Melatonin does not inhibit estradiol-stimulated proliferation in MCF-7 and BG-1 cells

William S. Baldwin, Gregory S. Travlos, John L. Risinger and J. Carl Barrett

Laboratory of Molecular Carcinogenesis and 1Experimental Pathology, National Institute of Environmental Health Sciences, National Institutes of Health, PO Box 12233, Research Triangle Park, NC 27709, USA

2To whom correspondence should be addressed
Email: barrett@niehs.nih.gov

Melatonin, an indolic pineal hormone, is produced primarily at night in mammals and is important in controlling biological rhythms. Previous research suggested that melatonin can attenuate proliferation in the estrogen-responsive MCF-7 breast cancer cell line. We tested whether these anti-proliferative effects may have physiological consequences upon two estrogen-responsive cell lines, MCF-7 (a breast cancer cell line) and BG-1 (an ovarian adenocarcinoma cell line). Melatonin (10⁻⁹–10⁻⁵ M) attenuated proliferation of MCF-7 and BG-1 cells by >20% in the absence of estrogen. However, 17β-estradiol exposure negated the ability of melatonin to inhibit proliferation. To substantiate this finding, cells were estrogen starved followed by multiple treatments with estradiol and melatonin. Melatonin did not inhibit estradiol-stimulated proliferation under this protocol. Estradiol increased MCF-7 and BG-1 cell cycle transition from G₁ to S phase, however, melatonin did not inhibit this transition nor did it down-regulate estradiol-induced pS2 mRNA levels measured by northern blotting, further indicating that melatonin was unable to attenuate estradiol-induced proliferation and gene expression. We also examined the effects of melatonin on estradiol-induced proliferation in MCF-7 cell xenografts in athymic nude mice. Melatonin at a dose of 28 times greater than 17β-estradiol did not inhibit estradiol-induced proliferation in vivo. Furthermore, pinealectomy did not increase proliferation. Therefore, we conclude that melatonin does not directly inhibit estradiol-induced proliferation.

Introduction

Several studies suggest that melatonin can inhibit cancer. For example, melatonin (500 µg/day) administered during the promotion phase reduced the incidence and number of mammary tumors in N-nitroso-N-methylurea-treated rats (1). Co-administration of tamoxifen and melatonin (20 mg/day/os) caused regression of metastatic breast cancer in women (2). The above studies provide evidence that high doses of melatonin can act to attenuate proliferation of estrogen-responsive tumors. Melatonin (2.5 mg/kg/day) is also reported to inhibit tumor formation in 7,12-dimethylbenz[a]anthracene-treated rats and pinealectomy increased tumor incidence in this estrogen receptor-independent model (3). It should be noted that in many of these studies melatonin was used at doses well above those that the body normally produces, therefore, the effects may be due to pharmacological actions of melatonin. Additionally, these studies generally address the actions of melatonin addition on tumor development, not the loss of melatonin that occurs during aging (4).

The role of melatonin in cancer has several public health implications. Melatonin is widely used by the US population despite the uncertainties of its physiological effects. In addition, concern about the potential carcinogenic hazard of electromagnetic fields is in part due to the electromagnetic field/melatonin hypothesis that suggests that electromagnetic fields and/or light at night reduce melatonin concentrations and thus cause breast cancer (5).

Melatonin has also been reported to be oncostatic in the estrogen receptor-positive breast cancer cell line MCF-7 (6). Melatonin was shown to directly attenuate growth in estrogen receptor-positive cells at physiological concentrations (~3 × 10⁻¹⁰ M at night), but not in estrogen receptor-negative cell lines (7), by increasing the delay from G₁ to S phase in the cell cycle of MCF-7 cells (8). Further studies have suggested that melatonin acts by down-regulating estrogen receptor number (9,10); however, melatonin has also been shown to increase estrogen receptor activity (11). Furthermore, others have presented evidence suggesting that attenuation of growth by melatonin does not occur at physiological concentrations or does not occur at all (12).

