Parous rats regain high susceptibility to chemically induced mammary cancer after treatment with various mammotrophic hormones

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Parity in humans and rats provides significant protection against mammary tumor development. This study was carried out to investigate whether treatment of parous rats with mammotrophic hormones would affect methyl-\(N\)-nitrosourea (MNU)-induced mammary carcinogenesis. Parous rats were treated with \(17\beta\)-estradiol (E2), progesterone (P4) and thyroxine (T4) alone or in combination. E2 (20 \(\mu\)g/60 days) and P4 (20 mg/60 days) were administered by silastic tubing and T4 in the drinking water (3 \(\mu\)g T4/ml). Hormonal treatments commenced 7 days before MNU injection and continued for 33 weeks. Animals were palpated weekly for tumor detection. The effects of the hormonal treatments on the circulating concentrations of E2, P4, growth hormone (GH), prolactin (PRL), T4 and insulin-like growth factor-I (IGF-I) after 7 days of treatment, the time of MNU injection, was assessed. Animals treated with E2 had significantly elevated circulation concentrations of GH, PRL and P4, and serum levels of E2 were more consistent in this group than in the other animal groups. P4 treatment caused elevation in P4 concentration in serum but did not affect the circulating levels of other hormones. The proliferation of the mammary gland at the time of MNU injection was elevated in animal groups treated with E2 either alone or with P4 and T4 and in animals treated with P4 alone, but the mammary gland was most differentiated in untreated parous rats and least in animals treated with E2 either alone or with P4 and T4. Mammary tumor incidence was 10\% in parous rats that did not receive any hormonal treatment. Treatments with E2 or P4 alone significantly increased the susceptibility of parous animals to 67 and 50.0\%, respectively; a tumor incidence similar to that of untreated AMV rats (64\%). Parous rats treated with E2 plus P4 had tumor incidence higher than 90\%. T4 administered did not affect mammary carcinogenesis.

Introduction

It has long been known that early parity is a very effective protector against breast cancer development in women (1). It is also well established that parity in rats provides almost complete protection against chemically induced mammary carcinogenesis (2,3). However, a clear understanding of how parity protects the mammary gland against cancer development is still lacking. For a good many years, it has been generally accepted that the pregnancy-associated refractoriness of the mammary gland to carcinogenesis is caused by lasting phenotypic alterations of the mammary epithelia that occur during pregnancy and possibly lactation (4–6). However, in the light of some recent reports, this theory appears to be insufficient to satisfactorily explain this phenomenon. For example, it has recently been shown that when isolated virgin rat mammary epithelial cells, previously exposed to \(N\)-methyl-\(N\)-nitrosourea (MNU), are transplanted into isogenic virgin and parous hosts the cancer development is significantly lower in the parous animals when compared with the virgin rats (7). This finding indicates that the physiological milieu may be the determining factor in the parity-associated refractoriness to mammary carcinogenesis. Indeed, we, and others, have shown that the hormonal environment is altered in parous rats as compared with that in age-matched virgin (AMV) animals. Specifically, it was found that both GH and PRL were reduced after parturition (8–10). Similarly, women who have carried at least one pregnancy to term have lower circulating levels of PRL and blunted PRL response to secretagogue (11). Based on these findings, we have hypothesized that this altered hormonal environment after parturition, which is less favorable for stimulation of mammary gland development, is, at least in part, causing the reduction in the susceptibility of the mammary gland to carcinogenesis.

The developmental stage of the mammary gland at the time of exposure to carcinogen has an important effect on the susceptibility of the gland to carcinogenesis (12,13). However, the effects of hormones on mammary carcinogenesis may be even more prevalent for the promotional period of cancer development. It has, for example, been shown that although the incidence of palpable mammary tumors is very low in parous rats after exposure to MNU, the incidence of latent carcinomas is quite high and approaching that of AMV rats (14). This finding supports our hypothesis in that although tumor initiation takes place in the mammary gland of parous rats upon MNU exposure, their hormonal environment appears to be sufficiently altered to prevent the development of frank tumors. However, it has never been directly determined if a defined hormonal treatment will counteract the protective effects parity provides against mammary carcinogenesis.

In the present study, we continue to examine the role hormones play in the parity-associated protection against breast cancer. The objectives were to investigate whether treatments of parous rats with hormones known to affect normal mammary gland development would alter the susceptibility of these animals to MNU-induced cancers.

