Influence of Melatonin on Invasive and Metastatic Properties of MCF-7 Human Breast Cancer Cells

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ABSTRACT

Melatonin, the principal pineal gland hormone, exerts a direct antiproliferative effect on estrogen-responsive MCF-7 cells in culture. The purpose of the current study was to investigate the effects of melatonin on the invasion capacity of MCF-7 cells.

In vitro, melatonin at physiological doses (1 nM) reduced (P < 0.001) the invasiveness of tumoral cells measured in Falcon invasion chambers. Subphysiological (0.1 µM) and pharmacological concentrations (10 µM) of melatonin failed to inhibit cell invasion. Melatonin was also able to block 17β-estradiol-induced invasion (P < 0.001). Pretreatment of MCF-7 cells with 1 nM melatonin increased the response of tumoral cells to the anti-invasive effects of this indolamine. To explore possible mechanisms by which melatonin reduces invasiveness, we measured the attachment of MCF-7 cells to a basement membrane, the chemotactic response of the cells, and their type IV collagenolytic activity. The presence of melatonin (1 nM) in the culture medium significantly reduced the ability of MCF-7 cells to attach to the basement membrane; this effect was enhanced by pretreating the cells with the same indolamine (P < 0.001). Melatonin also counteracts the stimulatory effects of 17β-estradiol on cell adhesion (P < 0.001). The chemotactic response of MCF-7 cells also decreased in the presence of 1 nM melatonin, and this melatonin-induced reduction of cell migration was more effective on cells that were previously incubated for 5 days with melatonin than it was on nonpretreated cells (P < 0.001). The simultaneous addition of 17β-estradiol and melatonin resulted in a significantly lower chemotactic response than that of 17β-estradiol-treated cells (P < 0.001). However, type IV collagenolytic activity was not influenced by melatonin. Our results demonstrate that melatonin reduces the invasiveness of MCF-7 cells, causing a decrease in cell attachment and cell motility, probably by interacting with the estrogen-mediated mechanisms of MCF-7 cell invasiveness. In addition, we also studied the influence of melatonin on the expression of two cell surface adhesion molecules (E-cadherin and β1 integrin) and an intermediate filament protein (vimentin), the expression of which has been correlated with the relative invasive capacity of human breast cancer cells. The culture of tumor cells in the presence of melatonin (1 nM) increased the membrane staining for E-cadherin and β1 integrin as well as the number of E-cadherin and β1 integrin immunoreactive cells (P < 0.01). Neither control MCF-7 cells nor those treated with melatonin stained for vimentin.

Preliminary in vivo experiments carried out on ovariectomized athymic nude mice implanted with 17β-estradiol pellets and inoculated with 5 x 10⁶ MCF-7 cells in the inguinal mammary fat pad suggest that melatonin could decrease the tumorigenicity of these tumor cells. However, these results need further confirmation.

Taken together, our results suggest that melatonin shifts MCF-7 human breast cancer cells to a lower invasive status by increasing the β1 integrin subunit and E-cadherin expression and promoting the differentiation of tumor cells. Finally, our study points out the existence of the anti-invasive actions of melatonin as a part of the oncostatic action of melatonin.

