Melatonin Inhibition and Pinealectomy Enhancement of 7,12-Dimethylbenz(a)anthracene-induced Mammary Tumors in the Rat

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ABSTRACT

The effects of the pineal hormone, melatonin, and of pinealectomy on the incidence of mammary adenocarcinoma in Sprague-Dawley rats treated with 7,12-dimethylbenz(a)anthracene (DMBA) were investigated. Melatonin (2.5 mg/kg), begun on the same day as DMBA (15 mg) treatment and given daily in the afternoon for 90 days, significantly reduced the incidence of mammary tumors from 79% (control) to 20% (treated) (p < 0.002). Rats pinealectomized at 20 days of age and treated with 7 mg of DMBA at 50 days of age had a higher incidence of tumors (88%) compared to control animals (22%). Fifteen mg of DMBA, which resulted in a higher incidence of tumors, reduced the difference between pinealectomized and control animals. Melatonin only partially reversed the effects of pinealectomy, reducing the incidence from 87% (pinealectomized alone) to 63% (pinealectomized plus melatonin); however, the tumor incidence was still lower (27%) in nonpinealectomized, melatonin-treated animals. Assessment of plasma prolactin, luteinizing hormone, follicle-stimulating hormone, estradiol, and cortisol in DMBA-treated tumor-free and tumor-bearing animals revealed a significantly lower plasma prolactin concentration [27 ± 5 (S.E.) ng/ml] in melatonin-treated animals as compared to vehicle-treated animals [65 ± 8 ng/ml]. The concentration of plasma prolactin was less in melatonin-treated, pinealectomized rats (55 ± 10 ng/ml) as compared to vehicle-treated, pinealectomized animals (101 ± 13 ng/ml). Other hormones were not affected by melatonin treatment. These data support the hypothesis that melatonin inhibits the development of DMBA-induced mammary tumors in the rat while removal of the pineal gland stimulates development of such tumors. Additionally, these experiments provide evidence that these effects may be mediated by a suppression of plasma prolactin levels.

INTRODUCTION

Rat mammary tumors induced by DMBA respond to a number of hormone manipulations (3, 8). DMBA-induced tumors are often prolactin dependent; experimental manipulations that reduce circulating levels of prolactin, such as hypophysectomy or treatment with 2-bromocryptine, inhibit tumor induction by DMBA (24, 25). Conversely, transplantation of a pituitary or withdrawal of 2-bromocryptine promotes tumor growth (24, 25).

Ovarian steroids also influence tumorigenesis following DMBA administration. For the initiation of mammary tumors, the presence of estrogens may be required (3, 4, 8). However, once induced, most mammary tumors can grow in the absence of ovarian steroids if prolactin levels are elevated; estrogen replacement by itself is usually insufficient to maintain tumor growth in hypophysectomized-ovariectomized DMBA-treated rats (3, 18). These observations suggest a permissive role for estrogens and an active role for prolactin in the promotion of DMBA mammary tumors.

Another line of investigation has suggested an inhibitory effect of the pineal gland on reproductive function and prolactin secretion. In the Syrian hamster, the pineal gland and its hormone melatonin induce gonadal collapse (7, 14, 16, 17, 19, 21, 22). Long-term daily afternoon injections of melatonin can induce anoestrus that essentially mimics the pineal-mediated response to a lighting schedule that inhibits reproductive function (19). The pituitary hormone affected most rapidly by melatonin treatment is prolactin, which is markedly suppressed in the circulation (14, 16).

In the rat, long-term daily afternoon injections do not induce a change in reproductive function (5). However, removal of the olfactory bulbs may change the sensitivity of rats to exogenous melatonin, since anosmic female rats treated daily with melatonin have lowered levels of pituitary prolactin (15). The possible inhibitory role of the pineal gland and melatonin on reproductive function and prolactin secretion led to this series of experiments which test the effects of the pineal gland and its hormone melatonin on induction of mammary tumors in the rat by DMBA.

MATERIALS AND METHODS

Animals. Sprague-Dawley rats were supplied by Zivic-Miller Co., Allison Park, Pa., and were housed in windowless rooms maintained on a lighting schedule of 12 hr of light per day (lights on at 6 a.m. for Experiments 1 and 2; lights on at 4 a.m. for Experiment 3). All animals were allowed access to food and water ad libitum.

Surgery. Pinealectomies were performed at 20 days of age according to the procedure of Hoffman and Reiter (6). At 2 weeks following surgery, the animals were shipped to our laboratories.

