Effect of Melatonin on Mammary Carcinogenesis in Intact and Pinealectomized Rats in Varying Photoperiods

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ABSTRACT

Exposure of female Holtzman rats to constant light (24 hr/day) immediately after birth significantly increased 9,10-dimethyl-1,2-benzanthracene-induced mammary cancer. Such "functionally pinealectomized" animals also revealed significant increase in the circulating level of prolactin and exaggerated development and proliferative activity of mammary epithelium, as measured by quantitation of terminal end buds and alveolar buds from the whole mounts and by DNA synthesis, respectively. Administration of melatonin (500 µg/day/animal i.p. given from 52 to 145 days of age) completely abolished the effect of functional pinealectomy by sharply reducing 9,10-dimethyl-1,2-benzanthracene-induced cancer incidence from 95% to 25% during the post-9,10-dimethyl-1,2-benzanthracene observation period which lasted up to 180 days. On the other hand, administration of melatonin to surgically pinealectomized animals exposed to constant light reversed the effect only partially by reducing the cancer incidence from 83% to 53%. Further, melatonin treatment in intact and surgically pinealectomized animals exposed to a short photoperiod revealed qualitatively similar differences in suppression of the cancer incidence. From these results, it is concluded that, to have an impressive antitumor effect, presence of the pineal gland is essential, and the probable site of melatonin action appears to be at both the pineal gland and the hypothalamus.

INTRODUCTION

Over the past few years, attention has been focused on the impact of melatonin, the principal hormone of the pineal gland, on the development of mammary cancer (1, 9, 24). Our laboratory has recently reported significant alterations in the cardinal features of mammary cancer, namely, high incidence, shorter mean latency period of tumor appearance in days, and multiplicity of tumors per animal, following carcinogen administration in "functionally pinealectomized" female Holtzman rats (12). Functional pinealectomy was brought about by exposing the female animals to constant light for 24 hr/day immediately after birth when a near total deprivation of melatonin is achieved (10, 27). The functionally pinealectomized animal therefore appears to be a unique endocrine model in which to study the effect of replacement therapy as well as the probable mechanism of melatonin action. This paper deals with our findings on these aspects.

MATERIALS AND METHODS

Animals. A group of 10- to 12-day-old pregnant Holtzman rats, randomly bred in the Institute, were kept in an air-conditioned room with controlled light 10 hr/day from 8 a.m. to 6 p.m. The litters were weaned on Day 21, and a group of 4 to 6 female rats/cage was exposed to the aforesaid LD2 schedule.

Another group of randomly bred 10 to 12-day-old pregnant Holtzman rats were kept in a small air-conditioned cubicle (1.83 x 1.81 x 2.74 cu m), lighted 24 hr/day with two 40-watt fluorescent tubes situated 30 cm above the top shelf of the racks. The cages were rotated from time to time to receive uniform light of 150 lux intensity. These pregnant rats delivered under the LL schedule, thus ensuring that their litters were exposed to constant light from birth. On Day 21 after birth, the female pups were weaned and randomly housed in different cages (4 to 6 animals/cage) exposed to constant light. These aforesaid groups of LD and LL animals were categorized as Groups 1 and 2, respectively. All these animals were kept on a balanced diet and given water ad libitum.

Additionally, 1-day-old female Holtzman rats from Groups 1 and 2 received surgical pinealectomy under deep hypothermia. On Day 21, these pups were weaned, kept 4 to 5 animals/cage, and exposed to respective LD and LL schedules. These pinealectomized animals belonging to LD and LL groups were categorized under Subgroups 1 and 2, respectively.

Morphology of Mammary Gland. From all of the groups, at least 5 to 7 animals were sacrificed on Day 55 of age for the study of morphological changes in the inguinal mammary glands from the whole-mount preparation. For this, the mammary glands attached to the skin pelt were fixed in 10% neutral formalin for 24 hr. Then, the glands were dissected from the skin, dehydrated through ascending grades of ethyl alcohol, defatted in chloroform, and thereafter stained with Kernechtrot (E. Merck, Darmstadt, Germany) and mounted. By using a microscopic graticule, the quantitation of TEB and AB was done in 20 different sites, and the average was expressed as the density of TEB and AB per sq mm.

Mammary Gland DNA Synthesis. A group of 5 to 7 female Holtzman rats (aged 55 to 60 days) in estrus from Group 1, Group 2, and Subgroup 1A were selected for this study. Two hr before sacrificing, each rat was given a single intraperitoneal injection of 100 µCi [3H]thymidine (LCT-3-Thymidine-methyl-T; specific activity, 6600 mCi/mmol) per 100 g body weight. The time of injection was adjusted in such a way that all these rats would be killed at about 5 p.m. Immediately after the animals were sacrificed, inguinal mammary glands from each animal were dissected out and weighed, and a 20% homogenate was prepared in 0.9% NaCl solution-EDTA. DNA content was determined by using the method of Welsch and Manus (26). Total DNA content was quantitatively determined by the diphenylamine colorimetric method of Burton (5). The [3H]thymidine content was determined by adding neutralized aliquots of the supernatant in Bray’s cocktail. The samples were counted in an LKB 1215 RACK BETA II liquid scintillation counter. The results were expressed as cpm or dpm of [3H]thymidine per µg of DNA.

