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Suppressive effect by melatonin on different phases of 9,10-dimethyl-1,2-benzanthracene (DMBA)-induced rat mammary gland carcinogenesis

Asha Subramanian and Lalita Kothari[CA]
Endocrinology Unit, Cancer Research Institute, Tata Memorial Centre, Parel, Bombay-400012, India.
Tel: 4123803; Fax: 9122 -4121089.

This comprehensive study examines the influence of oral melatonin on the initiation and promotion phases of DMBA-induced mammary tumorigenesis in intact and pinealectomized female Holtzman rats reared in short (light:dark schedule L:D 10:14) and long (L:D 24:0) photoperiods. Melatonin administration in the initiation phase significantly suppressed tumor incidence only in intact animals reared in both photoperiods, indicating that the presence of the pineal was obligatory. On the other hand, during the promotion phase, irrespective of the presence or absence of the pineal, the tumor-suppressive effect of exogenous melatonin was pronounced.

Key words: Initiation, mammary tumorigenesis, melatonian, promotion, suppression.

Introduction

Melatonin (*n*-acetyl-5-methoxytryptamine), an indoleamine secreted by the pineal gland, has been shown to suppress chemically induced mammary tumorigenesis in rats.[1–4] Further studies in our laboratory demonstrated for the first time that replacement or additive manipulations of oral melatonin from puberty to adulthood play a critical role in the suppression of DMBA-induced rat mammary tumors.[5]

In this paper, we report a comprehensive analysis of the influence of melatonin on the two major components of DMBA-induced mammary tumorigenesis: the initiation and promotion phases, in female Holtzman rats, intact and pinealectomized and reared in short and long photoperiods.

[CA] Corresponding Author

Materials and methods

Animals

Female Holtzman rats, intact and neonatally pinealectomized (PIX) were reared in short (light:dark schedule L:D 10:14) and long (L:D 24:0) photoperiods. It is pertinent to mention that since our earlier studies did not reveal any difference in tumor incidence between sham PIX animals and intact controls, sham surgery was not performed. The animals were housed 4–5 per cage in air-conditioned rooms with free access to food and drinking water. Cages were rotated from time to time for uniform light exposure.

Melatonin preparation and administration

Melatonin (Sigma Chemical Company, Batch No. 88F-7701) was dissolved in ethanol:drinking water 1:500 ml and the solution was stored in amber-colored bottles in the dark at 4°C. It was freshly prepared every other day and was orally administered to the experimental animals, round the clock, at a dose of 200 μg/rat/day in dark bottles. Control animals received only the vehicle (ethanol: drinking water 1:500 ml). For the initiation phase studies, melatonin was administered for a week prior to and for a week following carcinogen treatment, whereas in the case of promotion phase studies, it was begun a week following carcinogen treatment and was continued till the termination of the experiment.

Carcinogen administration and assessment of mammary tumorigenesis

DMBA (10 mg/ml sesame oil/rat) was administered once on day 55 of age to all animals by gastric

intubation. Beginning 1 month after carcinogen treatment, all rats were weighed monthly and palpated weekly for the presence of mammary tumors. The latency period of tumor appearance and tumor multiplicity (number of tumors per tumor-bearing animal) were recorded. The total observation period following DMBA administration was 27 weeks in all the groups.

At the end of the observation period, all animals were sacrificed under ether anesthesia. Mammary tumors were excised, fixed in Bouin's fluid and 5–6 μm paraffin sections of the tumors were stained with hematoxylin and eosin for histological examination and classification according to the criteria outlined by Young *et al.*[6]

Mammary gland morphology

Animals, both vehicle as well as melatonin-treated for 4 months in the promotion phase, were sacrificed and the whole-mount preparations of the mammary glands were used to study mammary gland morphology. For this, the mammary glands attached to the skin pelt were fixed in 10% neutral formalin for 24 h. 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.

Statistical analyses

Tumor incidence or the percentage of tumor-bearing animals among various groups was compared by the χ^2 test. All other statistical analyses were performed using the Student's t-test.