INTRODUCTION

The role of melatonin as an oncostatic gland has been studied in different animal models of tumorigenesis, especially in those concerning the mammary gland. The most common conclusion is that either experimental manipulations that activate the pineal gland or the administration of melatonin, the main pineal hormone, reduces the incidence and development of chemically induced mammary tumors, whereas pinealectomy usually stimulates breast cancer growth (1-4). Two different mechanisms have been proposed to explain how melatonin could reduce the development of mammary tumors: (a) indirect neuroendocrine mechanisms such as the melatonin regulation of some pituitary and gonadal hormones that control tumor growth (1, 3-5); and (b) modulation by melatonin of the immune response to the presence of a malignant neoplasm (6) and the action of melatonin as an endogenous hydroxyl radical scavenger (7, 8); on the other hand, direct antiestrogenic melatonin actions at the cellular level have been proposed (9, 10). In vitro, concentrations of melatonin (1 nM and 10 µM) corresponding to the physiological levels present in human blood during the night exert a direct antiproliferative effect on estrogen-responsive MCF-7 cells, including decreases in cell number, DNA content, and thymidine incorporation (9, 11, 12). To date, all of the studies (above all, in vitro studies carried out on MCF-7 cells) have focused on the antiproliferative actions of melatonin (10, 13-16), but some other possible mechanisms involved in the oncostatic properties of this hormone have not been explored. The MCF-7 breast carcinoma cell line, which was established from the pleural effusion of a patient with breast adenocarcinoma (17), contains estrogen receptors and requires estrogens to express a great malignant character (18, 19). It is well known that 17β-estradiol stimulates the invasive and metastatic potential of cancer cells, increasing the ability of MCF-7 cells to both form tumors and produce distant metastasis in nude mice (18, 20), and enhancing the ability of these cells to invade through an artificial reconstituted basement membrane in vitro (19). Because the anti-tumor action of melatonin is partially exerted by an interaction with 17β-estradiol (9, 10), we postulate that melatonin could also regulate the metastatic behavior of MCF-7 human breast cancer cells. To test our hypothesis, we studied: (a) the effects of melatonin on the invasion capacity of MCF-7 cells in vitro by evaluating its influence on cell invasion, cell adhesion, chemotaxis, and type IV collagenolytic activity of MCF-7 cells, which are associated with the processes involved in the metastatic behavior of MCF-7 cells; and (b) the effects of melatonin on the tumorigenicity and metastasis formation of MCF-7 cells in vivo. Furthermore, we also investigated the influence of melatonin on the expression of cell surface adhesion molecules, E-cadherin (a calcium-dependent membrane protein responsible for cell-cell contact) and β1 integrin (a subunit of integrins, which are receptors that regulate the interaction between cells and the extracellular matrix), as well as on the expression of vimentin (an intermediate filament protein whose expression has been previously related with the relative invasiveness of human breast cancer cells; Refs. 21-24).
MELATONIN AND TUMOR INVASION

Fig. 1. Schematic representation of the chambers used in the invasion and chemotaxis assays. A modified Boyden’s chamber was constructed by using Falcon multiwell cell culture plates (A) and removable Falcon cell culture inserts (B). When the inserts are placed into the well, two compartments, which are separated by a microporous filter, are defined (C). The filter was coated with Matrigel (invasion assay) or collagen IV (chemotaxis assay). In both types of assay, the cells and the substances tested were placed in the upper compartment, whereas fibronectin was placed in the lower compartment as a chemoattractant. Cells that traversed the reconstituted basement membrane (invasion studies) or the collagen IV layer (chemotaxis assays) crossed the microporous filter, adhered to the lower surface of the filter, and were fixed, stained, and counted (D).

MATERIALS AND METHODS

Cells and Culture Conditions. MCF-7 human breast cancer cells were purchased from the American Type Culture Collection (Rockville, MD) and maintained as monolayer cultures in 75-cm² plastic culture flasks in DMEM (Sigma Chemical Co., St. Louis, MO) supplemented with 10% FBS (Life Technologies, Inc., Erlāny, France), penicillin (20 units/ml), and streptomycin (20 μg/ml; Sigma Chemical Co.) at 37°C in a humid atmosphere containing 5% CO₂. Cells were subcultured every 3–4 days by suspension in 5 mM Na₂-EDTA in PBS (pH 7.4) at 37°C for 5 min.

Before each experiment, stock subconfluent monolayers (80%) of MCF-7 cells were incubated with 5 mM Na₂-EDTA in PBS (pH 7.4) at 37°C for 5 min, resuspended in DMEM supplemented with 10% FBS, and passed repeatedly through a 25-gauge needle to produce a single cell suspension. Cell number and viability were determined by staining a small volume of cell suspension with 0.4% trypan blue saline solution and examining the cells in a hemocytometer.

