Effects of Alterations in Early Hormonal Environment on Development and Hormone Dependency of Carcinogen-induced Mammary Tumors in Rats

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ABSTRACT

The purpose of this study was to determine the early role of estrogen and prolactin on subsequent hormone dependency of carcinogen-induced mammary tumors. Virgin 57-day-old Sprague-Dawley rats were given i.v. injections of 7,12-dimethylbenz(a)anthracene (DMBA). One day prior to and 7 days after DMBA administration, the rats were divided into separate groups and given: a daily 0.1-ml s.c. injection of vehicle (controls); haloperidol (0.5 mg/kg) to increase prolactin secretion; estradiol benzoate (1 µg/rat) to increase estrogen levels; bromocryptine (5 mg/kg) to inhibit prolactin release; tamoxifen (TAM, 20 µg/rat) to inhibit estrogen action; or the combination of TAM and bromocryptine. Drug and hormone treatments were terminated after 8 days. Sixteen weeks after DMBA administration, all animals were bilaterally ovariectomized, and 4 weeks later it was determined whether the mammary tumors were hormone dependent or independent.

Treatment with TAM resulted in a significant reduction in incidence of mammary tumors, but also a 3-fold increase in the percentage of these tumors that showed hormone independence as compared with that of control rats. Rats treated with the combination of TAM and bromocryptine also showed a significant reduction in tumor incidence and number, but a 5-fold greater percentage of hormone-independent tumors after ovariectomy. Rats given daily injections of haloperidol or estradiol benzoate showed only small differences in mammary tumor incidence or autonomy after ovariectomy, as compared with controls given injection vehicle alone. These results suggest that rats made deficient in estrogen and prolactin at the time of DMBA administration develop fewer tumors, but the tumors that develop are not dependent on these hormones for subsequent growth.

INTRODUCTION

Development and growth of mammary tumors induced in Sprague-Dawley female rats by administering DMBA are dependent on the presence of hormones, particularly PRL and estrogen (9, 17). Some DMBA-induced mammary tumors become hormone-independent or autonomous, as indicated by continued growth after ovariectomy. Ovariectomy not only removes the major source of estrogen in the body but also results in a significant reduction in PRL secretion by the pituitary (2). Estrogen is known to be a potent stimulator of PRL secretion. The mechanisms involved in development of hormone-independent mammary tumors are not well understood. Up to 20% of DMBA-induced mammary tumors in Sprague-Dawley rats show hormone independence shortly after their appearance, and the incidence of autonomy increases with age and size of the tumors (1, 7).

It has been established that the first week after carcinogen administration to Sprague-Dawley rats is "critical" for development of mammary tumors (6). Suppression of secretion of estrogen and/or PRL during this period results in permanent inhibition of mammary tumorigenesis (24, 25). These observations suggest that the hormonal milieu at the time of carcinogen-induced mammary tumors importantly influences mammary tumor dynamics. The purpose of this study was to determine whether the initial hormonal influence during the "critical" first week after DMBA administration is related to subsequent development and growth of autonomous mammary tumors.

