Influence of Melatonin on Mammary Gland Growth: *In Vivo* and *In Vitro* Studies[1] (43063)

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Abstract. **Our objective was to determine whether melatonin influenced mammary growth in response to mammogenic hormones. Prepubertal female BALB/c mice were injected for 9 days with 1 μg of 17β-estradiol and 1 mg of progesterone or 17β-estradiol/progesterone plus 50, 100, or 200 μg of melatonin. Area of the parenchyma and total DNA content of the second thoracic gland were similar between controls and melatonin-injected mice. However, μg of DNA/100 mg of mammary tissue were lower in animals treated with 17β-estradiol/progesterone plus 200 μg of melatonin than in controls. Triglyceride content of mammary glands from animals treated with 100 or 200 μg of melatonin/day increased relative to controls. In an *in vitro* experiment, thoracic mammary glands of 21-day-old mice were cultured for 6 days in a mammogenic milieu of hormones (17β-estradiol/progesterone, aldosterone, bovine prolactin, growth hormone, and insulin) with 0 (control), 10^{-6}, 10^{-9}, or 10^{-12} *M* melatonin. Relative to controls, 10^{-12} *M* melatonin increased and 10^{-6} *M* melatonin decreased mammary DNA and uptake of [*methyl*-^{3}H]thymidine. We conclude that high doses of melatonin reduce mammary development in normal mice and that some of this effect may be mediated directly at the mammary tissue.**
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Pinealectomy of rats at 21 days of age does not affect normal mammary development through 100 days of age or estradiol-induced mammary gland growth (1). However, normal rats exposed to constant light from birth have increased mammary development, and exogenous melatonin reverses this effect (2). Furthermore, melatonin is inhibitory and pinealectomy is stimulatory to growth of hormone-dependent mammary tumors (3–7).

We hypothesized that the pineal gland affects mammary gland growth either through direct actions of pineal secretions (primarily melatonin) on mammary tissue, or indirectly through pineal-dependent changes in the circulating concentrations of some of the pituitary and gonadal hormones that control mammogenesis (8, 9). Since gonadal and pituitary hormones that affect mammary tumor growth (10) are similar to those that cause development of normal tissue (11), we speculated that melatonin may negatively modulate normal mammary gland growth. Our objective was to study the influence of melatonin on normal mammary growth in response to mammogenic hormones and to determine whether direct actions of melatonin on mammary tissue may explain this effect.

Materials and Methods

Chemicals. Unless otherwise indicated, all reagents and hormones were purchased from Sigma Chemical Co. (St. Louis, MO).

Animals. In all experiments, female BALB/c mice (Charles River Laboratories Inc., Wilmington, MA) 21 days of age were housed in our vivarium at 25 ± 0.5°C and exposed daily to 12-hr light/12-hr dark. Animals had free access to food (Wayne Rodent Blox 8604-00; Continental Grain Co., Chicago, IL) and tap water. BALB/c mice have a genetic deficiency of enzymes essential for melatonin synthesis (12, 13). Thus, effects of exogenous melatonin can be distinguished without complications associated with endogenous melatonin.

***In Vivo* Studies.** Sixty 23- to 25-day-old mice were divided into four groups and received daily: 1 μg of 17β-estradiol plus 1 mg of progesterone (17β-estradiol/progesterone; control), 17β-estradiol/progesterone plus 50 μg of melatonin (17β-estradiol/progesterone + Mel-50), 17β-estradiol/progesterone plus 100 μg of melatonin

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(17β-estradiol/progesterone + Mel-100), or 17β-estradiol/progesterone plus 200 μg of melatonin (17β-estradiol/progesterone + Mel-200). Steroids were prepared by mixing 1 mg of 17β-estradiol and 1 g of progesterone with 100 mg of gum arabic dissolved in 0.9% NaCl (100 ml). Melatonin was dissolved in 95% ethanol and then diluted (1/9) in 0.9% NaCl. Steroids and melatonin were administered in two separate injections of 0.1 ml, given at 1600 hr (4 hr before onset of darkness) for 9 consecutive days. The day after the last injection, animals were sacrificed by cervical dislocation and the number four (inguinal) mammary glands were excised after removal of the intramammary lymph node. From each pair of glands, one was fixed in glacial acetic acid:ethanol (1:3, v/v), stained with alum-carmine, and examined in a whole-mount preparation. The contralateral mammary gland was weighed fresh and then frozen at −20°C until processed for determination of DNA and triglyceride content.

