters and/or lined luminal spaces that appeared to be acini and ductal structures. The shape of the nucleus was round, and the nuclear diameter was nearly as large as cell width. The shape of the cell was columnar under control conditions.

Myoepithelial cells were located at the periphery of the clusters, acinar, and ductal structures. Their nuclei were elliptical and homogeneously densely stained. Cell shape was also elliptical with cytoplasmic projections radiating from the nuclear center. These projections would lie on the borders of the glandular epithelial tissue sometimes forming a continuous boundary between epithelial and nonepithelial tissue.

The nonepithelial category consisted of all other cells not defined above. These cells were almost exclusively confined to the stromal matrix surrounding the epithelial tissue. Cell shape and size varied between the different classes of cell (i.e., endothelial, fibroblast, and mast cells).

Morphometric Analysis. Raw cell counts (Na) were obtained by scoring all observed nuclei, labeled as well as unlabeled, into one of the three categories. The final numerical density (Nv) of the cell types was obtained by correcting the raw counts (Na) using the Floderus equation (17) as detailed by Mori and Christensen (18), $Nv = Na/(D + T - 2h)$, where $T$ is average section thickness, $D$ is average nuclear diameter, and $h$ is the smallest discernible nuclear profile. In this study, $h$ was assumed to be $D/10$. The corrected value for $T$ was determined by measuring the diameter of reembedded sections. From these corrected counts the percentage of a population of cells per total cells in a unit area was calculated.

In order to understand the effects of the treatment on cell size, certain parameters of the cell had to be quantitated. These parameters included cell height and cell width because they could be directly measured. From these dimensions cell volume could be crudely estimated in order to assess the changes in cell size due to therapy. The formula $h \cdot w^2$ was used to estimate volume.

Cellular dimensions were measured with the × 100 oil immersion lens using a calibrated ocular micrometer. Glandular epithelial cell height was defined as the longest discernible axis. Width was then defined as the dimension 90° to the major axis. The myoepithelial cell size was not measured due to the difficulties introduced in estimation of volume brought on by the cytoplasmic projections. The cell size of nonepithelial tissue was not also measured due to the variation of cell types.

Hormonal Measurements. Blood was taken just prior to animal sacrifice. The sera were stored at −12°C until they were used for hormonal determinations. Estradiol and progesterone concentrations were measured using specific radioimmunoassays (19, 20) in routine use in the Core Endocrine Laboratory of the Hershey Medical Center.

Statistics. Data are expressed as the mean ± SEM. All results were tested for significance using a one-parameter analysis of variance with the Newman Keuls test (21).

RESULTS

Tumor Growth. Fig. 2 illustrates the effects of sequential hormone depletion and repletion on tumor growth. Tumor volume decreased by 60% 7 days following castration. Upon institution of hormonal repletion, tumor growth promptly resumed, and by the seventh day of repletion, tumor volumes had increased to their initial levels.

Treatment Effects on Tumor Cell Kinetics. Fig. 3 shows the
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Fig. 3. Changes in the labeling indices of all 3 tumor cell types combined induced by sequential hormone depletion and repletion. The number of tumors tested was 5 in each case. *, P < 0.01 versus intact; **, P < 0.01 versus intact and 7-day castration. Points, mean; bars, SEM.

Fig. 4. Changes in the labeling indices of the epithelial and nonepithelial tumor cells induced by our hormonal treatments. The number of determinations performed at each time was five. *, P < 0.05 versus intact; **, P < 0.01 versus intact; †, P < 0.05 versus 7-day castration; ‡, P < 0.01 versus 7-day castration; §, P < 0.01 versus intact. Points, mean; bars, SEM.

Fig. 5. Changes in the labeling indices of the glandular and myoepithelial cells following sequential hormone depletion and repletion. The number of determinations performed at each time was five. *, P < 0.01 versus intact for both cellular components; **, P < 0.05 versus intact; ‡, P < 0.01 versus intact. Points, mean; bars, SEM.

effect of our treatment on the LIs of all three tumor cell types combined. There was no difference in the LI of tumors of intact rats having volumes of 1600 mm³ and 600 mm³ (4.00 ± 0.87% and 3.97 ± 0.96%, respectively). Seven days post ovariectomy the LI decreased significantly to 0.54 ± 0.17%. When hormones were replaced the LI progressively increased and peaked at 10.0 ± 1.44% after 7 days of treatment.