Therefore, the present study was undertaken to examine the effects of melatonin in vitro and in vivo on estrogen-induced cell proliferation and some of the factors that may govern these effects in the estrogen receptor-positive human cancer cell lines of the breast (MCF-7) and the ovary (BG-1). MCF-7 cells are a well-established model for studying estrogen-stimulated growth in vivo (13). We have recently shown that BG-1 cells (14,15) are equally responsive to estrogen stimulation and represent a good alternative model to study estrogen effects in vitro. MCF-7 cells were previously examined for their responsiveness to melatonin (6–8) and shown to contain the putative melatonin nuclear receptor (RZRα) (16). The effect of melatonin upon BG-1 cells has not been examined previously.

Materials and methods

Cell culture

BG-1 cells are an ovarian adenocarcinoma from Wake Forest University (14). MCF-7 cells were originally obtained from ATCC (ATCC HTB-22; Rockville, MD). Other cell lines utilized for initial proliferation experiments include: 184 cells, which are a human mortal breast cell line; MDA-468 cells, which are a malignant human estrogen receptor-negative breast cell line; HBL100 cells, which are a human estrogen receptor-negative non-tumorigenic SV40 immortalized cell line; HeLa cells, which are an estrogen receptor-negative human cervical carcinoma cell line (15). All cells were cultured in phenol red-free Dulbecco’s modified Eagle’s medium/F12 (DMEM/F12) containing 5% fetal bovine serum and 0.1 mg/ml kanamycin sulfate (Gibco BRL, Gaithersburg, MD) at 37°C in a humidified atmosphere of 95% air/5% CO₂.
The medium was changed to phenol red-free DMEM/F12 containing 5% dextran-coated charcoal-filtered fetal bovine serum (DCC-FBS) to filter out organics, including steroid hormones. Cells were then trypsinized and plated for experimentation. All experiments were performed on cells passage less than eight.

**Non-radioactive cell proliferation assays**

HeLa, MDA-468, 184, HBL100, BG-1 and MCF-7 cells (20,000) were plated in 12 wells plates in DCC-FBS DMEM/F12 medium. Treated cells were exposed to 10⁻¹⁰, 10⁻⁹, 10⁻⁸ and 10⁻⁷ M melatonin (Sigma, St Louis, MO) and untreated cells were exposed to a corresponding amount of ethanol (0.01%) 24 h after plating. Some cells (BG-1 and MCF-7) were also growth stimulated with 10⁻⁷ M 17β-estradiol 48 h following plating and 24 h following the initial addition of melatonin. Cells were also pretreated with melatonin when treated with the fresh medium and estradiol. For consistency, cells that were not growth stimulated with estradiol were also given fresh medium and retreated with melatonin or ethanol. Cell proliferation was assayed colorimetrically at 570 nm 120 h following estradiol growth stimulation (Cell-Titer 96 Non-Radioactive Cell Proliferation Assay; Promega, Madison, WI). Briefly, medium was decanted and a solution containing 200 µl DCC-FBS DMEM/F12 and 30 µl 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added and the assay was scanned colorimetrically at 570 nm. 

**Coulter counting**

Cells (BG-1 and MCF-7) were prepared for experimentation and treated as described above following estrogen starvation for 4-7 days in phenol red-free DMEM/F12 medium containing 5% DCC-FBS according to previously published protocols (17,18). Cells were plated in 100 mm² dishes at 1-4 x 10⁶ cells/dish. Melatonin and subsequent estradiol treatments were as described above with the following exception. Cells received fresh medium and were re-exposed to melatonin and estradiol at 72 h. Cells were counted using a Coulter counter (Coulter Electronics, Hialeah, FL) following trypsinization at 0, 72 and 144 h. The addition of estradiol commenced the experiment (0 time), which was 48 h after cells were plated and 24 h after the initial addition of melatonin. 

**Cell cycle determination**

Cells (2x10⁵) were plated in 100 mm² dishes and exposed to 10⁻⁷ and 10⁻⁹ M melatonin as described above. Twenty-four hours following exposure to estradiol, cells were washed with PBS and scraped from the plates with 1.5 ml Tri-reagent (MRC, Cincinnati, OH). RNA was extracted using the acid guanidinium–phenol–chloroform procedure (21) and extracted using the acid guanidinium–phenol–chloroform procedure (21) and extended these observations to BG-1 cells, which were reported to inhibit the ability of melatonin to attenuate proliferation in MCF-7 cells (22).