Invasion Assay. Invasion assays were carried out by methods based on others described previously (25–27) in modified Boyden’s chambers constructed with Falcon multiwell cell culture plates and Falcon cell culture inserts (see Fig. 1). Cell culture inserts were converted into invasion chambers by applying a layer of basement membrane onto the surface of microporous filters present in each unit. Briefly, 6.4-mm-diameter filters (8 μm pore) of Falcon cell culture inserts were coated with 25 μg/filter of reconstituted basement membrane Matrigel (Collaborative Biomedical Products, Bedford, MA). Matrigel was diluted to the desired concentration with ice-cold distilled water, applied to filters, dried overnight at room temperature, and reconstituted with DMEM for 90 min at room temperature. Uniformity of the coating was checked by Coomassie Blue staining and low-power microscopic observation. The concentration of Matrigel used in the experiments (25 μg/filter) was determined in a previous study in which we varied the amount of Matrigel placed on the filters (0, 6.25, 12.50, 25, and 50 μg/filter) until finding the concentration that allowed a discriminating assay (data not shown).

Exponentially growing MCF-7 cells were harvested with 5 mM Na₂-EDTA in PBS (pH 7.4); washed in DMEM + 10% FBS by centrifugation; resuspended in DMEM supplemented with 10% FBS, penicillin (20 units/ml), and streptomycin (20 μg/ml); and passed repeatedly through a 25-gauge needle to produce a single cell suspension. After determination of the cell count and viability in a hemocytometer by the trypan blue exclusion test, the cells were added to the upper compartment of the modified Boyden’s chamber (1.5 × 10⁶ cells/chamber; see Fig. 1C). Fibronectin (16 μg/chamber) was placed in the lower compartment as a chemoattractant. At the beginning of the assay, melanotin (Sigma Chemical Co.; 10 μM, 1 μM, or 0.1 μM), 17β-estradiol (Sigma Chemical Co.; 1 μM or 10 μM), and/or the diluent (final ethanol concentration/plate, 0.0001%) were added to the upper compartment. In some experiments, the cells were preincubated for 5 days with melanotin (10 μM, 1 μM, or 0.1 μM) before being tested for invasiveness. After incubation for 5 days at 37°C in 5% CO₂, the cells on the upper surface of the filter were completely removed by wiping with a cotton swab, and the cells that had traversed the Matrigel and attached to the lower surface of the filter were fixed, stained with H&E, and counted in 15 randomly selected microscopic fields (>400) per filter. Experiments were performed three times with three dishes for each experimental condition each time.

Cell Adhesion Assay. The 24-well tissue culture plates were coated with 25 μg/well Matrigel and left to air dry in a hood overnight. The concentration of Matrigel used in the experiments was determined in a previous study in which we varied the amount of reconstituted basement membrane placed on the plates. To block nonspecific binding sites, all wells were also incubated with DMEM containing 0.1% BSA for 1 h at 37°C and then washed with the same medium. MCF-7 cells were suspended at 150,000 cells/ml in DMEM supplemented with 0.1% BSA, incubated at 37°C for 1 h to allow the restitutio

Chemoattractant (fibronectin)

Reconstituted Basement Membrane

(Matrigel) or Collagen IV

Upper Compartment

37°C, 5% CO₂

5 days

Invasive Cells

Plate Well

Complete Unit

3 The abbreviation used is: FBS, fetal bovine serum.

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supplemented with 10% FBS, penicillin (20 units/ml), and streptomycin (20 µg/ml) were placed in each well and incubated at 37°C in a humidified atmosphere (95% air:5% CO₂) for 5 days in the presence or absence of 1 nM melatonin. The incubation was terminated by chilling, and the culture supernatant was withdrawn and mixed with 50 µl of ice-cold 10% trichloroacetic acid and 0.5% tannic acid in a microcentrifuge tube. After a 30-min incubation at 4°C, the mixture was centrifuged at 10,000 × g for 10 min at 4°C to precipitate the undigested materials. The supernatant was withdrawn, and the 3H activity was counted.