Examination of the treatment effect on the LI of different tumor cell populations revealed that the responses of epithelial and nonepithelial cells were qualitatively similar (Fig. 4). Basal labeling indices were similar in epithelial and nonepithelial cells (4.04 ± 0.98% and 4.42 ± 1.43%, respectively). Within each cellular compartment no difference in labeling indices was observed between tumors of 600- and 1600-mm³ volume. Cas-

traction decreased the LI to 0.47 ± 0.15% in epithelial cells and to 1.16 ± 0.60% in nonepithelial cells. Following hormone repletion there was a progressive increase in the LI of both cellular components which peaked on Day 7 and subsequently declined by Day 10. However, the proliferative response of epithelial cells was greater with a LI on Day 7 (10.54 ± 2.17%), which was significantly higher than intact levels. In contrast, the LI of nonepithelial cells did not significantly exceed precastration levels (6.23 ± 1.72%).

The effects of the treatments on the labeling index of the two epithelial subpopulations are presented in Fig. 5. In intact rats the labeling indices of the glandular cells were similar in tumors of 600 and 1600 mm³ (3.87 ± 0.87% and 4.13 ± 1.0%, respectively). In contrast, the labeling indices of the myoepithelial cells were higher in the small tumors (3.01 ± 1.28% versus 1.42 ± 0.52%; P = not significant). This difference, however, could be accounted for by a single tumor with an unusually high LI (7.82%). If this were excluded the LIs of the myoepithelial cells in small and large tumors were similar (1.81 ± 0.56% and 1.42 ± 0.52%, respectively). The labeling indices of these two epithelial populations were similarly affected by our hormonal manipulations. A significant decline was observed 7 days after castration (0.51 ± 0.17% for glandular cells and 0.13 ± 0.08% for myoepithelial cells). Upon hormone repletion we observed a progressive rise in the labeling indices to levels on Day 7 significantly exceeding precastration values for both cell subpopulations (10.78 ± 2.13% for glandular cells and 7.06 ± 3.31% for myoepithelial cells).

Treatment Effects on Tumor Morphology. Castration caused a visible increase in the amount of necrosis within the tumor. In addition, the percentage of glandular epithelial cells within a unit area was reduced from 88.2 ± 0.9% to 76.0 ± 2.8% (Table 1), whereas the nonepithelial and myoepithelial components rose (from 8.0 ± 0.8% to 14.4 ± 1.8% and from 3.9 ± 0.9% to 9.6 ± 2.0%, respectively). These alterations in the cellular composition of the tumor were associated with the finding of larger areas of nonluminal spaces in tissue sections...
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Table 1 Effects of treatments on the percentage of the various subpopulations comprising the NMU-induced tumor

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Glandular epithelial</th>
<th>Myoepithelial</th>
<th>Nonepithelial</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>88.2 ± 0.9</td>
<td>3.8 ± 0.9</td>
<td>8.0 ± 0.8</td>
</tr>
<tr>
<td>7-Day castrate</td>
<td>76.0 ± 2.8</td>
<td>9.6 ± 2.0</td>
<td>14.4 ± 1.8</td>
</tr>
<tr>
<td>1-Day repletion</td>
<td>78.7 ± 4.1</td>
<td>7.1 ± 2.1</td>
<td>15.1 ± 2.7</td>
</tr>
<tr>
<td>3-Day repletion</td>
<td>77.4 ± 4.8</td>
<td>7.4 ± 3.3</td>
<td>15.2 ± 2.8</td>
</tr>
<tr>
<td>7-Day repletion</td>
<td>81.7 ± 3.1</td>
<td>6.6 ± 1.9</td>
<td>11.7 ± 2.9</td>
</tr>
<tr>
<td>10-Day repletion</td>
<td>80.8 ± 2.5</td>
<td>5.5 ± 0.8</td>
<td>14.6 ± 3.3</td>
</tr>
</tbody>
</table>

* Mean ± SEM percentage from a sample size of 5 tumors.

