Effects of the Pineal Hormone Melatonin on the Proliferation and Morphological Characteristics of Human Breast Cancer Cells (MCF-7) in Culture

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ABSTRACT

Since melatonin, the major hormone of the pineal gland, has been shown to inhibit the growth of mammary tumors in animal models of human breast cancer, we examined the hypothesis that this indoleamine has the potential to inhibit breast cancer growth by directly inhibiting cell proliferation as exemplified by the growth of the estrogen-responsive human breast cancer cell line MCF-7 in culture. Concentrations of melatonin (10⁻⁴ M; 10⁻¹⁰ M), corresponding to the physiological levels present in human blood during the evening hours, significantly inhibited (P < 0.001) cell proliferation by as much as 60% to 78% as measured by either DNA content or hemocytometer cell counts. Melatonin's inhibitory effect was reversible since the logarithmic growth of MCF-7 cells was restored after melatonin-containing medium was replaced with fresh medium lacking melatonin. Not only was the inhibitory effect of melatonin absent at either pharmacological (10⁻⁷ M; 10⁻³ M) or subphysiological (10⁻¹⁰ M; 10⁻⁷ M) concentrations, but melatonin also failed to inhibit the proliferation of either human foreskin fibroblasts or the estrogen receptor-positive human endometrial cancer cell line RL95-2. Both transmission and scanning electron microscopy revealed several morphological changes that correlated with melatonin's inhibition of cell growth. After just 4 days of exposure to melatonin, MCF-7 cells exhibited reduced numbers of surface microvilli, nuclear swelling, cytoplasmic and ribosomal shedding, disruption of mitochondrial cristae, vesiculation of the smooth endoplasmic reticulum, and an increase in the numbers of autophagic vacuoles. These results support the hypothesis that melatonin, at physiological concentrations, exerts a direct but reversible, antiproliferative effect on MCF-7 cell growth in culture. This antiproliferative effect is associated with striking changes in the ultrastructural features of these cells suggestive of a sublethal but reversible cellular injury.

INTRODUCTION

A number of years ago it was hypothesized that impaired pineal gland function might result in an increased incidence of breast cancer in women (1). Some support for a pineal-breast cancer nexus in humans is derived from clinical studies demonstrating that the amplitude of the nighttime peak of melatonin in either blood or urine is diminished in women with estrogen receptor-positive breast cancer versus those with estrogen receptor-negative disease or healthy, matched controls (2, 3). These data are intriguing in light of the fact that approximately two-thirds of primary breast cancers are estrogen receptor positive, and 50% to 70% of these respond to endocrine therapy (4-6).

Several studies have suggested that the pineal gland, presumably through its major hormone melatonin, may influence the development and growth of mammary tumors (7, 8) in addition to hormone-responsive reproductive tissues (9, 10). For example, melatonin administration has been shown to inhibit, while pinealectomy generally stimulates, mammary carcinogenesis (11-14). On the basis of these results, it has been proposed that the pineal gland, via the secretion of melatonin, inhibits mammary tumorigenesis by modulating the neuroendocrine regulation of PRL and 17β-estradiol secretion (7). However, as yet there is little evidence to suggest that this is the only or even the primary mechanism by which melatonin inhibits cancer growth (7, 12, 14).

The alternative hypothesis, that melatonin retards hormone-responsive breast cancer growth by directly inhibiting cell proliferation, has received no systematic investigative effort. Therefore, we decided to test this postulate by studying the effects of melatonin on the growth characteristics of these cells in vitro over a wide range of hormone concentrations including those present in human blood during the night (3, 15). Additionally, we tested the effects of melatonin on the surface morphology as well as the ultrastructural features of MCF-7 cells in culture. We chose this highly studied cell line inasmuch as these cells are estrogen responsive and possess receptors for a variety of hormones in addition to estrogen (16). Further impetus for our study was provided by a report that melatonin increases the number of both nuclear and cytoplasmic estrogen receptors in MCF-7 cells (17).