**Results**

**Inhibition of cell proliferation by melatonin**

Previous studies have shown that exposure to physiological levels of melatonin attenuates proliferation of estrogen receptor-positive cells, such as MCF-7 cells, but not estrogen receptor-negative cells (6). Our data confirmed that melatonin at >10⁻⁸ M attenuated the growth of MCF-7 cells by 50% and extended these observations to BG-1 cells, which were inhibited by 20%. These results were reproduced using the MTT assay and Coulter counting method (Table I and Figure 1). The proliferation of other cell lines was reduced slightly, but only at supraphysiological concentrations of melatonin (Table I). Melatonin attenuated proliferation of BG-1 and MCF-7 cells in a dose-dependent fashion starting at 10⁻⁹ M melatonin, with 10⁻⁷ M melatonin providing the maximum attenuation of growth. However, if the cells were pre-treated with melatonin and then stimulated with 17β-estradiol, growth was not significantly attenuated in MCF-7 and BG-1 cells (Table II), suggesting that melatonin attenuation of proliferation may not be physiologically relevant in estrogen-responsive cells and tissues. There was also no attenuation of proliferation during experiments performed in which melatonin and estradiol were added to the medium and the cells at the same time (data
Melatonin and estradiol-stimulated proliferation

Table I. Proliferation of different cell lines following melatonin exposure

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Growth (%)</th>
</tr>
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<tbody>
<tr>
<td>HBL100</td>
<td>100 ± 7</td>
</tr>
<tr>
<td>MDA468</td>
<td>100 ± 6</td>
</tr>
<tr>
<td>184</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>HeLa</td>
<td>100 ± 5</td>
</tr>
<tr>
<td>MCF7</td>
<td>100 ± 6</td>
</tr>
<tr>
<td>BG-1</td>
<td>100 ± 5</td>
</tr>
</tbody>
</table>

Y 10–11 M melatonin b 10–9 M melatonin b 10–7 M melatonin b 10–5 M melatonin

Values are expressed as means ± standard deviation (n = 6).

Table II. Proliferation of MCF-7 and BG-1 cells in untreated, 10–8 M 17β-estradiol-stimulated and melatonin pretreated, estradiol-stimulated cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Growth (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF7</td>
<td>100 ± 8</td>
</tr>
<tr>
<td>BG-1</td>
<td>100 ± 4</td>
</tr>
</tbody>
</table>

Y 10–11 M melatonin + E2 b 10–9 M melatonin + E2 b 10–7 M melatonin + E2 b 10–5 M melatonin + E2

Values are expressed as means ± standard deviation (n = 6).

Cell proliferation following multiple treatments with estradiol and melatonin

Because melatonin did not attenuate proliferation of cells following estradiol stimulation, the effects of multiple treatments with melatonin and estradiol were examined upon cells that were estrogen starved for 7 days to increase estrogen sensitivity (17,18). Cells were starved for 7 days to adhere to previously described protocols (17,18). It was hypothesized that if the difference in growth between melatonin exposure and no exposure was small, then multiple treatments may enhance the melatonin effect. Furthermore, enhancing estrogen sensitivity through estrogen starvation may manifest the ability of melatonin to reduce estradiol-induced proliferation. An estradiol concentration of 10–8 M was chosen because it has been found to exhibit greater proliferative activity in MCF-7 cells than lower concentrations (15). BG-1 cells show no dose dependence with estradiol between 10–8 and 10–10 M concentrations (15). Twenty-four hour melatonin pre-treatments were performed to down-regulate estrogen receptors (9,10). Using this protocol, melatonin did not attenuate estradiol-induced proliferation in MCF-7 or BG-1 cells at 10–9 or 10–7 M melatonin (Figure 2). Cells not treated with estradiol did not proliferate; therefore, melatonin could not attenuate proliferation of 7 day estrogen starved cells not exposed to estradiol. This work also demonstrates that melatonin does not attenuate growth of MCF-7 cells due to low level toxicity.