* P < 0.01 versus "intact.

* P < 0.01 versus "7-day castrate.

Castration also affected the size of the glandular epithelial cell (Table 2). Both height and width were reduced following ovariectomy. This was associated with a reduction in volume of 35%. Hormonal repletion seemed to reverse the effects of castration. Cell height and width as well as volume were increased during the repletion phase and reached intact levels by Day 3 of treatment. Subsequently, there was a slight decrease in all these parameters which remained significantly higher than castrate levels (Table 2).

Treatment Effects on Serum Hormone Levels. As expected, castration induced a profound reduction in the circulating levels of estradiol and progesterone (Fig. 8). Our hormonal replacement regimen was able to restore estradiol levels to control values. Since we did not replace progesterone, the levels of this hormone continued to be low during our treatment period (Fig. 8).

Discussion

The stimulative effect of hormones on human (22, 23) and experimental (24) breast cancer cell proliferation is well known. However, the differential proliferative responses of heterogeneous tumor cell subpopulations to modifications of the hormonal environment have not been adequately evaluated. This is a critical issue under a biological and potentially therapeutic point of view, since cellular heterogeneity is emerging as a cardinal feature of human malignancies including breast cancer (25). Consequently, our experiments were designed to evaluate, in detail, the effects of sequential hormone depletion and repletion on the cell kinetic characteristics of distinct subpopulations of tumor cells, using the hormone-responsive NMU rat mammary cancer. Our results indicate that both the epithelial and nonepithelial component of the tumors manifested a similar proliferative response to our hormonal manipulations (Fig. 4). In both cellular counterparts we observed, in fact, a drastic reduction in the labeling index after castration which was from ovariectomized animals (Fig. 6). These spaces appear to be areas where glandular epithelial cells had died while the surrounding myoepithelial cells and nonepithelial tissue remained apparently unaffected. Hormonal administration partially reversed the effects of castration in this regard (Fig. 7). Only the fraction of myoepithelial cells was significantly reduced by our treatment to levels similar to those observed in tumors of intact rats (Table 1). The fraction of glandular cells only slightly rose over castrate levels, whereas the percentage of nonepithelial cells remained unchanged throughout the hormone repletion phase (Table 1).

Castration also affected the size of the glandular epithelial

Fig. 6. Photomicrograph of tumor tissue from a 7-day castrated animal. Arrows, nonluminal spaces frequently observed following castration. × 520.

Fig. 7. Photomicrograph of cell types in tumors from animals repleted for 7 days demonstrating a general reversal of the effects of castration. × 520.

Fig. 8. Treatment effects on circulating levels of estradiol and progesterone. The number of determinations in each case was 5. Points, mean; bars, SEM.
followed by a progressive increase upon institution of hormone repletion (Fig. 4). The observed proliferative responses were, however, greater in the epithelial component of the tumor. While, in fact, the peak labeling index of nonepithelial cells simply returned to basal levels, that of the epithelial cells significantly exceeded intact values (Fig. 4). Such “overshoot” above control was observed both in the glandular and myoepithelial cells (Fig. 5).

The effect of our hormonal treatments on the cell kinetic characteristics of nonepithelial cells is of interest and deserves some comment. This observation raises the possibility of a direct mitogenic effect of hormones on nonepithelial cells. Although, however, both estrogen and progesterone receptors have been described in the adipose and connective tissue of the normal mammary gland (26), the potential presence of hormone receptors in the stromal compartment of experimental breast cancer has not been investigated. Recent immunocytochemistry studies utilizing monoclonal antibodies to the estrogen receptors have addressed this issue in human breast cancer specimens (25). Preliminary evidence suggests that nonepithelial cells do not contain estrogen receptors except for hormone-sensitive fibrocytes of the specialized lobular stroma (27). As an alternative to a direct receptor-mediated effect, hormones could influence the proliferative activity of the nonepithelial cells indirectly through their effects on the epithelial component of the tumor. This hypothesis is supported by the recent observation that heterogeneous populations of tumor cells can influence each other’s growth through the elaboration of secretory growth factors which act locally in a paracrine fashion (28). More specifically in this regard, several human breast cancer cell lines have recently been found to synthesize and secrete growth factors whose receptors are preferentially located on mesenchymal cells (29).