To measure the type IV collagenolytic activity in tumor cell-conditioned media, MCF-7 cells were cultured for 5 days in the presence or absence of 1 nM melatonin in the Falcon invasion chamber. After this period, the conditioned media were harvested by centrifugation. Aliquots of the culture supernatant (100 µl) were treated with trypsin (5 µg/ml) at 37°C for 10 min, followed by the addition of soybean trypsin inhibitor (40 µg/ml). Aliquots (100 µl) of the tryptsinized or nontrypsinized conditioned media were incubated with the radioactive type IV collagen (5 µg/9000 cpm/tube) in 100 µl of 10 mM CaCl₂, 0.1 M NaCl, and 50 mM Tris-HCl buffer (pH 7.5) at 37°C in 5% CO₂ for 24 h. The undigested materials were precipitated with 10% trichloroacetic acid and 0.5% tannic acid and then centrifuged, and the radioactivity in the supernatant was measured in a β scintillation counter.

Immunohistochemical Study of E-Cadherin, β₁ Integrin, and Vimentin.

Exponentially growing MCF-7 cells were plated on glass coverslips in DMEM supplemented with 10% FBS, penicillin (20 units/ml), and streptomycin (20 µg/ml) for 24 h. To achieve a partial synchronization of the cell cycle, the culture medium was then replaced by fresh supplemented DMEM containing 2 mM thymidine. Twenty-four h later, the cells were released from the thymidine block by rinsing the plate twice with DMEM and allowed to grow in supplemented DMEM containing either 1 nM melatonin or the melatonin diluent (ethanol; final concentration/place, 0.0001%). At 6, 12, and 24 h after the release from thymidine block, the culture media were aspirated, and the cells were washed in PBS and fixed for 10 min with 3.7% paraformaldehyde in PBS (pH 7.4) at room temperature. Cells were then incubated with the corresponding primary antibodies: (a) anti-E-cadherin (mouse monoclonal antibody to E-CAM/uvomorulin; Boehringer Mannheim, Indianapolis, IN) used at a 1:20 dilution; (b) rat monoclonal antibody reactive with the β₁ subunit (CD29) common to all members of the β₁ integrin family (Becton Dickinson, Bedford, MA) used at a 1:200 dilution; or (c) mouse antivimentin (Boehringer Mannheim) used at a 1:10 dilution. Incubations with the primary antibodies were performed either overnight at 4°C (E-cadherin and β₁ integrin) or for 1 h at room temperature (vimentin). After washing in PBS, coverslips were incubated for 60 min at room temperature with antimouse FITC-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) at a 1:100 dilution and mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Samples were studied with a confocal laser microscope (Bio-Rad 1024) using an argon ion (488 nm) to excite FITC. A semiquantitative estimation based on the relative amount of E-cadherin, vimentin, and/or β₁ integrin expressed by cells and a quantification of the number of immunoreactive cells were performed. The number of immunoreactive cells was counted in 15 randomly selected microscopic fields (×400) per sample.

In Vivo Tumorogenesis and Metastasis.

Homozygous athymic nude mice (nu/nu ; BALB/c strain) were obtained from Iffa-Credo (Barcelona, Spain). Animals were housed and maintained under pathogen-limited conditions in filtered laminar air flow hoods in standard vinyl cages with air filter tops at 21°C to 25°C with a photoperiod of 14 h of light and 10 h of darkness. Cages and bedding were autoclaved before use. Food and water were autoclaved and provided ad libitum. Six-week-old mice were used to initiate the experiments. Mice were bilaterally ovariectomized by standard surgical techniques 1 week before cell inoculation, and estrogen supplementation was given in the form of 17β-estradiol slow-release s.c. pellets (90-day release; 0.72 mg/pellet; Innovative Research, Sarasota, FL) placed in the interscapular region at the time of cell inoculation.