MATERIALS AND METHODS

Materials. Melatonin (N-acetyl-5-methoxytryptamine) was purchased from Sigma Chemical Co. (St. Louis, MO). DMEM with or without phenol red was obtained from Irvine Scientific (Santa Ana, CA), while FCS was purchased from Flow Laboratories, Inc. (McCLean, VA). The concentration of endogenous melatonin in FCS prior to dilution with DMEM was 20 pg/ml as measured by a sensitive radioimmunoassay. MCF-7 human breast cancer cells as well as human foreskin fibroblasts were obtained from American Tissue Type Culture Collection (Rockville, MD). The human endometrial carcinoma cell line (RL95-2) was received as a gift from Dr. John Davis, Department of Pathology, University of Arizona, College of Medicine.

Cell Culture. MCF-7 and RL95-2 cells were routinely cultured in 60-mm tissue culture plates containing DMEM with 10% FCS, bovine insulin (0.6 µg/ml), and penicillin/streptomycin (25 IU/25 µg per ml) at 37°C in a humidified atmosphere with 5% CO₂. Human foreskin fibroblasts were also grown in DMEM with 10% FCS according to previously described methods (18, 19).

In order to determine the effects of melatonin on cell proliferation, cells from stock plates were suspended by treatment with 0.25% trypsin, buffered with 0.2% EDTA (pH 7.3), and counted using a hemocytometer. Cells were adjusted to a density of 3.0 x 10⁵ cells/plate to the required volume of plating medium supplemented with 10% FCS. After 4 h, the plating medium was replaced with fresh medium containing the appropriate concentrations of melatonin (10⁻¹² M, 10⁻¹⁰ M, 10⁻⁸ M, 10⁻⁶ M) dissolved in ethanol (final concentration per plate, 0.001%). On various days following the initiation of either a 7- or 10-day growth experiment, cells were harvested by treatment with trypsin-EDTA, passed several times through a 21-gauge sterile needle, and counted on a hemocytometer or pelleted. Cell pellets were assayed for their DNA content using the diphenylamine DNA assay of Burton (20).

In an experiment to test the reversibility of melatonin's effects, cells...
were plated out as described above and treated with medium containing 10^{-9} M melatonin or vehicle. On either Day 2 or 7 of the growth period, in some groups of melatonin-treated cells, the cells were washed once with DMEM and then refed melatonin-deficient medium. Similarly, some groups of vehicle-treated cells were washed once and then refed with fresh medium on Day 7 of growth.

Transmission Electron Microscopy. MCF-7 cells were cultured in T-75 flasks containing DMEM with 10% FCS for TEM. When cells were light confluent, they were rinsed and refed with fresh medium containing, in addition to serum, either vehicle or melatonin (10^{-9} M) and incubated for a total of 4 days. After the incubation period, cells were harvested with a Ca^{2+}, Mg^{2+}-free phosphate-buffered saline/EDTA solution and pelleted by centrifugation (1000 x g) for 5 min. The cell pellets were then fixed with glutaraldehyde (3%) for 1.5 h and postfixed in osmium tetroxide (2%) for 1 h in phosphate buffer (pH 7.3) followed by embedding in Epox. Sections (70 nm) were stained with 5% uranyl acetate, counterstained with lead citrate, and viewed with a Phillips 300 electron microscope (60 kV).

Scanning Electron Microscopy. MCF-7 cells were cultured in plastic Petri dishes with coverslips and treated as outlined above. At the end of 4 days of culture, the cells grown on coverslips were fixed with glutaraldehyde (3%) for 1.5 h and postfixed with osmium tetroxide (2%) for 1 h in phosphate buffer (pH 7.3). The coverslips were then rinsed in phosphate buffer and dehydrated through a graded series of alcohols and isomyl acetate prior to critical point drying. The samples were mounted, shadowed with gold, examined, and then photographed on an ETEC autoscan scanning electron microscope at 20 kV.

Statistical Analysis. All cell growth data were analyzed by a two-way analysis of variance followed by the Student-Newman-Keuls multiple range test. Differences among group means were considered significant at P < 0.05.