Results

Initiation phase studies

When melatonin was administered to rats in the initiation phase (Figures 1 and 2), there was 62% suppression in mammary tumor incidence in intact animals reared in short photoperiod (L:D 10:14 intact) and 57% suppression in intact animals reared in long photoperiod (L:D 24:0 intact) as compared to their corresponding controls ($p < 0.05$). Exogenous melatonin did not seem to have any significant effect on tumor incidence in the pinealectomized (PIX) animals reared in either photoperiod.

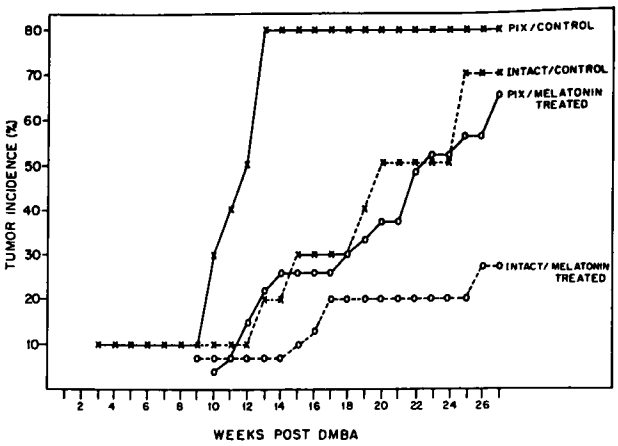

Figure 1. Initiation phase: effect of melatonin on mammary tumor incidence in rats exposed to L:D 10:14.

Intergroup comparison revealed that exogenous melatonin administration caused a significant reduction in mammary tumor incidence in L:D 10:14 intact animals when compared to L:D 10:14 PIX animals (60% reduction, $p < 0.01$) and when compared to L:D 24:0 PIX animals (59% reduction, $p < 0.01$).

The tumor multiplicity ranged from 1.3 to 1.5 tumors per tumor-bearing rat (Table 1) with no significant difference between any two groups of animals. The time taken to achieve median mammary tumor incidence (50% tumor incidence) was prolonged in all the melatonin-treated groups as compared to their corresponding controls (Table 1).

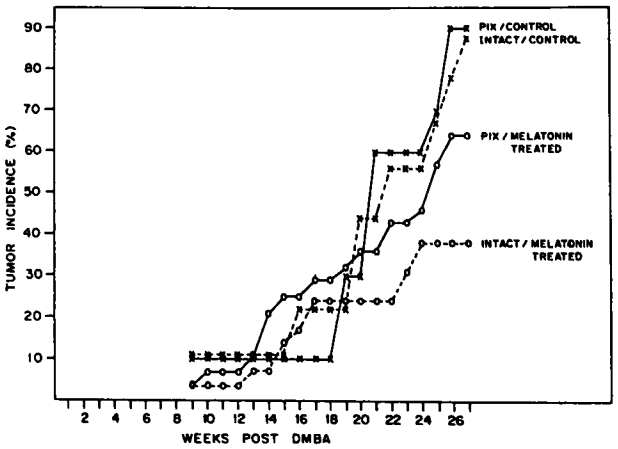

Figure 2. Initiation phase: effect of melatonin on mammary tumor incidence in rats exposed to L:D 24:0.

Table 1. Effect of melatonin administration during initiation on mammary tumor incidence in female rats treated with DMBA

Light:dark schedule L:D	Treatment		Mammary tumor incidence (%)	Tumor multiplicity, mean ± SEM	Time (week)s to achieve median tumor incidence
10:14	Intact/control	(20)[a]	70	1.4 ± 0.2	20
	Intact/melatonin	(30)	26.7[b]	1.9 ± 0.4	30
	PIX/control	(20)	80	1.4 ± 0.2	12
	PIX/melatonin	(26)	65.4	1.5 ± 0.2	23
24:0	Intact/control	(20)	87.5	1.4 ± 0.3	22
	Intact/melatonin	(29)	37.9[b]	1.3 ± 0.1	30
	PIX/control	(20)	90	1.4 ± 0.2	21
	PIX/melatonin	(28)	64.3	1.5 ± 0.2	25

[a] Number of rats.
[b] Vs control $p < 0.05$.