Materials and methods

Animals

Sprague–Dawley rats were used for all the experiments. The animals were purchased from Harlan Sprague–Dawley (San Diego, CA). To generate parous...
animals, virgin rats were mated at age 50–55 days. After parturition, the pups were removed and the mammary gland of the mothers were allowed to involute for 40 days. At that time, the parous rats were used for experimentation. Virgin rats age matched with the parous rats (AMV) were used as controls. All groups of animals in the experiments described here was approved by the Chancellor’s Animal Care Committee at the University of California, Santa Cruz.

Treatments with 17β-estradiol (E2), progesterone (P4) and thyroxine (T4)

Animals were treated with E2 (Sigma, St Louis, MO), P4 (Sigma), T4 (Sigma), E2 plus P4 (E2 + P4), and E2 plus T4 (E2 + P4 + T4). The steroids were administered by subcutaneous implants of silastic capsules (Helix Medical Inc, Carpinteria, CA). The capsules (2 cm long, 1.98 mm ID / 3.18 mm OD) contained 20 µg E2 mixed with cellulose, 20 mg P4 or 20 µg E2 mixed with 20 mg P4, when both steroids were administered simultaneously. T4 was administered in the drinking water (3 µg/ml). Controls, both parous and AMV rats received silastic capsules containing cellulose. Treatment commenced when the animals were ~114 days of age and was continued for 1 week when half of the animals in each group were killed for the assessment of circulating hormonal concentrations and mammary gland development at the time of carcigen injection. The remaining animals in each group that had received identical hormonal treatment to those killed after 1 week of hormonal exposure were injected with MNU (Ash, Stevens, Detroit, MI) and these animals continued to receive hormonal treatment for the next 7 months, when all animals were killed.

To control for the possible effects of the hormonal treatment, per se, on mammary tumorigenesis, an additional group of animals received the same hormonal treatment for the same period of time as the MNU-treated rats but these animals were not injected with the carcinogen (Figure 1).

Because MNU was administered to several groups of experimental animals at different times, a group of 50–60 day-old virgin female rats were also injected at each time with the same MNU solution as the experimental animals received to ascertain that the potency of the carcigen was the same or very similar for all the experimental groups. In all cases, the young virgin rats showed 100% mammary cancer incidences.

Implantation of capsules

Animals were anesthetized with a ketamine (Aveco, Fort Dodge, IO) and xylazine (Mobay, Shawnee, KA) mixture (30 mg ketamine + 6 mg xylazine/kg body wt). Approximately 1.5 cm dorsal incision was made through the skin. A small subcutaneous pocket was made with a pair of forceps where the capsule was implanted and the incision closed with wound clips. Every 2 months the capsules were replaced with freshly packed capsules.

Tumor induction, detection and removal

The carcigen was administered as described previously (15). Briefly, MNU was dissoluted in 0.9% saline, pH 5.0 and heated to 50–60°C. The animals were anesthetized as described above and then each received a single intraperitoneal injection of MNU (50 mg/kg body wt). Animals were palpated for detection of mammary tumors, commencing 1 month after the MNU injection. When the tumors had grown to 1.5–2.0 cm in diameter, rats were anesthetized as described above and the tumors surgically removed and processed for histology. At the end of each experiment, ~33 weeks after MNU injection, all animals were killed by CO₂ inhalation, followed by decapitation and trunk blood collection. The blood was centrifuged at 1000 g for 20 min, serum was harvested and stored at ~80°C until the hormonal concentrations of various hormones were measured. Mammary tissues were collected and either stored frozen for protein and DNA extraction or fixed in either Tellysničszky’s fixative for whole mounts and regular histological preparations, or in 4% paraformaldehyde for 3 h for immunocytochemistry.

Histology

Mammary gland samples obtained posterior to the lymph node of the right fourth mammary gland and mammary tumors were fixed, embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E) for inspection and classification of tumors. Whole mount preparations were made of the right first, second and third mammary glands. The glands were removed, fixed and stained with iron hematoxylin (16).