***In Vitro* Studies.** The technique of organ culture used was that of Banerjee *et al.* (14). Sixty 23- to 25-day-old female mice were primed with daily injections (9 days) of estradiol (1 μg) plus progesterone (1 mg) to stimulate lobulo-alveolar development of the mammary gland (15). Twenty-four hours after the last injection, mice were sacrificed by cervical dislocation, dipped in 70% ethanol; and pinned to a corkboard. The second pair of mammary glands (thoracic) were excised in a laminar air flow hood and placed on sterile Dacron rafts in 35-mm culture dishes (Corning Glass Works, Corning, NY) with 2 ml of Waymouth's MB 752/1 medium (GIBCO, Grand Island, NY) containing mammotrophic hormones and various doses of melatonin or ethanol. Penicillin (3.5 μg/ml) and L-glutamine (35 μg/ml) were also added to all culture dishes.

Mammotrophic hormones added to the basal medium were: 17β-estradiol (0.001 μg/ml), progesterone (1 μg/ml), aldosterone (1 μg/ml), bovine prolactin (5 μg/ml NIH-b5), bovine growth hormone (5 μg/ml NIH-b18), and bovine insulin (5 μg/ml). All mammotrophic hormones were prepared as concentrated stock solutions in either 95% ethanol (steroids), 0.001 *M* NaOH plus basal medium (1:4) (prolactin and growth hormone), or 0.005 *M* HCl plus basal medium (1:4) (insulin). The hormones were then filtered through sterile 0.22-μm filters (Millipore, Bedford, MA), stored at 5°C, and added to basal medium just prior to use. Medium containing the mammotrophic hormones was defined as growth-promoting medium.

In each mouse, one of the thoracic mammary glands was incubated in growth-promoting medium, to which melatonin in ethanol was added. The final concentration of melatonin in medium was 10^{-6}, 10^{-9}, or 10^{-12} *M*. The contralateral control gland was incubated in growth-promoting medium plus 0.02 μl of 95% ethanol/ml (equals the alcohol concentration in melatonin-containing media). Each mouse served as its own control. Results in the melatonin-treated glands were expressed as a percentage of control glands.

Tissue was incubated for 6 days in a humidified chamber at 37°C in an atmosphere of O_2 (95%) and CO_2 (5%). Medium was changed at Days 1, 3, and 5 of culture.

Four hours before the end of incubation [*methyl*-^{3}H]thymidine (80 Ci/mmol; New England Nuclear, Boston, MA) was added to the medium (1 μCi/ml). After incubation with [*methyl*-^{3}H]thymidine, medium was aspirated and tissues were rinsed twice with cold distilled water, weighed, and stored at −20°C until DNA content, triglyceride concentration, and [*methyl*-^{3}H] thymidine uptake were quantified.

Evaluation of Mammary Gland Growth. Mammary gland growth was evaluated from weight and area measurements in *in vivo* studies. Mammary area was measured with a planimeter traced around the periphery of the ducts.

DNA was measured as an index of cell number (16). Uptake of [*methyl*-^{3}H]thymidine (cpm/gland) *in vitro* was used as a mitotic index (17). Triglyceride content was used as a measure of the fat content of each mammary gland.

DNA, triglyceride, and thymidine uptake were measured by procedures adapted from various authors (16, 18–20). Each mammary gland was diluted with 20 volumes of ice-cold 2% perchloric acid (PCA) and then sonicated for 30 sec (Heat Systems/Ultrasonics, Inc., Plainview, NY). The homogenate was centrifuged for 5 min at 1500*g*. Pellets obtained after elimination of acid-soluble compounds were successively extracted for 45 min each with ethanol-2% sodium acetate, ethanol:chloroform (1:1), and ether. Between each extraction, contents were centrifuged for 5 min at 1500*g*. Extracts were subsequently dried under vacuum. After drying, activated alumina (800 mg) and isopropanol (5 ml) were added to each tube, contents were mixed, and then centrifuged for 5 min at 1500*g*. Duplicate 2-ml aliquots of the supernatant were assayed for triglyceride content (20). Triolein (glycerol trioleate) dissolved in isopropanol was used as the standard. Sensitivity of the method was 5 μg/ml.

Pellets obtained from each mammary gland after elimination of acid-soluble compounds and fat were incubated for 1 hr at 37°C in 0.5 ml of 1 *N* KOH. The digest was acidified with 6 *N* HCl (0.3 ml) and 10% ice-cold PCA (2 ml) to precipitate DNA. After centrifuging for 5 min at 1500*g*, the pellet was washed with 5% PCA. The remaining residue was extracted for 15 min with 1 ml of 5% PCA at 70°C, centrifuged, and the supernatant was saved. This supernatant, combined with those obtained by washing the pellet twice with 1 ml of 5% PCA, was analyzed in a spectrophotometer

at 268 μm. Highly polymerized calf thymus DNA was used as standard.