To verify the ability of melatonin to attenuate proliferation without estradiol present (Table I), a control was run with cells estrogen starved for only 4 days. Cells starved for 4 days still show proliferative capabilities without estradiol while still enhancing estradiol-stimulated growth. Melatonin at 10–7 M, which is the concentration of melatonin that provided the most potent attenuation of proliferation, was chosen for the study (Table I). Melatonin significantly attenuated proliferation (~20% inhibition) of MCF-7 and BG-1 cells in DCC-FBS (steroid hormone-free) under these conditions. However, melatonin did not inhibit growth in BG-1 or MCF-7 cells under this protocol if the cells were stimulated with estradiol (Figure 1).

Cell cycle alterations

To substantiate this work, we examined the effects of melatonin upon 17β-estradiol-enhanced cell cycle kinetics. 17β-Estradiol (10–8 M) increased the number of MCF-7 cells in S phase by 6% and the number of BG-1 cells in S phase by 31%, while causing a subsequent decrease in the number of cells in G1 phase of ~30% in both cell types (Table III). Melatonin (10–7 M and 10–9 M) was unable to alter the estradiol-induced decrease in G1 and subsequent increase in S phase (Table III). Thus, melatonin does not inhibit the estradiol-induced changes in the cell cycle.
Fig. 1. Proliferation of (A) MCF-7 and (B) BG-1 cells following multiple exposures to 17β-estradiol and melatonin. Cells were plated in 100 mm² dishes following 4 days estrogen starvation and then pre-exposed to 10^{-7} M melatonin 24 h prior to 17β-estradiol stimulus. Cells were re-exposed to melatonin and/or estradiol or Coulter counted every 72 h post-estradiol exposure. *, statistical significance of melatonin exposure compared with control at P < 0.05 by ANOVA followed by Scheffe’s multiple comparison test (n = 3); #, statistical significance of estradiol exposure compared with control at P < 0.05 by ANOVA followed by Scheffe’s multiple comparison test (n = 3).

Fig. 2. Proliferation of (A) MCF-7 and (B) BG-1 cells following multiple exposures to 17β-estradiol and melatonin. Cells were plated in 100 mm² dishes following 7 days estrogen starvation and then pre-exposed to 10^{-9} or 10^{-7} M melatonin 24 h prior to 17β-estradiol stimulus. Cells were re-exposed to melatonin and/or estradiol or Coulter counted every 72 h post-estradiol exposure.

PS2 message
PS2 mRNA is produced in response to 17β-estradiol in estrogen-responsive cancer cells (24). We examined pS2 mRNA levels by northern blotting and observed a significant increase in pS2 with exposure to estradiol. This increase in pS2 mRNA occurred within 24 h of estradiol exposure in BG-1 and MCF-7 cells. Melatonin at 10^{-7} M attenuated the production of pS2 mRNA by a small (13% in BG-1 and 17% in MCF-7 cells) but not significant level (P = 0.121 for BG-1 and P = 0.194 for MCF-7 cells) (Figures 3 and 4). This reduced production of pS2 corresponds well with the inhibition of proliferation following multiple melatonin treatments without estradiol present (Figure 1).

Melatonin effects on estrogen-dependent growth of MCF-7 cells in vivo
Only mice injected with MCF-7 cells and treated with exogenous 17β-estradiol formed significant tumor masses. The effects of pinealectomy on MCF-7 cell tumor mass in mice with intact ovaries could not be established, because significant tumor mass was not produced in these mice (data not shown).

Estradiol treatment (0.18 mg pellet) caused measurable proliferation of MCF-7 cells in athymic nude mice. Melatonin treatment (5 mg pellet) did not attenuate growth of MCF-7 cells in the mice treated with estradiol. Furthermore, loss of melatonin due to pinealectomy did not cause an increase in MCF-7 cell mass (Table IV). Neither treatment caused any obvious changes in histological appearance of the tumors. Hence, melatonin had no direct effects on MCF-7 cell proliferation in vivo.

Discussion
Estrogen receptor-positive cells, such as MCF-7 cells, show strict estrogen dependence for proliferation in vivo (25) yet may proliferate without estrogens present in vitro. Therefore, we were interested in examining whether melatonin could inhibit estradiol-dependent proliferation in vitro as well as...
Similar to previous in vivo xenograft experiments; MCF-7 and BG-1 cells in vitro did not proliferate following a 7 day estrogen starvation (Figure 2) and are dependent on 17β-estradiol for proliferation (Figure 2).