Immunocytochemistry

Paraformaldehyde-fixed mammary tissues were paraffin embedded and sectioned (5 μm). The sections were deparaffinized and rehydrated and then aldehyde groups and endogenous peroxidase activity blocked by incubation with 2% glycine and 0.3% hydrogen peroxide, respectively. Non-specific protein binding was blocked first with 2% dried milk followed by 5% goat serum both diluted in PBS. The tissue sections were then incubated overnight in primary antiserum to cyclin D (Upstate Biotechnology, Lake Placid, NY) diluted to 1:100. After thorough rinsing, the slides were incubated with the secondary antibody (biotinylated goat anti-rabbit IgG; Vector Laboratories, Burlingame, CA) diluted 1:200, followed by incubation with ABC reagent and diaminobenzidine (Vector Laboratories). After addition of a coverslip, the percentage cyclin D labeled mammary epithelial cells for all experimental and control groups was determined by counting immunostained and unstained cells. The cyclin D labeling was assessed at three to four different locations in two epithelial structures of the right fourth mammary gland pair. Therefore, the percentage cyclin D labeling in the mammary gland of each animal was the average of six to eight independent cell counts from different locations in the mammary gland, counting 635.8 ± 15.7 (mean ± SEM) cells at each location. Immunostaining with cyclin D to assess the percentage proliferating cells in the mouse mammary gland has been validated and the method was found to correlate very closely to that for BrdU labeling (17).

Precaution was taken to dissect histological samples from each mammary gland at as similar a location as possible, using the lymph node as a landmark.

Circulating concentrations of hormones

Concentrations in serum of E2, P4, T4, prolactin (PRL), growth hormone (GH) and insulin-like growth factor-I (IGF-I) were measured by radioimmunoassays (RIAs). Assay kits from Diagnostic Products (Los Angeles, CA) were used to measure E2, P4 and T4, modified for use with rat serum by diluting the reference standards in charcoal-stripped rat serum. PRL and GH were measured using reagents from NIDDK and IGF-I with an RIA kit from Diagnostic Systems Laboratories (Webster, TX).

Mammary gland differentiation

The α-lactalbumin (α-lac) content of the mammary gland was measured for the assessment of mammary gland differentiation at the time of MNU injection. The mammary tissues were finely ground in liquid nitrogen with mortar and pestle and then homogenized using a Polytron homogenizer in 2 vol (w/v) buffer (50 mM Tris–HCl, 5 mM MgCl₂, pH 7.5) containing 1 mM Pefabloc (Boehringer Mannheim, Indianapolis, IN) and 1 µM pepstatin A (Sigma). After homogenization, a 20 µl sample was collected for measuring total DNA content and the remainder of the preparation extracted for 1 h, followed by centrifugation at 20 000 g for 30 min. The supernatant was collected and assayed for total protein and α-lac concentrations. For measuring the α-lac concentration, an RIA was developed using antiserum generated against rat α-lac (generous gift from Donald E. Ebner, University of Kansas Medical Center). Rat α-lac was purified from rat milk using a previously developed procedure (18). This purified preparation of α-lac was used as a reference standard and as iodinated tracer. The within and between coefficient variation of the assay was 30 and 18.4%, respectively.

Total protein was measured using the BCA protein assay (Pierce, Rockford, IL) using BSA (Fraction V, Sigma) as a standard, and total DNA content of each mammary gland homogenate was measured with fluorometric assay (18) using calf thymus DNA (Sigma) as a standard.

Statistics

The effects of the various hormonal treatments on mammary cancer incidence was analyzed using 2×2 contingency tables and χ²-test. The effects of the treatments on tumor load, concentrations of the different hormones in serum, 

Fig. 1. Schematic representation of the experimental procedure. (See Materials and methods for more details.)
Parity and mammary carcinogenesis