Radioimmunoassays. Radioimmunoassays for estradiol, cortisol, prolactin, LH, and FSH were performed on plasma samples collected from animals used in Experiment 3. The reagents for the prolactin, LH, and FSH radioimmunoassays were provided by the National Institute of Arthritis, Metabolism, and Digestive Diseases. Plasma prolactin is reported in ng of prolactin Preparation RP-1, using rat prolactin antibody S-5. Plasma LH is reported in ng of LH Preparation RP-1, using rat LH Antibody S-4. Plasma FSH is reported in ng of FSH Preparation RP-1, using rat FSH Antibody S-9.

Statistics. Data from Experiments 1 and 2 were analyzed by the generalized Wilcoxon test. For Experiment 3, the overall equality of the 5 test groups was compared using the generalized Kruskal-Wallis test. Statistical differences of body weights and hormone levels in Experiment 3 were determined by Student's t test.

Experiment 1: Effect of Melatonin on the Incidence of DMBA-

1 The abbreviations used are: DMBA, 7,12-dimethylbenz(a)anthracene; LH, luteinizing hormone; FSH, follicle-stimulating hormone; i.g., intragastric.
induced Mammary Tumors. Sixty 50-day-old rats were given 15 mg of DMBA in peanut oil by i.g. intubation. On the same day, 30 of these animals received 500 µg melatonin (Sigma Chemical Co., St. Louis, Mo.) i.p. The remaining 30 animals received 0.25 ml vehicle (4% ethanol in phosphate-buffered saline). The melatonin and vehicle injections were administered at 4 p.m. and were continued daily for 90 consecutive days. Beginning 1 week following DMBA treatment and for 140 days, the animals were palpated twice weekly, and the incidence of tumors was recorded. Tumors were measured with vernier calipers, and when the tumors reached 2.5 cm in single diameter the animals were sacrificed.

Experiment 2: Effect of Pinealectomy on the Incidence of DMBA-induced Mammary Tumors. At 20 days of age, 36 rats underwent pinealectomy and 36 animals had a sham pinealectomy. When the rats were 50 days old, 7 mg of DMBA were administered to all rats by i.g. intubation. After 1 week, the surviving animals were examined twice weekly for tumors. The study was terminated 240 days following the administration of DMBA, and each animal was checked for successful pinealectomy at autopsy.

At 20 days of age, 30 rats were pinealectomized and 30 rats were sham pinealectomized. All animals were given 10 mg of DMBA at 50 days of age. One week following DMBA treatment, the presence of tumors was recorded in the remaining animals, and, as previously described, animals with tumors 2.5 cm in diameter were sacrificed, and the absence of the pineal gland was determined. The study was terminated 240 days following the administration of DMBA.

Experiment 3: Effect of Melatonin and/or Pinealectomy on the Incidence of DMBA-induced Mammary Tumors. When animals were 52 days old, 15 mg of DMBA were administered to 175 rats by i.g. intubation, and animals that died within 1 week following DMBA treatment were not included in the experiment. Seventy of the remaining animals had a pinealectomy at 20 days of age, and one-half of these received daily injections of 500 µg melatonin between 11 a.m. and 12 noon, beginning 1 day after DMBA treatment. The remaining 35 pinealectomized animals were given injections of 0.25 ml vehicle (4% ethanol in phosphate-buffered saline) at the same time of day. Thirty-five animals that underwent a sham operation at 20 days of age were also given injections of 0.25 ml vehicle daily at this same time of day. Seventy intact animals were divided into 2 groups: one receiving daily injections of 500 µg of melatonin between 11 a.m. and 12 noon; and the other receiving daily injections of 0.25 ml of vehicle at the same time of day. Treatment began 1 day after DMBA injection and was continued for 92 days. The 5 study groups were: Group 1, pinealectomy plus melatonin; Group 2, pinealectomy plus vehicle; Group 3, sham pinealectomy plus vehicle; Group 4, intact plus melatonin; Group 5, intact plus vehicle.

During the course of this experiment, animals that developed mammary tumors >1 cm in diameter were sacrificed at weekly intervals at 10 a.m. Tumor-free animals were sacrificed 92 days after DMBA treatment at the same time of day. Each animal was sacrificed by decapitation, and tumors were removed and stored in 10% formalin until processed for histological evaluation. Trunk blood was collected in heparinized tubes and centrifuged, and the plasma was harvested and stored at -20° until assayed by radioimmunoassay for prolactin, LH, FSH, estradiol, and cortisol.

RESULTS

Inhibition of Mammary Tumors by Melatonin in DMBA-treated Rats. During the first 90 days following a 15-mg dose of DMBA, 50% of the vehicle-injected rats developed tumors, while none of the melatonin-injected animals had tumors (Chart 1). Melatonin injections were discontinued on Day 90 following DMBA. By Day 140 (50 days after discontinuation of melatonin), 79% of the vehicle-injected animals had developed tumors, while significantly less, 20%, of the melatonin-injected animals developed breast tumors (p < 0.002).