MATERIALS AND METHODS

Tumor Induction and Drug Treatment. Virgin female Sprague-Dawley rats (Harlan Research Animals, Indianapolis, Ind.), 57 days old, were given a single i.v. injection of 1 ml lipid emulsion containing 5 mg DMBA (8). The rats were housed in plastic cages in a temperature- and light-controlled room (24 ± 0.5°C; 14 hr-light/10-hr dark cycle) and fed rat chow (Ralston-Purina Co., St. Louis, Mo.) and water ad libitum. Rats were divided into 6 groups (Groups A to F) and, beginning 1 day before and for 7 days after DMBA administration, were given the following treatments. Group A (controls) each received a daily 0.1-ml s.c. injection of the 2 injection vehicles, 0.3% ethanol and 0.87% NaCl solution. Group B rats were given a daily 0.1-ml s.c. injection of 0.3% ethanol and a s.c. injection of 5 mg HAL/kg body weight (McNeil Laboratories, Ft. Washington, Pa.), suspended in 0.1 ml 0.87% NaCl. HAL is a dopamine receptor blocker that increases pituitary PRL release. Group C rats each received a daily s.c. injection of 1 µg EB (Sigma Chemical Co., St. Louis, Mo.) dissolved in 0.1 ml of 0.3% ethanol, to raise blood estrogen and PRL levels, together with an injection of 0.1 ml 0.87% NaCl. Group D rats were each given a daily s.c. injection of 5 mg CB-154/kg body weight (Sandoz, Ltd., Basel, Switzerland) suspended in 0.1 ml 0.87% NaCl, and a 0.1-ml s.c. injection of 0.3% ethanol. CB-154 is an ergot drug that reduces PRL release from the pituitary. Group E rats each received a daily s.c. injection of 20 µg TAM (ICI, Rotterdam, The Netherlands) suspended in 0.1 ml 0.3% ethanol, together with a 0.1-ml s.c. injection of 0.87% NaCl. TAM is an anti-estrogenic drug. Group F rats each were given a daily s.c. injection of 0.1 ml TAM (20 µg/rat) and CB-154 (5 mg/kg body weight) to inhibit both estrogen and PRL action. All injections were given between 8 and 10 a.m. for 8 days only.

Tumor Measurements and Classification. Tumor measurements and body weights were recorded at weekly intervals from the beginning until termination of the experiment. Average tumor diameter for each palpable...
tumor was determined by using the mean of the 2 largest perpendicular diameters as measured with vernier calipers. Average latency period was calculated for all tumors in a group.

A tumor that decreased by 5 mm or more in average tumor diameter was classified as regressing. A tumor that increased by more than 5 mm in average tumor diameter was classified as growing, and a tumor that changed less than 5 mm in average tumor diameter was considered as stable. Upon termination of the experiment, tumors were removed for routine histological examination.

**Evaluation of Hormone Dependency of Mammary Tumors.** Sixteen weeks after DMBA administration, all animals were bilaterally ovariectomized to determine hormonal dependency of the mammary tumors. This period of time after DMBA administration was chosen because at least 94% of the tumors could be classified as adenocarcinomas at this time, and most are hormone dependent (7). The percentage of mammary adenocarcinomas decreases progressively by 16 weeks after carcinogen administration (7). Tumor growth was followed for 4 weeks after ovariectomy.

**Blood Collection and Hormone Assay.** Blood was collected under light ether anesthesia by orbital sinus puncture on the last day of drug and hormone treatment (7 days after DMBA administration), prior to ovariectomy (16 weeks after DMBA administration), and upon termination of the experiment (4 days after ovariectomy). In all 3 periods, blood was collected between 10 and 11 a.m., when serum PRL levels in female rats are approximately the same during the estrous cycle. Serum was separated by centrifugation and stored at −20°C until assayed for PRL by a standard radioimmunoassay method (20).

**RESULTS**

The effects of the various hormone and drug treatments on mammary tumorigenesis at the end of 16 weeks are shown in Table 1. Tumor incidence in vehicle-treated controls (Group A) averaged 72.2%, and the average number of tumors per tumor-bearing rat was 3.8. Spontaneous regression was found in 4 tumors in these rats. Rats that received daily injections of HAL (Group B) showed no significant difference in percentage of rats with tumors, but more total tumors and tumors per rat. Rats given EB (Group C) showed small but insignificant differences in mammary tumor development as compared with controls. Rats given injections of CB-154 showed decreases in all parameters of tumor development, and these differences approached statistical significance when compared with controls (Group A).

Rats given injections of TAM (Group E) showed very significant reductions in incidence of mammary tumors (22.2%) as compared with controls (72.2%). Rats given the combination of TAM and CB-154 (Group F) also showed significant reductions in tumor incidence (23.4%) and number of tumors per tumor-bearing rat (2.0), when compared with controls (Group A). However, the combined treatment was only slightly more effective in inhibiting mammary tumor development than TAM treatment alone (Group E). Animals in Groups E and F had no tumors that displayed spontaneous regression.