Two 0.4-ml aliquots from each tube containing the extracted DNA were transferred to scintillation vials containing 5 ml of Safety Solve (Research Products International, Mount Prospect, IL) and radioactivity was measured in an Isocap 300 liquid scintillation counter (Searle Analytic, Inc., Elk Grove Village, IL) and results were expressed as cpm (total [*methyl*-^{3}H] thymidine uptake) and cpm/μg of DNA.

Statistical Analysis. For *in vivo* studies, one-way analysis of variance and Dunnett's *t* test for differences between each group and control were performed (21). For *in vitro* studies, analysis of variance and the Student-Newman-Keuls test were used to analyze the differences among means of the three experimental groups (21). Within each group, differences between glands incubated with and without melatonin were analyzed by Student's *t* test for paired samples (22).

Results

Melatonin Effects on 17β-Estradiol/Progesterone-Induced Mammary Growth *In Vivo*. Weights of mammary glands from animals treated 9 days with 17β-estradiol/progesterone plus Mel-100 were greater than those from animals receiving only 17β-estradiol/progesterone (Table I). However, when mammary weight was corrected for differences in body weight, no differences were found among the four treatments. Similarly, there was no difference in mammary area between control and melatonin-treated animals.

Numbers of mammary cells, measured as total DNA/gland, were not different between 17β-estradiol/progesterone controls and melatonin-injected mice. However, DNA expressed as μg/100 mg of mammary tissue was lower in glands from animals treated with 17β-estradiol/progesterone + Mel-200 than in controls ($P < 0.01$).

The total triglyceride content and triglyceride concentration per mammary gland significantly increased in mice receiving either 17β-estradiol/progesterone plus Mel-100 or 17β-estradiol/progesterone plus Mel-200, as compared with control animals (Table I).

Melatonin Effects on Mammary Gland Growth *In vitro*. Mammary DNA (expressed as either μg/gland or μg/100 mg of tissue) in glands cultured in 10^{-12} *M* melatonin was greater ($P < 0.05$) than in glands cultured in the absence of melatonin (Fig. 1). However, when the concentration of melatonin was increased to 10^{-6} *M*, mammary DNA decreased ($P < 0.05$) in relation to controls. A concentration of 10^{-9} *M* melatonin in medium did not change mammary DNA relative to glands cultured without melatonin. Both total DNA and DNA concentration in mammary tissue were lower at 10^{-9} and 10^{-6} *M* melatonin than at 10^{-12} *M* ($P < 0.02$ and $P < 0.005$, respectively).

Analogous to changes in mammary DNA, uptake of [^{3}H]thymidine per mammary gland increased at 10^{-12} *M* melatonin and decreased at 10^{-6} *M* melatonin as compared with uptake in the contralateral control glands ($P < 0.05$, Fig. 2). However, increasing the dose of melatonin to 10^{-9} and 10^{-6} *M* decreased the uptake of [^{3}H]thymidine per gland relative to uptake in glands incubated with 10^{-12} *M* melatonin ($P < 0.05$ and $P < 0.01$, respectively, Fig. 2). Uptake of [^{3}H]thymidine per 100 μg of DNA was similar in cultures of control and melatonin-treated glands.

Triglycerides (mg/gland or μg/100 mg of tissue) in melatonin-treated glands were not different from that in control glands, ranging from 93 to 105% of the value in controls.

Discussion

Previous reports suggest that melatonin reduces growth of hormone-dependent mammary tumors (3–

Table I. Wet Weight, Area, DNA, and Triglycerides of Inguinal Mammary Gland from Immature Mice after 9 Days of Treatment with 1 μg of Estradiol + 1 mg of Progesterone or 17β-Estradiol + Progesterone and 50, 100, or 200 μg of Mel[a]

Mammary characteristic	17β-estradiol/progesterone	17β-estradiol/progesterone + Mel-50	17β-estradiol/progesterone + Mel-100	17β-estradiol/progesterone + Mel-200
Weight (mg)	58.1 ± 2.4	59.1 ± 3.6	69.6 ± 3.8[b]	66.9 ± 2.6
Weight (mg/g body wt)	3.1 ± 0.1	3.4 ± 0.2	3.7 ± 0.2	3.6 ± 0.1
Area (mm^2)				
DNA (μg)	8.2 ± 0.3	9.9 ± 0.6	9.3 ± 0.7	8.3 ± 0.4
DNA (μg/100 mg tissue)	60.2 ± 1.9	60.8 ± 0.9	65.8 ± 3.2	55.9 ± 1.7
Triglyceride (mg)	108.6 ± 5.5	107.9 ± 7.1	97.9 ± 5.8	85.5 ± 3.3[c]
Triglyceride (mg/100 mg tissue)	40.0 ± 1.6	39.1 ± 2.8	51.4 ± 2.7[c]	48.7 ± 1.9[c]

[a] Values are mean ± SE.
[b] Different from 17β-estradiol/progesterone control ($P < 0.05$).
[c] Different from 17β-estradiol/progesterone control ($P < 0.01$). Number of animals = 10–15/group.