Plasma Estradiol and PRL. Estradiol and PRL were estimated before and after 5 days of melatonin (500 µg/day) treatment, from animals in estrus from all of the groups (ages 55 to 60 days) by the radioimmunoassay procedures of Korenman et al. (11) and Midgley (16), respectively. The reagents for PRL radioimmunoassay were provided by the National Institute of Arthritis, Metabolism, and Digestive Diseases. The reagents for estradiol radioimmunoassay were bought from Serono Diagnostics, Biodata S. P. A., and melatonin was obtained from Sigma Chemical Co.

Tumor Induction. A total of 275 animals were used for this study. Of...
these, Group 1 comprised 115 animals and Group 2 consists of the remaining 180 animals. Fifty-six animals from Group 1 and 69 animals from Group 2 were neonatally pinealectomized. At about 55 days of age and at the time of estrus, all these aforesaid animals categorized under Groups LD and LL were given a single dose of DMBA, 10 mg/100 g body weight i.g. The overall mortality following DMBA was about 20%. Melatonin treatment was given i.p. (500 μg dissolved in 4% ethanol-0.01 M phosphate-buffered saline (pH 7) buffer per day, in the late afternoon) from Days 52 to 145 of age. Further details of these animals are as shown in Table 1.

One month after DMBA administration, animals were palpated for tumor once weekly for up to 180 days post-DMBA treatment. Latency period of tumor appearance in days was also recorded. After the tumor had grown to 1 cm in diameter, these animals were sacrificed after being weighed, and the weights of different organs were recorded. Tumors were fixed in Bouin’s solution and 5-μm sections were prepared and stained with hematoxylin and eosin.

Vaginal Smears. Vaginal smears stained with methylene blue were examined daily in both the treated and untreated groups and subgroups of animals.

Statistical Analyses. Data on the morphology, DNA synthesis, hormone levels, organ weights, and latency period in days of tumor appearance were analyzed by Student’s t test; statistical differences in tumor incidence were compared using the χ² test. Comparison of degree of suppression was done using Student’s t test.

RESULTS

Structures in mammary gland, TEBs and ABs quantitated from the whole-mount preparation at the age of 55 to 60 days from different groups are shown in Chart 1. It is evident that, in both intact LL and PX LL animals, the morphological changes in the mammary gland are advanced, and the density in both TEB and AB in LL animals is significantly higher (p < 0.01) than that seen in intact LD animals, ages 55 to 60 days, a period considered to be the most vulnerable for chemical induction of cancer (Chart 1). When morphological changes in PX LD animals during the aforesaid period are compared with those in the intact LD animals, whereas TEB are significantly increased (p < 0.001), AB are shown to be significantly low. Circulating plasma PRL levels during 55 to 60 days in LL groups are significantly elevated when compared to those seen in LD (intact pineal or PX) animals.

Chart 1 summarizes the incidence of mammary carcinoma (histologically categorized as adenocarcinoma) in all 4 experimental groups. It is evident that not only the incidence is significantly high in intact LL and PX LL groups as compared to that in the intact LD group of animals, but also the latency period of tumor appearance is significantly shorter in LL animals. On the other hand, PX LD animals show comparable incidence and latency period of tumor appearance.

The DNA-synthetic pattern in the mammary gland on the day of estrus in intact pineal (LD), functionally pinealectomized, and PX (LD) animals at 55 to 60 days of age shows a clear positive correlation with tumor incidence (Chart 2).

Chart 3 reveals the effect of short-term administration (5 days) of melatonin on the plasma concentration of PRL and estradiol.
on the day of estrus in all experimental groups of animals. Plasma PRL levels reveal significant differences in LL and LD animals. There was, however, no significant change observed in the level of circulating plasma estradiol in these groups. When melatonin was administered at about 4 p.m. for 5 consecutive days, the inhibitory effect on plasma PRL and estradiol levels was dramatic in "intact pineal" animals irrespective of whether they are exposed to LD or LL schedules. However, in PX animals exposed to LL or LD schedules, melatonin treatment was completely ineffective in bringing about reduction in PRL or estradiol.

Table 1 summarizes our observations on the incidence of mammary cancer following melatonin treatment in intact and pinealectomized animals exposed to varying photoperiods. It is clear from Table 1 that there is a significant suppression in the incidence of mammary cancer in the intact LL group, but such suppression in pinealectomized (LL) animals is not significant. A significant difference was found in degree of suppression in functional versus PX groups under the LL schedule. In animals under the LD schedule, on the other hand, there is an apparent difference in the degree of suppression in the incidence, but this difference is not statistically significant.

Charts 4 and 5, using the life table approach, not only depict striking differences in the suppression of the incidence in intact and pinealectomized LL animals but also indicate the long latency period of tumor appearance in response to melatonin treatment.