Sixteen weeks after DMBA administration, rats with tumors in all treatment groups were ovariectomized to determine mammary tumor hormone independence. During the 4-week period after ovariectomy, one rat in Group A, 3 rats in Group B, one rat in Group C, 2 rats in Group D, one rat in Group E, and no rats in Group F died. These rats were not included in calculations of mammary tumor hormone dependence in their respective groups.

The effects of ovariectomy on mammary tumor regression in the various treatment groups are shown in Table 2. In the control rats (Group A), ovariectomy resulted in regression of 75% of the mammary tumors, whereas 13.9% were stable and 11.1% showed continued growth. Over 80% of the tumors in rats treated with HAL (Group B) or EB (Group C) showed regression 4 weeks after ovariectomy, while less than 10% of the tumors were stable or continued to grow. The total number of tumors in the HAL-treated group at that time was almost twice that of the control or EB-treated groups. Rats given injections of CB-154 (Group D) showed little difference in tumor response to ovariectomy as compared with controls (Group A). On the other hand, rats given injections of TAM (Group E) showed less regression of mammary tumors after ovariectomy (52.4%) than did controls and a 3-fold greater number of tumors that continued to grow (33.3%) as compared to controls (11.1%). Rats treated with the combination of TAM and CB-154 (Group F) exhibited only a 27.3% regression of mammary tumors after ovariectomy, or only about one-third as many regressing tumors as controls. The incidence of autonomous tumors in these rats (Group F) was 54.5% or about 5 times more than in the controls (11.1%).

The percentage change in average tumor diameter in the 4-week period after ovariectomy is shown in Chart 1. Ovariectomy significantly decreased average tumor diameter by 50% in control rats (Group A) as compared to initial preovariectomy values. A significant reduction of average mammary tumor diameter was also found in rats treated with HAL (Group B), EB (Group C), or CB-154 (Group D). Ovariectomized rats previously treated with TAM (Group E) showed an insignificant reduction in average tumor diameter as compared with preovariectomy values. In

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment†</th>
<th>No. of rats/group</th>
<th>No. of rats with tumors</th>
<th>% of rats with tumors</th>
<th>Total no. of tumors</th>
<th>No. of tumors/tumor-bearing rat</th>
<th>Av. tumor diameter (cm)</th>
<th>Av. latency period (days)</th>
<th>No. of regressing tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Controls (0.87% NaCl)</td>
<td>18</td>
<td>13</td>
<td>72.2</td>
<td>49</td>
<td>3.8</td>
<td>1.1</td>
<td>77.8</td>
<td>4</td>
</tr>
<tr>
<td>B</td>
<td>HAL (0.5 mg/kg)</td>
<td>21</td>
<td>17</td>
<td>77.3</td>
<td>78</td>
<td>4.6</td>
<td>1.3</td>
<td>80.3</td>
<td>8</td>
</tr>
<tr>
<td>C</td>
<td>EB (1 μg/rat)</td>
<td>22</td>
<td>16</td>
<td>72.7</td>
<td>57</td>
<td>3.7</td>
<td>1.5</td>
<td>82.4</td>
<td>3</td>
</tr>
<tr>
<td>D</td>
<td>CB-154 (5.0 mg/kg)</td>
<td>25</td>
<td>15</td>
<td>57.1</td>
<td>43</td>
<td>2.6</td>
<td>1.2</td>
<td>89.0</td>
<td>5</td>
</tr>
<tr>
<td>E</td>
<td>TAM (20 μg/rat)</td>
<td>45</td>
<td>10</td>
<td>22.2°</td>
<td>25</td>
<td>2.5</td>
<td>1.6</td>
<td>77.4</td>
<td>0</td>
</tr>
<tr>
<td>F</td>
<td>TAM (20 μg/rat) + CB-154 (5.0 mg/kg)</td>
<td>47</td>
<td>22</td>
<td>23.4°</td>
<td>22</td>
<td>2.0°</td>
<td>1.3</td>
<td>90.6</td>
<td>0</td>
</tr>
</tbody>
</table>