RESULTS

Effect of Melatonin on Cell Proliferation. As shown in Fig. 1, control cultures of MCF-7 cells incubated with the vehicle exhibited logarithmic growth and reached confluency by Day 7 of culture. Cell growth in an untreated control group (data not shown) was identical to that in the ethanol-vehicle control group. Hemocytometer counts revealed that cell growth in the presence of a pharmacological concentration of melatonin (10^{-9} M) corresponding to peak nighttime serum levels observed in humans (3, 15) was markedly inhibited, such that the cell number was 75% less than in the vehicle-treated controls. Similarly, another concentration of melatonin in the physiological range (10^{-11} M) significantly slowed the rate of proliferation during the first 72 h of the experiment. By the fifth day of the experiment, cell growth plateaued with cell density remaining markedly reduced such that, by Day 7 of growth, cell number in the melatonin-containing plates was 62% less than in the controls (Fig. 1). Seedling efficiency in the untreated controls, vehicle-treated controls, and melatonin (10^{-9} M)-treated culture plates was 78.1 ± 1.7%, 76.1 ± 2.3%, and 75.5 ± 1.8%, respectively. Neither the presence nor absence of phenol red in the incubation medium had any influence on melatonin’s ability to inhibit cell proliferation (data not shown).

Melatonin’s inhibitory effect on the growth of MCF-7 cells was further substantiated by measuring the total DNA content of each culture plate. Using this criterion of measurement, 10^{-9} M melatonin proved to be effective in significantly suppressing the increase in DNA content observed in the control plates over the 7-day growth period, such that by Day 7, DNA content was nearly 60% less than the controls (Table 1).

In another set of experiments, we tested the effects of melatonin on MCF-7 cell proliferation over a wide range of concentrations including the pharmacological (10^{-5} M and 10^{-7} M), physiological (10^{-9} M and 10^{-11} M), and subphysiological (10^{-13} M and 10^{-15} M) ranges. Fig. 2 reveals that only at concentrations corresponding to the physiological range was melatonin capable of suppressing cell proliferation.

The antiproliferative effect of 10^{-9} M melatonin persisted for up to 7 days of continuous culture (Fig. 3). However, when melatonin-treated MCF-7 cells were washed and refed with fresh, melatonin-deficient medium, cell proliferation increased significantly, regardless of the previous duration (2 or 7 days) of melatonin treatment (Fig. 3).

Melatonin had no effect on the growth characteristics of either nontransformed human foreskin fibroblasts (Table 2) or on the proliferation of either mouse 3T3 or 3T6 or Rat-1 fibroblasts (data not shown). Similarly, physiological levels of melatonin do not inhibit the proliferation of the estrogen-responsive, human endometrial adenocarcinoma cell line RL95-
2 (Fig. 4); however, micromolar concentrations of this indoleamine resulted in significantly \( (P < 0.01) \) lower cell counts on both Days 5 and 7 of growth.

Effects of Melatonin on Cellular Surface Morphology and Ultrastructure. The surfaces of control MCF-7 cells (grown in DMEM with 10\% FCS) as viewed with SEM were almost uniformly covered with a dense network of microvilli (Fig. 5A), whereas melatonin-treated cells typically exhibited a prominent qualitative decrease in the density and length of surface microvilli (Fig. 5B). Additionally, the density of cells observed on the coverslips after 4 days of incubation in melatonin-containing medium was markedly lower than in the controls.

As revealed by TEM, MCF-7 cells from control plates were most often ovoid with a centrally placed nucleus containing one or more prominent nucleoli (Fig. 6A). In general, the cells appeared to be metabolically active as evidenced by a number of large clear mitochondria with regular thin cristae. The cytoplasm also possessed considerable quantities of both rough and agranular endoplasmic reticulum (Fig. 6, A and B).

In approximately one-fourth of the untreated cells, numerous small electron-dense membrane-bound vesicles (0.2-\( \mu \)m diameter) were found in close proximity to the inner surface of the plasma membrane; some vesicles appeared to be detaching from the plasma membrane (Fig. 6B). Fig. 6B also reveals other prominent ultrastructural features including numerous surface microvilli as well as the presence of relatively few autophagic vacuoles.