Table I. Mammary carcinogenesis in MNU-injected parous rats treated with estradiol (E2), progesterone (P4), thyroxin (T4) or different combinations thereof as compared with carcinogenesis in untreated parous and age-matched virgin (AMV) rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mammary cancer incidence</th>
<th>Percentage rats with mammary cancer</th>
<th>Cancer load number/rat* (mean ± SEM)</th>
<th>Latency range (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parous</td>
<td>1/10</td>
<td>10.0</td>
<td>0.1 ± 0.1</td>
<td>106</td>
</tr>
<tr>
<td>Parous + E2</td>
<td>4/6*</td>
<td>66.7</td>
<td>1.0 ± 0.4</td>
<td>58–228</td>
</tr>
<tr>
<td>Parous + P4</td>
<td>7/14*</td>
<td>50.0</td>
<td>0.8 ± 0.3</td>
<td>130–223</td>
</tr>
<tr>
<td>Parous + T4</td>
<td>3/11</td>
<td>27.3</td>
<td>0.4 ± 0.2</td>
<td>122–181</td>
</tr>
<tr>
<td>Parous + E2 + P4</td>
<td>12/13*</td>
<td>92.3</td>
<td>2.3 ± 0.4</td>
<td>71–230</td>
</tr>
<tr>
<td>Parous + E2 + P4 + T4</td>
<td>9/13*</td>
<td>81.8</td>
<td>1.2 ± 0.3</td>
<td>77–230</td>
</tr>
<tr>
<td>AMV</td>
<td>14/22*</td>
<td>63.6</td>
<td>2.1 ± 0.6</td>
<td>57–229</td>
</tr>
</tbody>
</table>

*This analysis contains all animals in each group, including those that never developed palpable mammary cancer.

**Significantly different from untreated parous animals (P < 0.05).

†Significantly different from all other groups (P < 0.05).

Table II. Concentrations in serum of hormones at the time of MNU administration

<table>
<thead>
<tr>
<th>Groups</th>
<th>PRL (ng/ml)</th>
<th>E2 (pg/ml)</th>
<th>P4 (ng/ml)</th>
<th>GH (ng/ml)</th>
<th>T4 (ng/ml)</th>
<th>IGF-I (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parous</td>
<td>32.1 ± 10.7b</td>
<td>4.9 ± 2.4b</td>
<td>15.4 ± 4.8b</td>
<td>21.8 ± 6.0b</td>
<td>62.4 ± 2.7b</td>
<td>1293 ± 26.9c</td>
</tr>
<tr>
<td>Parous + E2</td>
<td>80.0 ± 8.7c</td>
<td>8.9 ± 1.5b</td>
<td>32.3 ± 3.2c</td>
<td>94.7 ± 26.0c</td>
<td>50.0 ± 1.5b</td>
<td>1109 ± 58.8b</td>
</tr>
<tr>
<td>Parous + P4</td>
<td>14.9 ± 2.4b</td>
<td>1.0b</td>
<td>22.8 ± 6.7bc</td>
<td>46.6 ± 18.0b</td>
<td>51.5 ± 4.0b</td>
<td>1276 ± 41.0bc</td>
</tr>
<tr>
<td>Parous + T4</td>
<td>16.1 ± 4.0b</td>
<td>3.4 ± 1.9b</td>
<td>13.8 ± 4.8b</td>
<td>54.6 ± 16.0b</td>
<td>85.3 ± 3.5d</td>
<td>1323 ± 37.8c</td>
</tr>
<tr>
<td>Parous + E2 + P4</td>
<td>96.1 ± 14.4c</td>
<td>5.3 ± 0.5b</td>
<td>32.4 ± 3.6c</td>
<td>93.4 ± 19.2c</td>
<td>54.2 ± 2.4b</td>
<td>1278 ± 72.9c</td>
</tr>
<tr>
<td>Parous + E2 + P4 + T4</td>
<td>81.8 ± 15.1c</td>
<td>5.4 ± 1.4b</td>
<td>36.7 ± 3.2c</td>
<td>88.1 ± 17.2bc</td>
<td>88.1 ± 3.7d</td>
<td>1338 ± 78.6c</td>
</tr>
<tr>
<td>AMV</td>
<td>32.8 ± 8.0b</td>
<td>2.6 ± 1.1b</td>
<td>18.1 ± 4.4b</td>
<td>46.1 ± 6.6b</td>
<td>54.9 ± 2.0b</td>
<td>1271 ± 35.8c</td>
</tr>
</tbody>
</table>

*The values represent mean ± SEM. Means with different superscripts within each column differ significantly from each other (P < 0.05).