† Treatment for 1 day before and 7 days after DMBA.
° p < 0.05 as compared to controls (Group A).
Effect of ovariectomy 16 weeks after DMBA on mammary tumor regression 4 weeks later

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of rats</th>
<th>Total no. of tumors</th>
<th>Regressed (%)</th>
<th>Stable (%)</th>
<th>Grew (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Controls (0.87% NaCl)</td>
<td>12</td>
<td>36</td>
<td>27 (75.0)</td>
<td>5 (13.9)</td>
<td>4 (11.1)</td>
</tr>
<tr>
<td>B</td>
<td>HAL (0.5 mg/kg)</td>
<td>14</td>
<td>67</td>
<td>56 (83.6)</td>
<td>5 (7.5)</td>
<td>6 (9.0)</td>
</tr>
<tr>
<td>C</td>
<td>EB (1 μg/rat)</td>
<td>14</td>
<td>35</td>
<td>29 (82.8)</td>
<td>3 (8.6)</td>
<td>3 (8.6)</td>
</tr>
<tr>
<td>D</td>
<td>CB-154 (5.0 mg/kg)</td>
<td>10</td>
<td>24</td>
<td>17 (70.8)</td>
<td>3 (12.5)</td>
<td>4 (16.7)</td>
</tr>
</tbody>
</table>
| E     | TAM (20 μg/rat) | 9 | 21 | 11 (52.4) | 3 (14.3) | 7 (33.3)
| F     | TAM (20 μg/rat) + CB-154 (5.0 mg/kg) | 11 | 22 | 6 (27.3) | 4 (18.2) | 12 (54.5)

* Treatment for 1 day before and 7 days after DMBA.

p < 0.05 as compared to controls (Group A).

**DISCUSSION**

This study demonstrates that estrogen and PRL at the time of DMBA-induced mammary tumor initiation not only influences the development and number of mammary tumors, but also the degree of autonomy shown by these tumors subsequently. Control animals which received injections of vehicle 1 day prior to and 7 days after DMBA administration (Group A) showed a 75% incidence of mammary tumors, and only 11% of these tumors showed continued growth after ovariectomy. In contrast, rats which received daily injections of both CB-154 and TAM for the same 8 days (Group F), inhibiting estrogen action and depressing PRL release, showed a significantly lower incidence of mammary tumors (23%) than in control rats, but 54.5% of these tumors exhibited autonomy after ovariectomy. These results indicate that hormonal independence of carcinogen-induced mammary tumors is determined during early development of these tumors.

It has been reported that, at 3 months after DMBA administration, at least 94% of the mammary tumors found in rats can be classified as adenocarcinomas; at 5 months, this drops to 80%, and by 9 months only 40% of the tumors are adenocarcinomas. We chose to examine tumor response to ovariectomy 4 months after DMBA administration, since tumors at this early stage of development are nearly all frank adenocarcinomas and are highly hormone dependent (1).

Our results are in agreement with previous reports showing that removal of estrogen influence by use of antiestrogenic drugs (12) or ovariectomy (6) shortly after carcinogen administration in rats results in significant inhibition of mammary tumorigenesis. Suppression of serum PRL for several weeks prior to and after carcinogen administration also was reported to inhibit mammary tumorigenesis (3, 13). In the present study, CB-154 reduced the incidence of tumors, total number of tumors, and number of animals had long since been removed from drug or hormone treatment, no differences in serum PRL levels were found among the different treatment groups (Chart 2). The last blood collection was taken upon termination of the experiment, 4 weeks after ovariectomy. All rats showed reduced PRL levels in response to ovariectomy, and there were no differences among treatment groups.