Cells from subconfluent monolayers of MCF-7 cells were harvested, suspended in DMEM supplemented with 10% FBS, washed in DMEM by centrifugation, resuspended in DMEM, and passed repeatedly through a 25-gauge needle to produce a single cell suspension. Once the cell number and viability were determined, 5 × 10⁶ cells in 0.1 ml of culture medium were inoculated directly into the inguinal mammary fat pad of the mouse by means of a syringe and a 25-gauge needle.

Melatonin administration (5 µg/g body weight/day) dissolved in drinking water was begun at the time of tumor inoculation. Controls were given drinking water containing 0.05% ethanol. There were 10 animals/group. Tumor size was measured weekly and at autopsy. Mice were sacrificed on or before week 10 after inoculation, depending on physical condition. Some mice were lost due to unexpected deaths and could not be analyzed. The volume of the tumor was calculated according to the following formula:

\[
\text{Tumor volume} = \frac{(\text{width})^2 \times \text{length}}{2}
\]

At autopsy, mice were sacrificed by ether anesthesia, and brain, lymph nodes, heart, adrenal glands, muscle (chest wall), bone (ribs), kidney, liver, lung, spleen, and all proliferating tumors were removed postmortem and fixed in 10% formalin for histological examination. The embedding, mounting, and staining of tissues with H&E were performed in the standard way.

Statistical Analysis. Data were analyzed by a one-way ANOVA followed by the Student-Newman-Keuls multiple comparisons test. Tumoral incidence in mice and the percentage of immunoreactive cells in the immunohistochemical study were analyzed by a nonparametric ANOVA (Mann-Whitney test). Differences among the groups' means were considered significant at \( P < 0.05 \).

RESULTS

Effects of Melatonin on the Invasive Activity of MCF-7 Cells.

When melatonin at physiological concentrations (1 nm) was added to the upper compartment of the invasion chamber, the number of cells that invaded the Matrigel membrane decreased significantly (−44%; \( P < 0.001 \)) as compared with that of the control group. Subphysiological (0.1 µM) or supraphysiological (10 µM) concentrations of melatonin lacked this effect (Fig. 2A). Pretreatment of MCF-7 cells with 1 nM melatonin for 5 days before the invasion assay increased (Fig. 2B) the response to the anti-invasive effects of this indolamine (\( P < 0.001 \)). The highest anti-invasion effects were found when MCF-7 cells were first incubated for 5 days in the presence of 1 nM melatonin in culture media, and then the same concentration of melatonin was added to the upper compartment of the invasion chamber. In this way, a reduction of up to 74% of the invasiveness of MCF-7 cells was observed.

Fig. 2. Effect of melatonin on the invasiveness of MCF-7 cells. A. exponentially growing MCF-7 cells were harvested and counted, and 1.5 × 10⁶ cells were added to each invasion chamber in the presence of melatonin (10 µM, 1 nM, or 0.1 µM) or the diluent (ethanol; final concentration, 0.0001%). B. Cells were preincubated for 5 days with melatonin (10 µM, 1 nM, or 0.1 µM) before being placed in the invasion chamber and exposed to the same concentration of melatonin. Data are expressed as the percentage of the control response (mean ± SE). a, \( P < 0.001 \) versus control; b, \( P < 0.001 \) versus other melatonin treatments.
The addition of 17β-estradiol (1 μM or 10 nM) to the upper compartment of the invasion chamber (Fig. 3) increased the number of cells invading the Matrigel membrane, and melatonin (1 nM) was able to counteract the stimulatory effects of 17β-estradiol when both hormones were added simultaneously (P < 0.001).