Results

Cancer incidence

Table I shows the overall cancer development in all the MNU-treated animal groups of the study. Parous animals not receiving any hormonal treatment were refractory to MNU-induced mammary cancer development with only 10% incidence. All hormonal treatments of the parous animals, except T4 alone, increased significantly mammary cancer incidence when compared with parous rats that received no hormonal treatment. The hormonal treatment that had the greatest effect on MNU-induced mammary carcinogenesis was that of E2 and P4 administered simultaneously, where the incidence went up to 92.3%. The tumor load was also higher in this group of animals than any other group except AMV, where tumor load was similar. Treatment of the parous rats with E2 or P4 alone both significantly increased MNU-induced mammary cancer incidence, as compared with parous rats that did not receive hormonal treatment, to 66.7 and 50%, respectively. Parous rats treated with T4 alone had 27.3% incidence of MNU-induced mammary tumors, which was not significantly different from hormonally untreated parous rats, and addition of T4 treatment to the E2 + P4 treatment did not increase the tumor incidence over treatment with E2 + P4 together. Latency to the appearance of first palpable mammary tumor tended to be shorter in all groups that received E2 treatment either alone or in combination and was comparable to that of AMV (Table I).

Of the rats that received hormonal treatments only but not MNU injection, one animal in the E2 + P4-treated group and another in the E2 + P4 + T4-treated group developed mammary adenocarcinoma. The total number of animals in both of these groups was six and therefore the tumor incidence for each group was 17%. No other hormonal treatment, i.e. E2, P4, T4 administered alone or controls that carried capsules containing cellulose, developed mammary carcinomas.

The number of animals carrying only non-malignant mammary tumors, mostly fibroadenomas, was ~10–20% and this did not differ significantly between groups.

Circulating concentration of hormones

The concentrations of E2 in serum of animals treated with E2 were on average ~5–9 pg/ml. These concentrations were not significantly different from the average circulating concentrations of E2 for the other groups (Table II). However, the circulating concentrations of E2 in treated rats were much more consistent than those in untreated animals, where the E2 concentrations in blood were very erratic and frequently below detectable levels for the assay (0.4 pg/ml). In any event, the physiological effects of the E2 treatment were quite obvious, as the concentrations in serum of other hormones were effected.

In particular, the circulating levels of PRL, GH and P4 were significantly higher in parous compared with all other groups, except P4 concentration of P4-treated rats and the GH concentration of T4-treated rats, where these hormonal concentrations were also increased; however, to a lesser extent than in the E2 treatment groups. T4 treatment elevated the circulating concentrations of T4 significantly over all other groups and, interestingly, the parous rats that did not receive any hormonal treatment had significantly higher T4 levels in serum than the parous rats treated with E2, P4 and E2 + P4 and the AMV. The concentration of IGF-I in blood was significantly lower in parous animals treated with E2 alone than in all other groups,
except parous rats treated with P4 alone. The serum concentration of GH in parous, untreated rats tended to be lower than in other groups of animals, but in contrast to what we had seen before (8), this trend did not reach a significant level. Also, no reduction was found in the circulating levels of PRL in the parous animals although parity has previously been shown to blunt PRL release in rats (9,10). This discrepancy was probably caused by difference in protocol used for the blood sampling in the present study in that the status of the estrous cycle was not monitored, and insufficient handling of the rats prior to collection of the blood and, therefore, the blood sampling may have caused stress-related release of pituitary hormones.

**Mammary gland development at the time of MNU administration**

**Histology**

Inspection of histological mammary gland sections revealed that parous animals that received E2 treatment, alone or in combination with other hormones, had more extensive lobulo-alveolar structures than other groups (Figure 2). P4 treatment alone appeared to stimulate ductal development of the mammary gland as evidenced by denser mammary ductal structures in this group of rats. However, endbuds were clearly present in both P4 and untreated parous animals (Figure 2). No obvious histological differences were seen between the mammary epithelia of the untreated parous and AMV rats and the parous rats treated with T4 (data not shown).

**DNA content**

The DNA content of the mammary gland, expressed as mg DNA/g wet weight tissue, was also calculated. All animal groups treated with E2 either alone or with other hormones had significantly higher DNA content in their mammary tissues than the other groups. In these groups, the average mammary gland DNA content was ~2.0 mg/g wet wt tissue. The DNA contents of the mammary gland in other groups were similar or on average just over 1.0 mg/g wet wt tissue (Figure 3A).