Enhancement of Mammary Tumor Development by Pinealectomy in DMBA-treated Rats. To induce a lower percentage of intact animals developing tumors, low doses of DMBA were administered. After a dose of 7 mg of DMBA, 48% of the pinealectomized animals developed tumors within 2 months, while none of the intact animals developed tumors during this same period of time (Chart 2). By the 240th day after DMBA treatment, 88% of the pinealectomized animals had tumors, a figure which was significantly greater than the 22% tumor incidence in intact animals (p < 0.002).

In a second study, in which 10 mg of the DMBA were administered, the differences between pinealectomized and control animals were not as striking (Chart 3). By the 230th day following treatment, 46% of the pinealectomized animals developed tumors compared to 21% of the intact animals (p < 0.03).

A final comprehensive experiment was designed to determine the effect of pinealectomy and melatonin administration on plasma hormone concentrations and on the incidence of mammary tumors. A high dose of 15 mg of DMBA was administered, and by the end of the study 87% of the intact animals developed tumors (Chart 4). There was no further increase in the incidence of tumors in the pinealectomy group. Pinealectomized animals which received daily melatonin injections had
a lower incidence of tumors (63%) than did either intact or pinealectomized animals; however, the difference was not statistically significant. Only 27% of the pineal-intact animals receiving melatonin had tumors by the end of the experiment, which was significantly less than that observed in the control or pinealectomy groups ($p < 0.002$). Irrespective of treatment group, the mean number of tumors per tumor-bearing rat was 1.4.

In Experiment 3, for each animal possessing at least one mammary tumor, a single 4-μm hematoxylin-eosin-stained section of the dominant mammary tumor nodule was examined and classified according to standard histological criteria (28). Of the 86 tumors examined, 84 were malignant epithelial tumors, one was a fibroadenoma, and in one section no tumor was identified. The tumors were almost exclusively papillary carcinomas, with occasional cribriform and/or comedocarcinoma variants in portions of the tumors. Mitoses were readily apparent, and necrosis as well as an infiltrating component were apparent in some of the sections. The carcinomas were usually intersected by fibrous septae which were frequently infiltrated by a mild to moderate chronic inflammatory infiltrate. The low incidence of benign fibroepithelial tumors in this study is probably attributable to both the relatively young age of the animals at necropsy and the selection of the dominant, rapidly growing mass for histological evaluation. Mammary tumors were not analyzed histologically in Experiments 1 and 2.

The body weight of each animal in Experiment 3 was determined 5 weeks after DMBA treatment. At this point in the experiment, there was no significant difference in body weight among the groups. During the course of the experiment, body weights of the animals from the 5 groups were determined and compared at the first appearance of tumor. No significant difference was noted in the body weights of the animals from the 5 treatment groups.

We examined the effect of these experimental manipulations on the plasma concentrations of various pituitary and steroid hormones known to influence tumor growth and gonadal function. No significant differences among the groups were observed in the concentrations of LH, FSH, cortisol, or estradiol (Table 1). Plasma prolactin was significantly suppressed in animals given daily injections of melatonin compared to vehicle-injected animals ($p < 0.01$). Pinealectomized animals given injections of melatonin also had lower levels of plasma prolactin than did pinealectomized, vehicle-treated animals ($p < 0.01$).

**DISCUSSION**

We have recently summarized epidemiological and anecdotal clinical evidence which supports the hypothesis that the pineal gland and its secretions may inhibit the development of breast carcinoma in humans (2). In order to study this possible relationship in an animal model, we have investigated the effect of the pineal gland and melatonin in the development of DMBA-induced tumors in the rat. The present studies provide evidence for a modulating role of melatonin and the pineal gland in the development of breast cancer in this system. Long-term daily administration of melatonin inhibited tumorigenesis, whereas pinealectomy increased the incidence of breast tumors. Mea-

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**Table 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats</th>
<th>Prolactin (ng/ml)</th>
<th>LH (ng/ml)</th>
<th>FSH (ng/ml)</th>
<th>Estradiol (pg/ml)</th>
<th>Cortisol (μg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melatonin</td>
<td>16</td>
<td>27 ± 5</td>
<td>54 ± 8</td>
<td>80 ± 7</td>
<td>29 ± 4</td>
<td>13 ± 2</td>
</tr>
<tr>
<td>Vehicle</td>
<td>26</td>
<td>65 ± 8</td>
<td>75 ± 10</td>
<td>112 ± 23</td>
<td>32 ± 3</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>Pinealectomized-melatonin</td>
<td>23</td>
<td>55 ± 10</td>
<td>65 ± 10</td>
<td>80 ± 11</td>
<td>42 ± 5</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>Pinealectomized</td>
<td>18</td>
<td>101 ± 13</td>
<td>71 ± 16</td>
<td>82 ± 15</td>
<td>43 ± 10</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>Sham operated</td>
<td>21</td>
<td>81 ± 9</td>
<td>90 ± 16</td>
<td>120 ± 25</td>
<td>40 ± 6</td>
<td>12 ± 2</td>
</tr>
</tbody>
</table>

*a* Mean ± S.E.