Previous research has demonstrated that melatonin down-regulates estrogen receptor mRNA levels and estradiol binding in MCF-7 cells (9,10); thus, it was hypothesized that enhanced estrogen sensitivity due to estrogen starvation (17,18) may provide conditions that increase the efficacy of melatonin as an inhibitor of estradiol-induced proliferation. However, 10–7 and 10–9 M melatonin did not inhibit estradiol-induced proliferation under these conditions (Figure 2). As a positive control, the effects of melatonin upon basal cell growth and estradiol-stimulated cell growth were examined in MCF-7 and BG-1 cells following only 4 days of estrogen starvation. Under these conditions cells continued to grow throughout the experiment and melatonin inhibited basal cell growth but not estradiol-induced proliferation (Figure 1). Additionally, estradiol increased transition from G1 to S phase in MCF-7 and BG-1 cells, but melatonin exposure did not attenuate the estradiol-induced increase in S phase (Table III). Melatonin also did not cause a significant decrease in pS2 mRNA levels in MCF-7 or MCF-7 cells (Figures 3 and 4). The minimal decrease seen is interesting, because it suggests an antitumorogenic effect of melatonin on cancer cells that may be due to a decrease in estrogen receptor induced by melatonin. However, this response was not statistically significant nor consequential in attenuating estradiol-induced proliferation (Figures 1 and 2). This work demonstrates that melatonin does not work directly to suppress estradiol-induced proliferation in vitro. Therefore, we examined the ability of melatonin to attenuate proliferation in nude mice. Under these conditions, melatonin did not attenuate proliferation in athymic nude mice treated with estradiol and the loss of melatonin due to pinealectomy had no effect on MCF-7 cell proliferation (Table IV).

Recent studies have demonstrated that MCF-7 cells contain the putative melatonin receptor RZKa (16). Melatonin may transduce its in vitro antiproliferative and DNA synthesis-inhibiting (26) effects through this receptor. However, the estrogen receptor gene promoter does not contain an RZR response element (27) and thus melatonin would not be expected to directly inhibit estradiol receptor-mediated processes. However, it is contradictory that most of the cell lines whose growth is attenuated by melatonin are estrogen receptor-positive (7; this paper). Melatonin may work through other methods to down-regulate proliferation in vitro in estrogen receptor-positive cells, such as through growth factors, prolactin or prostaglandins (16). Alternatively, the small effect melatonin has on in vitro growth may be suppressed by the vast effects estradiol has upon these cells, suggesting that melatonin would not have a direct effect upon estradiol-induced proliferation in vivo.

This work does not support the hypothesis that loss of melatonin would have a direct effect on estradiol-induced cancers. However, multiple researchers have demonstrated...
that melatonin acts through high affinity plasma membrane receptors in the brain (superchiasmatic nucleus and hypothalamus) and hypophysal pars tuberalis (28,29). Activation of these receptors (Mel1A, Mel1B, and Mel1C) inhibits cAMP production in a manner consistent with the superfamily of G protein-coupled receptors (30). Inhibition of cAMP could inhibit release of gonadotropin releasing hormone from the hypophysalms and luteinizing hormone from the pituitary. Gonadotropin releasing hormone is essential in the subsequent release of luteinizing hormone and FSH from the pituitary. Subsequent down-regulation of gonadotropin release would effect 17β-estradiol release and may attenuate proliferation of estrogen-responsive cancers during the promotional phase, as hypothesized by Stevens and Davis (5) and seen by Blask et al. (1). We were unable to examine this hypothesis in vivo because ovary-intact mice without estradiol pellets did not produce MCF-7 cell tumors (data not shown).

In conclusion, although melatonin does attenuate proliferation of MCF-7 and BG-1 cells in serum, it does not inhibit estradiol-induced proliferation under these conditions. This was demonstrated using both proliferation studies and biomarkers of estrogen exposure in vitro, as well as proliferation studies in vivo. Consequently, this work suggests that reduced concentrations of melatonin would not cause a direct increase in proliferation of estrogen receptor-positive cells and tissues and does not support a role of melatonin in the etiology of estrogen-responsive cancers.

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References