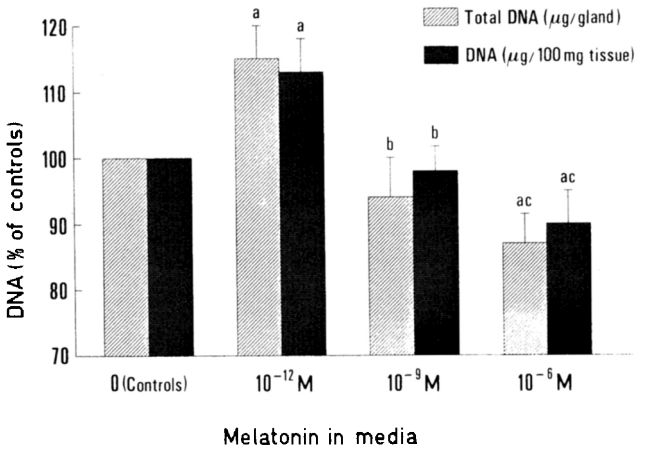

Figure 1. DNA in mammary glands incubated with and without melatonin. Differences between means: a, $P < 0.05$ versus controls; b, $P < 0.02$; and c, $P < 0.005$ versus glands incubated in 10^{-12} *M* melatonin. Number of pairs per group were 10–14.

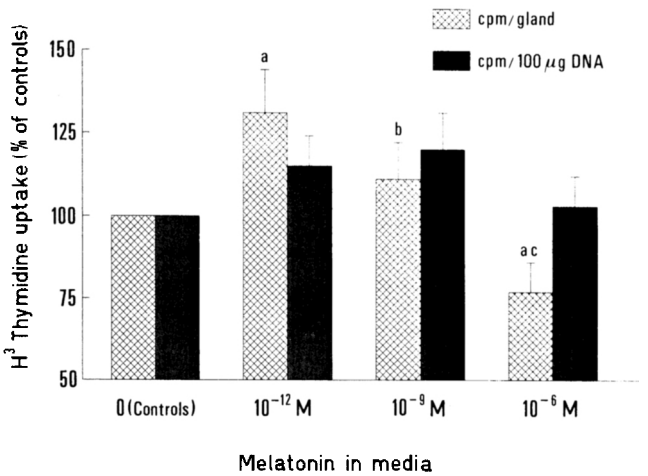

Figure 2. [*methyl*-H³]Thymidine uptake in mammary glands incubated with and without melatonin. Differences between means: a, $P < 0.05$ versus controls; b, $P < 0.05$; and c, $P < 0.01$ versus glands incubated in 10^{-12} *M* melatonin. Number of pairs per group were 10–14.

7). In the present study, melatonin *in vivo* (injections of 200 μg/mouse/day) as well as *in vitro* (incubation with 10^{-6} *M* melatonin) suppressed the growth of normal mammary tissue induced by exogenous mammogenic hormones. It should be noted that the 10^{-12} *M* dose of melatonin added to mammary tissue *in vitro* stimulated mammary cell numbers above that of controls. The mechanisms whereby 10^{-12} *M* melatonin was stimulatory to mammary development, but higher doses were inhibitory are unknown.

Our observation of increased fat content of the mammary tissue of mice in response to increasing doses of melatonin agrees with whole body fattening responses observed in hamsters (23) and cattle (24) given exogenous melatonin. Increased extraparenchymal fat in the mammary fat pad has been implicated as a potential inhibitor of mammary parenchymal growth in dairy cattle (25, 26). Therefore, in addition to reducing endogenous mammogenic hormones, we speculate that melatonin-induced increases in extraparenchymal fat of the mammary gland may also play a role in mediating the negative effects of melatonin on mammary parenchymal growth. In any case, changes in extraparenchymal fat pads should be investigated further since the stromal component of the mammary gland affects the mammary growth response to mammotrophic hormones *in vivo* (27) as well as *in vitro* (28).

Results of the *in vitro* study suggest that at least a part of the action of melatonin is mediated directly at the level of the mammary gland. However, results from the *in vitro* study do not support our speculation that increased lipid plays a role in reducing mammary growth since triglyceride content was unaffected by melatonin. Direct action of melatonin on gonads, accessory sexual organs, and human mammary adenocarcinomas have been demonstrated previously (5, 29, 30). In most cases melatonin counteracts the effects of steroids (30). The possibility that melatonin actions may be mediated by changes in mammary gland steroid receptors within the mammary parenchymal cells is supported by results published for human breast cancer cells (31). We conclude that high doses of melatonin suppress mammary development in normal BALB/c mice and that some of this effect may be mediated directly at the mammary gland.

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