**Effects of Melatonin on the Attachment of MCF-7 Cells to the Basement Membrane.** To evaluate the influence of melatonin on the attachment of MCF-7 cells to the basement membrane, tissue culture plates were coated or not coated with an extract of basement membrane components (Matrigel), and cells were added to the plates and incubated for 10, 30, or 60 min in the presence of different concentrations of melatonin, 17β-estradiol, or the hormone diluent. The number of cells that adhered to the wells was then counted. As expected, the nonspecific attachment of MCF-7 cells to Matrigel-coated wells was higher than that to the uncoated wells (data not shown). Melatonin did not influence the adhesiveness of cells to uncoated tissue culture plates, whereas it reduced the adhesion of cancer cells to the Matrigel basement membrane (P < 0.001). This reduction was greater at 1 nM melatonin than it was at 10 μM or 0.1 pm (Fig. 4A). The melatonin-induced reduction of the adhesiveness of MCF-7 cells was significantly higher in cells pretreated with the indolamine (10 μM, 1 nM, or 0.1 pm) than it was in nonpretreated cells (P < 0.001; Fig. 4B). 17β-Estradiol (1 μM or 10 nm) increased the adhesion of cells to the Matrigel membrane. The simultaneous addition of 17β-estradiol and melatonin resulted in a significantly lower (P < 0.001) cell adhesiveness than did the addition of 17β-estradiol alone and was similar to that obtained with melatonin alone (Fig. 5).

**Effects of Melatonin on the Chemotactic Migration of MCF-7 Cells.** The introduction of fibronectin as a chemoattractant into the lower compartment of the invasion chamber sped up, in a linear manner, the movement of the cells from the upper compartment to the lower compartment through the collagen IV membrane separating both. This chemotactic response of MCF-7 cells was inhibited by the addition of 1 nM melatonin; other concentrations of this indolamine were ineffective (Fig. 6A). The actions of melatonin in lowering cell migration were stronger in cells that were previously incubated with 1 nM melatonin than they were in nonpretreated cells (P < 0.001; Fig. 6B). 17β-Estradiol (1 μM or 10 nm) stimulated the chemotactic migration of MCF-7 cells. The simultaneous addition of 17β-estradiol and melatonin resulted in a significantly (P < 0.001) lower chemotactic responsiveness than that of the 17β-estradiol-treated cells and was similar to the chemotactic migration observed in control (untreated) cells (Fig. 7).
DISCUSSION

Direct antitumor actions of melatonin at the cellular level have been described using the in vitro antiproliferative actions of melatonin as a basis. Because only human breast cancer cell lines that express estrogen receptors have been found to be responsive to the antimitogenic effects of melatonin, the current hypothesis is that the oncostatic actions of melatonin are mediated via its effects on the tumor cells’ estrogen-response pathway (10, 11, 13). The link between the antiproliferative effect of melatonin on the growth of MCF-7 cells and the estrogen-response pathway is further supported by: (a) the ability of melatonin to block the mitogenic effect of 17β-estradiol (9, 10) in different culture systems (monolayer and clonogenic soft agar); (b) the melatonin blockade of the estrogen rescue of tamoxifen-inhibited cells in clonogenic agar and monolayer culture (10); (c) the down-regulation by melatonin of estrogen receptor expression in MCF-7 cells (11, 30); and (d) melatonin modulation of estrogen-regulated proteins, growth factors, and proto-oncogenes in human breast cancer cells (31, 32). This concept is also supported by a study in which women with estrogen receptor-positive breast cancer were found to have decreased nocturnal plasma levels of melatonin when compared with women with estrogen receptor-negative breast tumors and with age-matched controls (33). Because human breast cancer cells show a pleiotropic response to estrogens (34, 35), and, as noted previously, the malignant character of these cells increases with estrogens, we considered it of interest to study whether melatonin may or may not modify the metastatic behavior of breast cancer cells.