**DISCUSSION**

To study the effect of melatonin on mammary tumorigenesis, a classical endocrinological maneuver is used in this study by exposing female rats to constant light immediately after birth (10, 27). In such "functionally pinealectomized" young virgin rats, melatonin deprivation removes its inhibitory regulatory control over the hypothalamic-hypophysial axis leading to constant availability of estrogen and elevated circulating PRL (15). In response to these changes in the hormonal profile, there occur premature sexual maturation, prolonged estrus, and prolonged mammary gland stimulation (15). We have reported elsewhere that DMBA administration to these animals resulted in a significant increase in mammary cancer when compared to intact controls exposed to short photoperiod (LD) schedule (12). The observed striking difference in the 2 groups appears to be due mainly to changes in the differentiating capacity of TEB and in the DNA-synthetic index in the mammary gland, and we have been able to establish a clear positive correlation between its concentration in plasma and the aforesaid changes in the mammary epithelium (15). Nagasawa (17) has hypothesized that PRL plays the cardinal role in regulating the morphogenic and the mitogenic properties of the mammary epithelium. Our data on both the differentiating capacity of TEB as well as DNA synthesis not only corroborate this finding but also indicate that the preferential route for protective effect is through DNA synthesis (Charts 1 and 2).

Taking into account the possible inhibitory role of melatonin...
on mammary cancer, neonatal surgical pinealectomy was expected to show similar effect but, as shown by Lapin (14) and our laboratory (13), the mammary cancer incidence in pinealectomized rats kept in short photoperiod and given DMBA was similar to that seen in LD intact controls. Therefore, it is highly probable that, during the dark phase, some amount of melatonin is synthesized by extrapineal sources such as retina and Harderian gland (4, 18-20) in these animals, and that amount of melatonin is capable of inhibiting prolactin synthesis and release (Chart 1). Furthermore, high estrogen activity suggested by the morphological features of the mammary gland with particular reference to increase in TEB (8) and by the presence of prolonged estrus as seen from the vaginal cytology of these animals supports the finding of Reiter et al. (23), that, in the absence of the pineal gland, the antigonadotropic potential of melatonin cannot be manifested. In other words, melatonin per se does not seem to be antigonadotropic, but by acting at the pineal gland and releasing bioactive principle(s), brings about an antigonadotropic effect (2, 3, 7, 22).

The role of melatonin on chemical induction of mammary cancer can also be studied by replacement therapy after functional or surgical pinealectomy in LL animals or by additive treatment in animals exposed to LD schedule.

Tamarkin et al. (24) have shown that long-term daily administration of melatonin inhibits tumorigenesis in animals in normal LD schedules, and this effect is mediated by modulating the circulating concentration of PRL. In our study, daily melatonin administration in the late afternoon from Days 52 to 145 of age (DMBA was usually given during estrus between 55 to 60 days) decreased mammary cancer in both groups and subgroups (Table 1). An unexpected finding was that, in the melatonin-treated group of LL animals, the percentage of suppression in cancer incidence was significantly higher in intact animals when compared to that seen in the PX animals under the LD schedule. Similar results in intact LD and PX LD animals showed differences in response to melatonin, but neither the observed difference in incidence nor the percentage of suppression appeared to be significantly different (Table 1). In brief, our data suggest that intact LL animals are highly responsive to melatonin replacement therapy and that the presence of the pineal gland is essential in order for melatonin to have an impressive antitumor effect.

Although the precise mechanism and mode of action of melatonin are not known, it is relevant to mention that next to medial basal hypothalamus in bovine brain, it is the pineal gland which has high affinity melatonin binding (6). Furthermore, as reported by Wurtman et al. (28), pineal exhibits the highest uptake of radioactive melatonin after its i.v. administration. Therefore, the plausible explanation for the observed results in the intact pineal LL group could be that the density of melatonin receptors in these areas would increase in apparent compensation for continuous deprivation of the melatonin during constant light. In our working hypothesis then, unless melatonin is the neurotransmitter for releasing bioactive pineal proteins and/or peptides or unidentified substances from the pineal gland, its antitumor action is bound to be less effective in PX animals. In this respect, the observation of Pavel (21) that melatonin serves as the releasing hormone for AVT mainly from the pineal gland is important. Recently, Vijayan et al. (25) have shown that AVT has both an antigonadotropic and an anti-PRL effect. If AVT has a physiological role in suppression of luteinizing hormone and PRL secretion, the mechanism of action should be at the hypothalamic level though as mentioned previously, the presence of the pineal gland is essential for release of AVT (22). We have reported elsewhere that melatonin treatment from Days 20 to 55 of age in the intact pineal LL group reversed the changes in mammary epithelium and DNA synthesis besides significantly decreasing both the plasma PRL and estradiol concentration (15). In fact in the present study in the presence of the pineal gland, even 5 days of treatment showed a dramatic decline in the plasma PRL level and a trend towards inhibition of estrogen. On the other hand, such treatment in pinealectomized animals, whether exposed to short or long photoperiods, failed to show any changes in the aforementioned hormonal levels (Chart 3).

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