By contrast, cells propagated in the presence of melatonin showed striking degenerative changes as compared with controls (Fig. 6C). For example, after just 4 days of melatonin exposure, the majority of cells exhibited an enlarged nucleus with dispersed chromatin and at least one prominent nucleolus. The enlarged nuclei were typically enveloped by a very thin rim of cytoplasm which contained reduced numbers of mitochondria, most of which possessed disrupted cristae and very small or absent Golgi complexes. Additionally, melatonin-treated cells showed a prominent dilation and vesiculation of the smooth endoplasmic reticulum, ribosomal shedding and scattering throughout the cytoplasm, and a virtual disappearance of secretory granules chararctistic of the control cells. Melatonin-exposed cells also contained a substantially increased number and size of autophagic vacuoles (cytosegrosomes) and myelin figures (Fig. 6, C and D).

Some melatonin-treated cells (Fig. 6D) appeared to have a morphology which was intermediate between control cells (Fig. 6, A and B) and the melatonin-treated cells depicted in Fig. 6C. For example, the cell in Fig. 6D contains a large number of mitochondria and a normal nucleus reminiscent of the control cells. These cells also exhibited several other interesting features which included moderate amounts of endoplasmic reticulum and prominent whorls of myofilaments located in the juxtanuclear cytoplasm. However, the prominent autophagic vacuoles are similar to those seen in the cell depicted in Fig. 6C.

DISCUSSION

The potential involvement of the pineal gland, and more particularly melatonin, in the regulation of the growth of malignant disease has been suggested by reports that the induction and growth of experimentally induced, hormone-responsive

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Table 2: Effects of melatonin (10\(^{-5}\) M) on the proliferation of human foreskin fibroblasts in culture expressed as the mean number of cells per plate

<table>
<thead>
<tr>
<th>Day</th>
<th>Treatment</th>
<th>1</th>
<th>3</th>
<th>5</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated controls</td>
<td>0.5 x 10(^5) ± 1,137(^*)</td>
<td>5.0 x 10(^5) ± 12,683</td>
<td>10 x 10(^5) ± 30,169</td>
<td>11 x 10(^5) ± 29,504</td>
</tr>
<tr>
<td></td>
<td>Vehicle controls</td>
<td>0.5 x 10(^5) ± 939</td>
<td>5.0 x 10(^5) ± 10,194</td>
<td>10 x 10(^5) ± 17,416</td>
<td>11 x 10(^5) ± 21,507</td>
</tr>
<tr>
<td></td>
<td>Melatonin</td>
<td>0.5 x 10(^5) ± 994</td>
<td>5.0 x 10(^5) ± 11,105</td>
<td>9 x 10(^5) ± 17,198</td>
<td>10 x 10(^5) ± 21,566</td>
</tr>
</tbody>
</table>

\(^*\) Mean ± SEM.
mammary cancers are inhibited by pharmacological levels of melatonin, while the elimination of the endogenous melatonin signal by pinealectomy generally promotes mammary tumorigenesis (7, 8, 11, 13). Furthermore, these studies suggest that the pineal gland releases an oncostatic substance(s) which acts through indirect neuroendocrine mechanisms controlling the release of pituitary and/or gonadal hormones that endogenously promote tumor growth (12, 14). In the present study, we addressed the alternative hypothesis that the pineal hormone melatonin acts directly on human breast cancer cells to inhibit their growth in culture.

We have demonstrated that melatonin can act directly at the cellular level to inhibit the proliferation of an estrogen receptor-positive human breast cancer cell line for as long as 10 days in continuous culture. Furthermore, melatonin’s growth-inhibiting activity on MCF-7 cells not only appears to be a reversible phenomenon, but it is restricted to concentrations that correspond to physiological levels present in human blood during the melatonin nocturnal surge (3, 15).