Promotion phase studies

As evident from Figures 3 and 4, when melatonin was administered in the promotion phase, there was significant suppression in mammary tumor incidence in all groups of animals as compared to their corresponding controls, irrespective of the presence or absence of the pineal and the photoperiod in which they were reared. The reduction was 77% in L:D 10:14 intact group ($p < 0.005$), 55% in L:D 10:14 PIX group ($p < 0.05$), 72.5% in L:D 24:0 intact group ($p < 0.05$) and 65.8% in L:D 24:0 PIX group ($p < 0.05$).

Tumor multiplicity ranged from 1 to 1.7 with a significant reduction in L:D 24:0 PIX animals treated with melatonin as compared to melatonin-treated L:D 24:0 intact animals ($p < 0.01$; Table 2). The time taken to achieve median tumor incidence was prolonged in all the melatonin-treated groups as compared to their corresponding controls (Table 2).

Mammary gland morphology

Prolonged melatonin administration brought about a distinct alteration in the architecture or morphology of the mammary glands as evident from the whole-mount preparations. Control mammary glands of intact groups of animals exposed to either photoperiod showed extensive ductal branching with terminal end buds (TEBs) and alveolar buds (ABs; Figure 5A, C, E, G), whereas melatonin-treated mammary glands show-

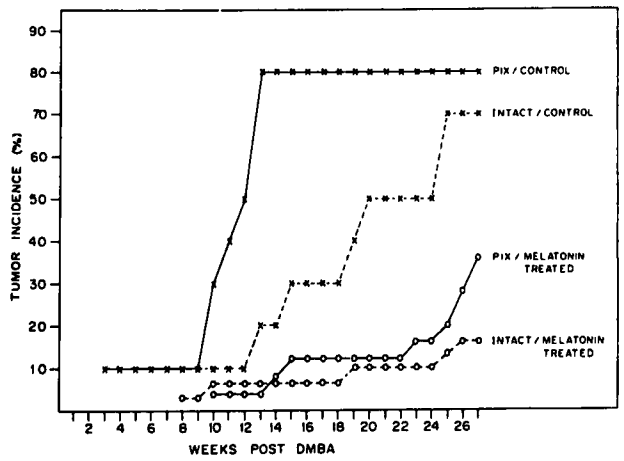

Figure 3. Promotion phase: effect of melatonin on mammary tumor incidence in rats exposed to L:D 10:14.

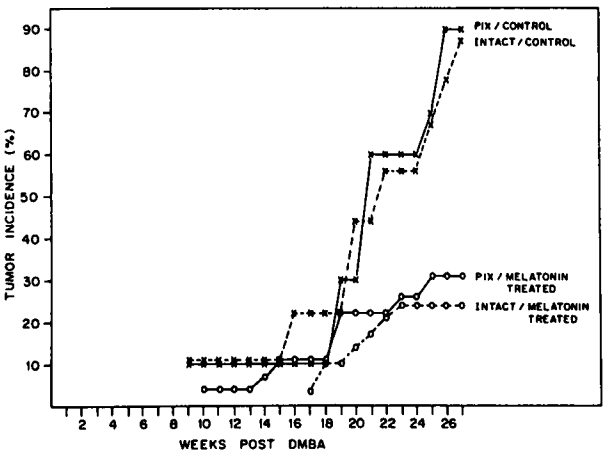

Figure 4. Promotion phase: effect of melatonin on mammary tumor incidence in rats exposed to L:D 24:0.