*b* $p < 0.01$ compared to vehicle group.

*c* $p < 0.01$ compared to pinealectomized group.
measurement of various hormones suggests that these effects may be mediated by changes in circulating levels of prolactin. Melatonin administration depressed prolactin concentrations in the plasma, while pinealectomized rats had the highest prolactin levels and the highest tumor incidence of all groups studied. However, these experiments do not conclusively indicate that regulation of plasma prolactin is the sole or necessary mechanism by which the pineal gland and melatonin may influence tumorigenesis following DMBA administration.

Earlier studies indicate that, contrary to inhibiting prolactin secretion, melatonin administration actually causes an increase in circulating prolactin (14). More recent studies have demonstrated that daily afternoon melatonin treatment of anomic female rats caused a reduction in serum and pituitary prolactin levels whereas the same treatment given to intact controls had no effect (15). Perhaps the effect of anosmia is to increase the sensitivity of the animal to melatonin, a possibility which may also apply to the DMBA-treated rat.

It should be noted that the paradigm for the daily injection of melatonin was drawn from previous studies in Syrian hamsters, in which melatonin inhibited reproductive function only when administered at specific times of day (21). Long-term administration of melatonin to hamsters induces testicular regression and decreases levels of prolactin in males and induces anovulation in females only if administered 1 to 4 hr prior to the onset of the dark period of the day; injections of melatonin in the morning have no effect on reproductive function (21). In the present study, the animals were given injections during this period of maximum melatonin sensitivity. However, we chose a 20-fold higher daily dose of melatonin to be administered to the DMBA-treated rats to ensure a long period of elevated levels of circulating melatonin.

The pineal gland itself may influence the sensitivity of target organs to exogenous melatonin. Melatonin is synthesized by the pineal and secreted into the circulation in a diurnal rhythm characterized by low levels during the day and elevated levels at night (9–11, 20, 26). The presence of this daily rhythm may affect the sensitivity of specific target tissues to the hormone. Consequently, administration of melatonin prior to the nocturnal increase in endogenous melatonin may be most effective because melatonin target tissues are most sensitive to the hormone at this time.

Removal of the pineal gland has been shown to abolish the melatonin rhythm in the circulation of the rat (9). In the present study, pinealectomy enhanced mammary tumorigenesis in DMBA-treated rats, suggesting that the pineal gland, through the daily melatonin rhythm, may have an inhibitory effect on tumor induction by DMBA. However, hormone replacement by a single daily injection of melatonin to pinealectomized rats partially restored the tumor incidence to that of untreated animals, but not to the even lower incidence observed in intact, melatonin-treated rats.

One explanation of this observation is that removal of the pineal gland, which results in the loss of the melatonin rhythm, might cause a loss in end organ sensitivity to melatonin and may abolish the time-of-day sensitivity to exogenous melatonin. A single daily injection of melatonin might not be sufficient to reinitiate hormonal sensitivity. This hypothesis is supported by studies in the hamster, in which a single daily injection of melatonin was not sufficient to induce gonadal collapse in pinealectomized animals; rather, 3 daily injections of melatonin were necessary to induce gonadal collapse in pinealectomized animals (19, 21). A parallel study of multiple melatonin injections in DMBA-treated rats has not been performed.

Alternatively, removal of the pineal gland might result in the loss of another substance(s) in addition to melatonin that might also have a role in the inhibition of tumorigenesis. One hypothesis is that melatonin functions within the pineal gland to initiate the release of other pineal products, most notably arginine vasotocin, which has been shown to act on gonadal tissue (12, 13). The hypothesis is supported by studies in vivo which revealed the uptake of \(^{3}H\)melatonin by the pineal gland and by another study demonstrating the release of arginine vasotocin into cerebrospinal fluid by melatonin (12, 27). However, arginine vasotocin has been shown to increase prolactin levels in the rat (23), and a role for this molecule in tumor induction has not been shown. Alternatively, other pineal products, possibly regulated by melatonin, may play a role in DMBA-mediated mammary tumors.

A previous study concluded that melatonin inhibited tumor induction in DMBA-treated rats (1). Additionally, this study demonstrated that melatonin treatment did not reduce tumor incidence in rats whose pineal melatonin rhythm was suppressed by constant exposure to light (1). Thus, this previous study provides additional evidence that mammary tumorigenesis can be inhibited by daily melatonin treatment provided that the melatonin rhythm is present. These studies suggest that a possible role of pineal function and the daily melatonin rhythm should be examined as an etiological factor in human breast cancer.

REFERENCES

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