The antiproliferative effect of melatonin was not evident with respect to a number of normal fibroblastic cell lines, including human foreskin fibroblasts, suggesting that melatonin may not directly influence the division of nontransformed cells. Furthermore, the failure of physiological levels of this indoleamine to inhibit the growth of another epithelially derived, estrogen receptor-positive human neoplastic cell line (RL95-2 endometrial cancer cells) (21) indicates that melatonin’s full oncostatic effect at physiological concentrations in vitro may be specific only for certain types of hormone-responsive cancers. For other types of hormone-responsive cancer, such as the RL95-2 endometrial cancer, supraphysiological concentrations of melatonin may be required to achieve a significant antiproliferative effect. This postulate is supported by recent evidence showing that melatonin is incapable of inhibiting the growth of human melanoma cells in culture unless it is present in the micro- to millimolar concentration range (22).

Both scanning and transmission electron microscopic techniques revealed that melatonin treatment significantly alters many of the ultrastructural features of MCF-7 cells. For example, the marked qualitative reduction in the density and size of surface microvilli of melatonin-treated cells is noteworthy, particularly in light of the fact that estrogen-induced mitogenesis of MCF-7 cells is associated with a rapid increase in the length and number of microvilli (23). Melatonin-treated cells also displayed alterations in cellular organelles associated with protein synthesis, such as Golgi complexes, which were either very small or not observable as compared with control.
cells. Additionally, the notable distortion and vesiculation of the smooth endoplasmic reticulum are ostensibly associated with an influx of sodium and water into the cells (24).

Other striking degenerative features include a melatonin-induced increase in the number of autophagic vacuoles and membranous whorls called myelin figures. The appearance of myelin figures signifies that widespread damage has occurred to all cell membranes as a result of a dissociation of lipoproteins. An increase in the appearance of autophagic vacuoles is characteristic of cells that have experienced a protracted period of sublethal injury and represents a protective mechanism by which cells sequester focal cytoplasmic injury within autophagic vacuoles, thereby separating the injured area from undamaged organelles (24). That the marked increase in autophagic vacuoles in melatonin-treated cells is a characteristic of reversible injury is supported by the fact that melatonin's inhibition of cell proliferation was indeed reversible even after 7 days of exposure to this indoleamine.

To our knowledge, this is the first clear-cut demonstration that physiological levels of melatonin have the capacity to act directly on hormone-responsive human breast cancer cells to inhibit their proliferation and alter their morphological characteristics in culture. This suggests that perhaps melatonin concentrations normally present in human blood, particularly during the night, may offer some degree of direct oncostatic protection. Conversely, a deviation in blood levels of melatonin from the physiological range might release once dormant estrogen-responsive breast tumor stem cells from this inhibitory state. Although speculative, this idea receives some credence from the work of Tamarkin and coworkers (3), who observed a significant decrease in the amplitude and duration of the nocturnal plasma melatonin peak, albeit still within the physiological range, in women with estrogen receptor-positive breast cancer as compared with either normal matched controls or women with estrogen receptor-negative breast cancer. Moreover, both the amplitude and duration of the nocturnal melat-
cancer are somewhat greater than in normal women. This is interesting in light of the fact that supraphysiologic concentrations of melatonin were ineffective in suppressing cell proliferation in the present study, while melatonin at any concentration fails to inhibit the growth of estrogen receptor-negative human breast cancer cells in culture.4

It is seemingly paradoxical that melatonin would inhibit MCF-7 cell proliferation in our study and transiently increase the levels of 17β-estradiol receptors in the investigation of Danforth et al. (17). This could be explained by the markedly different culture conditions used in the Danforth et al. (17) study in which melatonin had no effect on cell growth in charcoal-stripped, steroid-free medium. Apparently, under certain culture conditions, other hormones such as PRL can increase 17β-estradiol receptor levels in MCF-7 cells without affecting cell growth (6). The exact mechanism(s) by which melatonin inhibits breast cancer growth directly at the cellular level is unknown; however, it appears that the presence of serum itself as well as hormones, such as 17β-estradiol and PRL, may play an important role in mediating the inhibitory effect of melatonin on MCF-7 cell growth (Footnote 4; Ref. 25). The results presented here may provide an important basis for eventually understanding the cellular-molecular endocrine mechanisms by which the pineal gland and its hormone melatonin inhibit breast cancer growth in both in vivo and in vitro models of human breast cancer.

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REFERENCES


*S. M. Hill and D. E. Blask, unpublished results.