Histological examination of tumors removed from all rats at the end of the experiment showed that 98% of the tumors were adenocarcinomas. These tumors contained characteristic columns of epithelial cells many cell layers thick. Little fibrosis was present, and only one carcinosarcoma was found in Group E, and one sebaceous cell carcinoma was found in Group B.
tumors per tumor-bearing rat, as compared with controls. Although these differences barely missed statistical significance, they are believed to be physiologically significant. Previous investigators induced hypoprolactinemia for several weeks prior to as well as after DMBA administration (3, 13), which delayed maturation of the mammary glands and rendered them susceptible to the action of the carcinogen (4). Under the conditions of the present study, the increase in estrogen action produced by EB administration, and of PRL secretion by HAL injections, did not significantly alter mammary tumor development. In a previous report, we found that injection of EB or of HAL for 20 days before and 20 days after DMBA administration significantly depressed mammary tumor development (13). This may reflect the longer duration of treatment with these agents and perhaps the doses used as well.

Insofar as the effect of the early treatments on subsequent autonomy of the mammary tumors is concerned, it appears that depression of estrogen action by TAM may be somewhat more important than inhibition of PRL release by CB-154. Daily injections of CB-154 during this early period before and after carcinogen administration produced significant reduction in serum PRL levels, but mammary tumor regression after ovariectomy did not differ significantly from controls. By contrast, treatment with TAM during the same early period did not significantly alter basal PRL values, but it significantly decreased mammary tumor development and resulted in greater autonomy of tumors after ovariectomy. The combined treatment of TAM and CB-154 was no more effective in inhibiting mammary tumor development than TAM treatment alone, but these tumors subsequently displayed the greatest degree of autonomy after ovariectomy. Presumably, hormone independence of these tumors was greatest because they were the least dependent on estrogen and PRL for growth after development.

In the present study, both hormone-dependent and hormone-independent tumors were found in the same animals regardless of the treatment given. It is known that mammary gland susceptibility to carcinogen induction of tumors is high when the mammary gland contains a large number of undifferentiated mitotically active terminal end buds (21, 22), such as exists in female rats at about 57 days of age (9, 11). Estrogen and PRL both stimulate mitotic activity in normal and neoplastic mammary tissue (14, 27). DMBA-induced tumors contain a heterogeneous cell population, and it has been suggested that, within a single tumor, growth in response to stimulatory hormones depends on the rate of cell division in the hormone-dependent cells within that tumor (15, 18). It was found that, at the time of DMBA administration, the greater the rate of mitotic activity in the terminal end buds, the greater was the rate of DNA synthesis. This was correlated positively with carcinogen binding and tumor incidence (10, 19). In the present study, combined TAM and CB-154 treatment at the time of tumor initiation may have resulted in reduced mitotic activity and fewer cells to be acted upon by DMBA.

Since cellular responses to a hormone are mediated by the binding of the hormone to a specific receptor on or within the cell, it is of interest that estrogen and PRL binding was found to be generally lower in hormone-independent than in hormone-dependent mammary tumors (16, 26). Identification of PRL receptor sites in DMBA-induced mammary tumors by autoradiography showed that, in some tumors, all cells contained PRL receptors whereas, in other tumors, up to 50% of the cells remained unlabeled (5). Thus, within a given mammary tumor, individual cells display wide variability in hormone binding and hormone dependence. These heterogeneous cell populations appear to be in a dynamic state, since mammary tumor responsiveness to ovariectomy declines with increasing age and size of tumors (1, 7).

In conclusion, we have demonstrated that the hormonal milieu in rats at the time of initiation of mammary tumorigenesis by DMBA determines not only tumor incidence, but also subsequent hormonal dependency of these tumors. Animals made deficient in estrogen and PRL activity at the time of DMBA administration develop fewer tumors, but the tumors that do develop are less dependent on these hormones for subsequent growth.
REFERENCES

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