**Cyclin D labeling**

The percentage cyclin D labeling of the mammary epithelia was highest in parous rats treated with E2 + P4 and E2 + P4 + T4 and reached average levels of 19.9 ± 1.1 and 22.6 ± 1.3% (mean ± SEM), respectively in these groups. Animals treated with E2 and P4 alone had 13.6 ± 0.9 and 15.4 ± 0.6% cyclin D immunostained cells, respectively, which were significantly higher than that of AMV and untreated parous rats, where cyclin D was found in 7.7 ± 0.6 and 9.1 ± 0.9% mammary epithelial cells, respectively. In parous rats treated with T4 alone 11.7 ± 1.1% cells were immunostained which was significantly higher than that of AMV (Figure 3B).

**Concentration of α-lactalbumin in mammary tissues**

The concentration of α-lact albumin was used to assess the level of differentiation of the mammary tissues (Figure 3C). Interestingly, the mammary glands of untreated parous rats showed significantly higher α-lac concentrations in the mammary gland than all other groups or 7.7 ± 1.3 μg α-lac/mg DNA (mean ± SEM). The least differentiated mammary tissues were found in animal groups treated with E2 alone or E2 in combination with other hormones. In these groups the content of the mammary gland was 1.67 ± 0.9 μg α-lac/mg DNA in E2-treated rats; 0.68 ± 0.2 μg α-lac/mg DNA in E2 + P4-treated rats and 0.57 ± 0.1 μg α-lac/mg DNA in parous animals treated with E2 in combination with P4 and T4. Parous rats treated with P4 and T4 alone had α-lac concentrations of 3.59 ± 0.8 and 4.27 ± 0.9 μg α-lac/mg DNA, respectively, and AMV had 3.23 ± 0.5 μg α-lac/mg DNA (mean ± SEM).

**Discussion**

The refractoriness of the mammary gland to carcinogenesis after full term pregnancy has long been known. The
It has been theorized, based on some experimental data from rats, that the mammary gland sustains permanent biochemical alterations by going through a full term pregnancy (4–6). A few years ago, we proposed that although the mammary gland might acquire physiological changes during pregnancy that caused protection against cancer, these protective effects could be obliterated in a hormonal environment that would stimulate mammary gland development (8). We based this hypothesis on our findings that the parous mammary gland appeared to be in a less stimulated state than the gland of virgin rats, and also on the observation that the circulating concentrations of GH and PRL were reduced in parous rats compared with the virgin animals. As GH and PRL are both known to be important for mammary gland development, we speculated that the reduced levels of mammotropic hormones in blood could cause the mammary gland to remain in a state of regression and, therefore, refractory to cancer development. We show here that this is the case; mammary glands in parous rats can be made highly susceptible to carcinogenesis in a hormonal environment favorable for mammary gland development.

We assessed the proliferation rate (percentage cyclin D-labeled and total DNA content) as well as the levels of differentiation (α-lac content) of the mammary glands of parous rats after 7 days of hormonal treatment. The same parameters were assessed in the mammary glands of untreated parous rats and AMV at the same time. Important findings emerged from these assessments. Firstly, the mammary glands of parous rats not receiving any hormonal treatment had the highest level of differentiation as compared with all the other animal groups, but any hormonal treatment of the parous rats reduced the level of differentiation. E2 treatment, either alone or, more dramatically, with P4 was the most effective in reducing mammary differentiation. At the same time, the rate of proliferation of the hormone-treated parous rats was increased. Therefore, it appears that some proportion of the mammary epithelia that survive the involution of the gland after weaning are differentiated. This is in keeping with what Russo and his associates maintain, namely that the mammary gland of the parous rat retains a higher level of differentiation after involution as compared with AMV rats (4–6). However, with a hormonal environment that stimulates mammary gland development, the gland becomes highly susceptible to carcinogenesis, so much so that it even exceeds the susceptibility in AMV rats. Therefore, the mammary epithelia from the parous...
animals is capable of rapid proliferation under the appropriate hormonal stimulation. It should be pointed out, in this context, that the whole mount preparations of the parous rats revealed structures associated with mammary gland proliferation (endbuds) even in animals that did not receive any hormonal treatment (Figure 2). Also supporting the proliferative capability of mammary epithelial cells from parous animals is the finding that isolated mammary cells and mammary explants from parous mice have only slightly reduced efficiency in filling cleared mammary fat pads of virgin female hosts as compared with virgin donors (19). Another finding worth noticing is that although the mammary glands of all parous rats treated with E2 alone or with E2 + P4 showed a significant increase in cell proliferation, the correlation between cell growth and mammary carcinogenesis was not always obvious when all the animal groups were considered. For example, no difference was found in the percentage cyclin D immuno-staining or total DNA content of mammary glands of parous untreated rats and AMV animals. However, the susceptibility of these two groups to mammary tumor development was significantly different. Therefore, it appears that a high cell proliferation rate, per se, is not essential for high susceptibility of the gland to tumorigenesis, rather that the activity of particular genes is the determining factor. These genes would then either be largely non-expressed or show a high level of expression in the parous glands without hormonal stimulation, but the opposite situation would be found in the mammary gland of AMV rats. We have, for example, shown that the expression of both the estrogen receptor and the epidermal growth factor receptor are significantly reduced in mammary glands of parous rats compared with the glands of AMV animals (8). It has also been shown that the expression of BRCA1 and BRCA2 genes is increased in the parous mouse mammary gland compared with age-matched virgin animals (20,21); a finding that may be of great importance as inactivation of these genes is associated with increased risk in developing familial breast cancer (22). Obviously, more work is needed here to determine in detail the differences in gene expression of parous versus virgin mammary glands and how they relate to the changes in gene expression after hormonal treatments that has been shown to increase mammary cancer development.