Table 2. Effect of melatonin administration during promotion on mammary tumor incidence in female rats treated with DMBA

Light:dark schedule L:D	Treatment		Mammary tumor incidence (%)	Tumor multiplicity, mean ± SEM	Time (week)s to achieve median tumor incidence
10:14	Intact/control	(20)[a]	70	1.4 ± 0.2	20
	Intact/melatonin	(31)	16.1[b]	1.6 ± 0.4	38
	PIX/control	(20)	80	1.4 ± 0.2	12
	PIX/melatonin	(25)	36[c]	1.2 ± 0.2	30
24:0	Intact/control	(20)	87.5	1.4 ± 0.3	22
	Intact/melatonin	(29)	24.1[b]	1.7 ± 0.2	33
	PIX/control	(20)	90	1.4 ± 0.2	21
	PIX/melatonin	(26)	30.8[b]	1.0 ± 0.0	29

[a] Number of rats.
[b] Vs control $p < 0.005$.
[c] Vs control $p < 0.05$.

ed sparse branching with fewer mammary gland structures (Figure 5 B, D, F, H). Since a similar picture was seen in the PIX groups of animals reared in either photoperiod, the data have not been included.

In both initiation and promotion phase studies, melatonin had no deleterious effect on the body weights of animals. More than 98% of the mammary tumors on histological examination were found to be adenocarcinomas, the remaining being benign fibroadenomas.

Discussion

This, to our knowledge, is the first report giving a comprehensive analysis of the influence of melatonin on the initiation and promotion phases of DMBA-induced mammary tumorigenesis in rats reared in varying photoperiods.

Even though a few earlier studies have examined the effect of melatonin on rat mammary tumorigenesis,[1-5] the exact phase of the tumorigenic process in which melatonin exerts its oncosuppressive effect has not been worked out. Moreover, these studies employed varying experimental protocols wherein the photoperiod to which the animals were exposed, the doses of DMBA and melatonin used, the route of administration of melatonin and time period over which it was administered were different, making comparison and interpretation of these data quite difficult.

Aubert *et al.*[1] studied the effect of twice-weekly, late-afternoon injections of melatonin (200 μg), beginning on the day of DMBA treatment (25 mg i.g.) on mammary tumor incidence in Sprague Dawley rats. The incidence of mammary tumors was decreased by 25 to 30% in both intact and PIX rats maintained on a L:D 12:12 lighting schedule (long photoperiod). Under conditions of constant lighting (L:D 24:0), melatonin had no effect on tumor incidence in either group. From these results, it appeared that the presence of the pineal was not required for the antitumorigenic effect of melatonin when treatment was initiated at the time of tumor induction. On the other hand, Tamarkin *et al.*[2] using Sprague Dawley rats, demonstrated that daily afternoon injections of melatonin (500 μg) starting on the day of DMBA administration (15 mg i.g.) and continued for 90 days thereafter, resulted in a 70–75% lower incidence of mammary tumors when compared with controls at the end of 20 weeks post-DMBA. However, melatonin had no significant effect in lowering mammary tumor incidence in PIX rats, suggesting that the presence of the pineal gland was required for melatonin to exert a maximal oncostatic effect.

We therefore felt the need to perform a detailed analysis of the influence of melatonin on the tumorigenic process, thus confirming and extending our consistently observed previous findings.[5]

In the present study, we employed a lower dose of DMBA (10 mg i.g., day 55 of age) to induce a lower percentage of tumors and a higher dose of melatonin (200 μg/rat/day, orally) as compared to our previous study.[5] We observed that when melatonin was orally administered to animals in the initiation phase (Figures 1 and 2), irrespective of