The alterations in the proliferative activity of the myoepithelial cells are also of interest (Fig. 5). In tumors of intact rats, the labeling index of these cells was considerably lower than that observed in the glandular (Fig. 5) and nonepithelial (Fig. 4) cells. This finding is consistent with the observation made in normal human breast tissue grown in culture or xenografted in nude mice, that the proliferative activity of the myoepithelial cells is minimal (30). In our experimental system, however, these cells were able to markedly accelerate replication upon hormonal exposure following an initial phase of hormone depletion (Fig. 5).

Induction of tumor cell growth synchronization has been proposed as a potential means to enhance the efficacy of phase-specific cytotoxic chemotherapy in breast cancer treatment (1–3). Although the “overshoot” in labeling indices observed in the epithelial components of the tumor on Day 7 of treatment (Figs. 4 and 5) indicates that a certain degree of cell cycle synchronization had been obtained, by that time the tumors had essentially regrown to their original sizes (Fig. 2). Under optimal conditions of synchronous growth, one would anticipate instead that, if all the cells start proliferating simultaneously upon exposure to a mitogen, there should be a peak in DNA synthesis (reflected by a rise in the labeling index) which should precede any expansion in tumor size. These conditions would obviously be ideal for practical therapeutic implications, since the tumor would be rendered sensitive to the action of phase-specific cytotoxic drugs without increasing in volume and thus posing a threat to the host. If one considers the complexity of hormone action on mitogenesis (i.e., the numerous events taking place between the initial binding of the hormone to its receptor and initiation of proliferation) and the heterogeneous hormone sensitivity of tumor cell subpopulations, it is not surprising that only a suboptimal synchronous growth occurred following hormonal administration. It is also intuitive that the more distally we can intervene in the hormone action pathway, the more likely we may be to optimize hormonally induced breast cancer cell growth synchronization. We are currently exploring this possibility by manipulating the polyanime pathway, which, according to our studies (31–33), may be a critical distal “second messenger” of hormonal action in this experimental breast cancer system.

Following ovariectomy we observed a significant decrease in the dimensions and volume of the glandular epithelial cells (Table 2). This finding is consistent with the ultrastructural study by Tseng (16) who observed a decrease in secretory activity of the luminal cells of NMU mammary tumors after castration. Such decrease in cell size contributed significantly to the observed reduction in tumor volume induced by ovariectomy. Since the glandular epithelial cells comprise 88% of the tumor cell population, a 35% decrease in their volume (Table 2) accounted for 31% reduction in overall tumor volume 7 days after castration. The overall decrease in tumor volume was, however, 60% (Fig. 2). Thus, cell loss must have been an additional major contributing factor, as suggested by areas of visible necrosis observed in tumors of castrated rats. Our data do not allow us to determine whether ovariectomy actually induced acceleration of cell death versus just inhibition of cell proliferation while leaving the rate of cell loss unaffected. Hormonal repletion was able to completely reverse the effects of ovariectomy on glandular cell dimension and volume by Day 3 of treatment (Table 2). Hormonal administration was also only partially able to reverse the effect of ovariectomy on the cellular composition of the NMU mammary tumors. Only the fraction of the myoepithelial cells was totally restored to control levels, while the fractions of glandular and nonepithelial cells were slightly or not affected at all, respectively (Table 1). It should be emphasized that our hormone repletion regimen was primarily aimed at providing optimal amounts of estradiol and prolactin, the two major hormones supporting NMU mammary tumor growth (8, 9), rather than attempting to reproduce the hormonal milieu of the intact rat. As can be seen in Fig. 8, although serum estradiol levels were restored to control intact values, serum progesterone levels remained suppressed since this hormone was not repleted. Furthermore, although serum prolactin levels were not measured in these experiments, we have previously shown that daily administration of 1 mg of perphenazine for 10 days raises serum prolactin levels to values 4-fold higher than those measured in intact rats (8). Consequently, these differences in the endocrine milieu between hormonally repleted and intact rats could potentially account for the above-mentioned differences in tumor morphology.

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