It is known that treatment with E2 stimulates the release of both PRL (23 and GH (24,25); and PRL, either alone or synergistically with E2, stimulates P4 production (26,27). In this study, we found that all animal groups treated with E2 had elevated concentrations in serum of PRL, GH and P4. This complicates all interpretations of the results, as E2 could be acting directly on the mammary gland to affect development and tumorigenesis, it could be acting by stimulating the release of other hormones that, in turn, stimulate the gland, but most likely both of these events are taking place, as evidence for the importance of all these hormones in mammary gland growth, differentiation and possibly carcinogenesis is numerous (28).

Administration of P4 alone did significantly increase mammary tumorigenesis in the parous rats. Here the effect on the mammary gland may be more direct than those of E2 because the P4 treatment did not obviously affect the circulating concentrations of other hormones. Progesterone treatment has been shown to stimulate chemically induced mammary cancer in rats (29,30), although the effects of P4 or other hormonal treatments on pregnancy-associated protection against mammary cancer were not known before. Accompanying the increase in mammary tumorigenesis of P4-treated rats was a significant increase in the cell proliferation and reduction in $\alpha$-lact content of the mammary gland. However, these changes in growth and differentiation of the mammary gland were not as pronounced as after E2 treatment. Nevertheless, association of mammary carcinogenesis with increase in proliferation and reduction in differentiation is apparent.

Reports on the effects of thyroid hormones on chemically induced mammary cancer in rats are conflicting, with some researchers reporting some stimulatory effects (31) but others very little effects (32) or even reduction with high doses of thyroxine (33). We found that treatment of parous rats with T4 alone caused a slight increase in mammary tumor incidence, but this was not significantly different from untreated parous rats. Here again, a reduction was seen in the $\alpha$-lact content of the mammary gland but no significant effects were seen in cyclin D immunostaining or DNA content of the mammary gland after T4 treatment.

Untreated control animals (carrying cellulose capsules) and animals that received hormonal treatment only, but were not injected with MNU did not, in most cases, develop palpable mammary tumors. The only exceptions were that one animal each in the parous rat groups treated with E2 + P4 and E2 + P4 + T4 developed mammary carcinoma. Although the tumor incidence in these two groups was not significantly different from that of untreated controls, this finding is noteworthy. For example, long-term treatment with high doses of 17$\beta$-estradiol and progesterone has been shown to cause mammary cancer in rats after 6–7 months of latency (34), and evidence suggests that estradiol or estradiol metabolites may be genotoxic carcinogens in their own right (35), quite aside from the hormonal effects of estradiol to stimulate tumor progression and perhaps cancer initiation (12,28). The results presented here, although not representing a large sample size, support this notion.

In conclusion, parous rats that show almost complete refractoriness to chemically induced mammary carcinogenesis acquire high susceptibility after hormonal treatment. The increase in mammary tumorigenesis was usually associated with a low level of differentiation and high rate of proliferation in the mammary gland, but this correlation was not always apparent, indicating that activation or inactivation of specific genes was required for high mammary cancer incidence.

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References


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