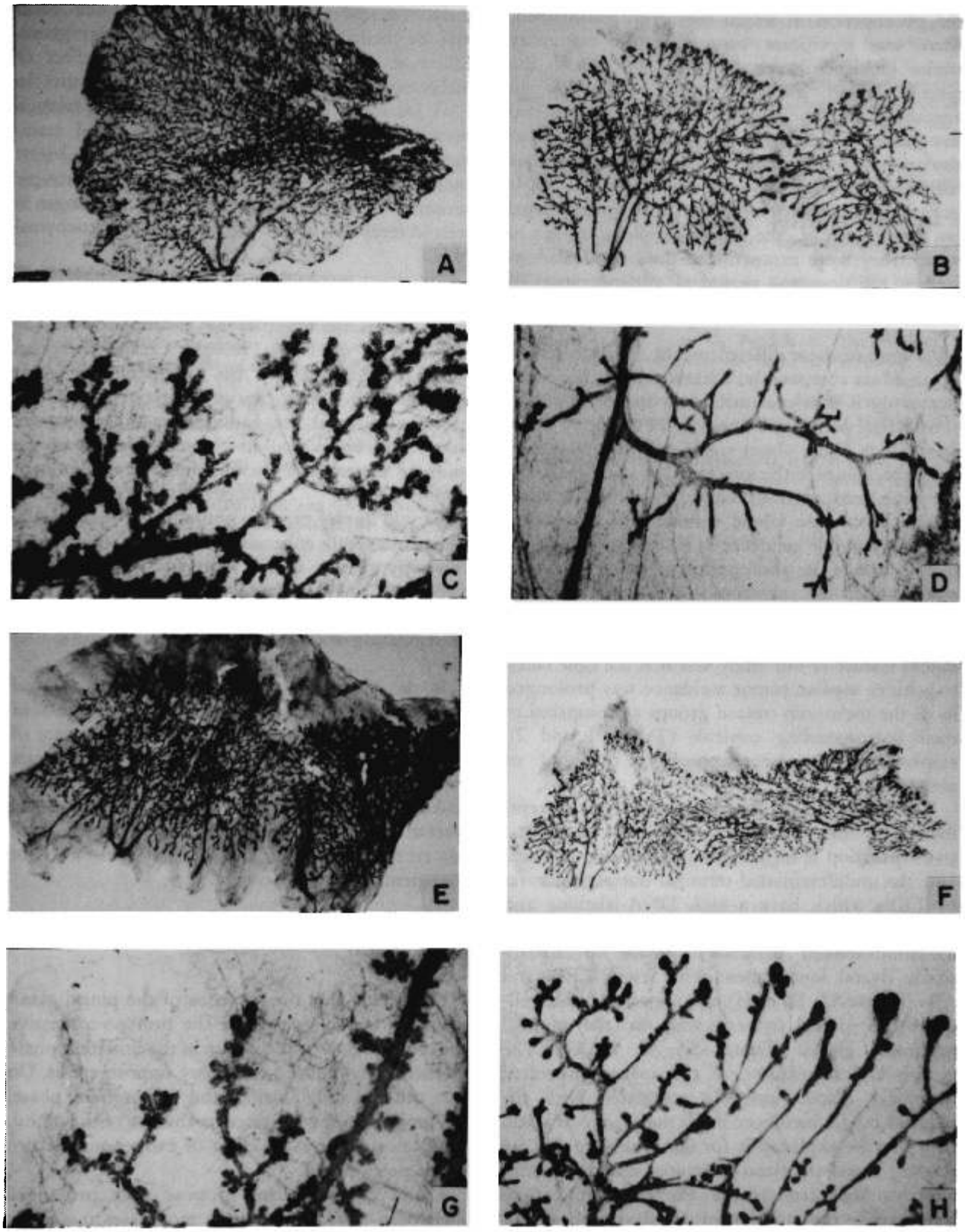

Figure 5. Photomicrographs of control and melatonin–treated mammary gland whole mounts stained with Kernechtrot. L:D 10:14 intact:control (A, C); melatonin-treated (B, D). L:D 24:0 intact:control (E, G); melatonin-treated (F, H). Magnification:(A, B, E, F) ×3. (C, D, G, H) ×19.

the photoperiod in which they were maintained, there was significant suppression in mammary tumor incidence in intact groups but not in the pineal ablated groups. These results are in agreement with the findings of Tamarkin *et al.* and in contrast to those of Aubert *et al.*, indicating that the presence of the pineal is essential for exogenous melatonin to bring about its oncostatic effect. It is important to stress that the strain of animals used by Tamarkin *et al.*, the photoperiodic schedules to which they were exposed, the dose of DMBA as well as the dose and period of administration of melatonin were different from those employed in our study.

In spite of these differences, the ultimate results obtained are comparable. What is noteworthy about our study is that oral melatonin treatment for as short a time period as 2 weeks in the initiation phase was able to bring about significant suppression in tumor incidence in the intact groups of animals. On the other hand, prolonged melatonin administration in the promotion phase significantly suppressed mammary tumor incidence in all groups of animals irrespective of the photoperiod in which they were reared and the presence or absence of the endogenous melatonin rhythm (Figures 3 and 4). These results corroborate our previous findings.[5] A salient feature of our study was that the time taken to achieve median tumor incidence was prolonged in all the melatonin-treated groups as compared to their corresponding controls (Tables 1 and 2), emphasizing the oncosuppressive potential of melatonin.

The characteristic that determines the susceptibility of the mammary gland to neoplastic transformation is its actively proliferating epithelium: the undifferentiated terminal ductal structures or TEBs which have a high DNA-labelling and mitotic index.[7] Whole-mount preparations of melatonin-treated mammary glands revealed a sparse ductal arrangement with fewer TEBs and ABs (Figure 5B, D, F, H) as compared to the well-developed ductal system seen in the control mammary glands (Figure 5A, C, E, G). The morphological pictures of the melatonin-treated mammary glands uniquely correlated with the reduced tumor incidence in all the groups studied. One possible explanation for this would be that, by altering mammary gland structures, chronic melatonin treatment renders the mammary epithelium refractory to the carcinogenic insult. It is pertinent to mention here that Sanchez-Barcelo *et al.*[8] observed a reduction in mammary gland development in normal BALB/c mice by high doses of melatonin and suggested that some of this effect may be mediated directly at the mammary gland. Likewise, in our recent study of the effect of melatonin on spontaneous mammary tumors in C3H/Jax mice, we observed a positive correlation between spontaneous tumor incidence and mammary gland development.[9] Studies on human breast cancer cells[10] suggest that the antigonadotropic action of melatonin may be mediated by changes in steroid receptors within the mammary parenchymal cells.

The exact mechanism through which the pineal gland in general and melatonin in particular exert their oncostatic effect is as yet unknown. The presence of melatonin receptors[11] and increased serum melatonin levels[12] has been demonstrated in patients with breast cancer. Moreover, both the pineal gland and the endogenous opioid system have been implicated in the modulation of the immune system and thereby in the regulation of tumor growth.[13] Administration of exogenous melatonin during primary immunization specifically and permanently enhanced the memory of immune reactivity against specific antigen.[14] Further, Kerenyi *et al.*[14] have recently hypothesized that light has a major role in tumorigenesis through its effect in regulating melatonin production by the pineal gland.

It is pertinent to emphasize that several preliminary findings indicate the effectiveness of melatonin therapy in the treatment of a variety of human malignancies including long-standing breast cancer as well as lymphomas, lymphoblastic leukemias and osteogenic sarcomas.[14] These data warrant the need for more intensive clinical research to recruit melatonin as a preventive and/or therapeutic agent for cancer.

Conclusion

We conclude that the presence of the pineal gland seems to be obligatory for the tumor-suppressive effect of exogenous melatonin in the initiation phase of chemically induced mammary tumorigenesis. On the other hand, during the promotion phase, irrespective of the presence or absence of the pineal, the tumor-suppressive effect of exogenous melatonin is pronounced.

Could we therefore propose that prolonged melatonin treatment perhaps modulates the action of various growth factors from both paracrine and endocrine organs, thus preventing the occult clones in carcinogen-administered animals from expressing

their full potential as viable tumors for a considerably long period of time?

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