The present study demonstrates that melatonin at physiological doses (1 nM) reduces the invasiveness of tumor cells in vitro, and this anti-invasive effect is increased when cells are pretreated with the same indolamine. On the contrary, subphysiological (0.1 pM) and pharmacological concentrations (10 nM) of melatonin failed to inhibit cell invasion. It is interesting to emphasize that the optimal melatonin concentration to reduce MCF-7 invasiveness is the same as that which gives the highest antiproliferative effects in an anchorage-dependent culture system (9, 13). As reported previously (19), estrogens induce a marked change in the interaction of MCF-7 cells with the basement membrane components, changes that are characteristic of the malignant phenotype. Estrogen-treated cells show a greater attachment to the basement membrane, a greater ability to migrate toward laminin, a greater proliferation in culture in the presence of the basement membrane matrix, and a much greater ability to invade the barriers of the reconstituted basement membrane (19). These changes have been related with an increase in 17β-estradiol in the number of laminin receptors in MCF-7 cells (19). In our invasion model, 17β-estradiol also increased the number of cells that invaded the reconstituted basement membrane, and, interestingly, melatonin was able to counteract the stimulatory effects of 17β-estradiol. The effects of some antiestrogenic drugs on MCF-7 cell invasiveness have been studied...
Melatonin and Tumor Invasion

Fig. 8. Immunohistological staining of cell surface E-cadherin and β1 integrin. Partially synchronized MCF-7 cells were incubated for 24 h with the melatonin diluent (A and D) or 1 nM melatonin (B and E) and fixed and stained with the mouse monoclonal anti-E-cadherin antibody (A and B) or the rat monoclonal anti-β1 integrin antibody (D and E). Quantification of the number of E-cadherin-immunoreactive cells (C) and β1 integrin-immunoreactive cells (F) in the presence (C or absence (D) of 1 nM melatonin after 6, 12, and 24 h of incubation is shown. Data are expressed as the percentage of the control response (mean ± SE). a, P < 0.05; b, P < 0.01; c, P < 0.005 versus control.

with different results that were apparently related to differential estrogen agonism. Tamoxifen and 4-hydroxytamoxifen, which are known to be partial agonists, increase the invasive behavior of tumor cells, whereas the antiestrogen ICI 164384, which lacks estrogen agonism, reduces the invasive behavior of tumor cells (36) and is able to counteract the estrogen invasion-stimulatory effect in a manner similar to that which we found with melatonin.

Having found that melatonin reduces the invasiveness of tumor cells, our aim was to define the role of this indolamine within each step of the metastatic process. Tumor invasion is a complex biological process that begins with the detachment of tumor cells from the main tumor mass and the subsequent invasion of the adjacent tissue and the surrounding blood and lymphatic vessels. A small but significant proportion of these detached tumor cells finally arrests in distant organs by attachment to their basement membrane, by the secretion of enzymes that cause a degradation of this membrane barrier, and by the migration of tumor cells into the target tissue, forming secondary tumor deposits (metastasis). Thus, we studied the influence of melatonin on some of these steps: (a) the attachment of MCF-7 cells to the basement membrane; (b) the secretion of enzymes by the tumor cells; and (c) the chemotactic response of the tumor cells in the target tissue.

We show here that melatonin reduces the ability of MCF-7 cells to attach to the basement membrane, with this effect being greater at a concentration of 1 nM than at 10 μM or 0.1 μM. It has been demonstrated that estrogen treatment induces a marked rearrangement of the cytoskeleton and adhesion structures and enhances the attachment of MCF-7 cells to laminin, a basement membrane component (19, 37). In agreement with these previous studies, 17β-estradiol, in our system, increased the adhesion of the cells to the basement membrane; at a physiological concentration, melatonin completely abolished this effect of 17β-estradiol.

The chemotactic response of MCF-7 cells toward fibronectin (used as a chemoattractant) was also reduced by melatonin at physiological concentrations (1 nM). It is well known that 17β-estradiol stimulates the chemotactic migration of MCF-7 cells (19); the simultaneous addition of 17β-estradiol and melatonin resulted in a significantly lower chemotactic response than that seen in 17β-estradiol-treated cells.

Although previous studies indicate that the regulation of the invasiveness of MCF-7 cells by antiestrogens may be mediated by an increase in collagenase IV activity (36), we did not find significant changes in the collagenolytic activity in cells treated with melatonin, which indicates that this is not among the mechanisms involved in its anti-invasive effects.

Taken together, our results demonstrate that melatonin reduces not only the proliferation of MCF-7 cells in vitro but also their invasiveness, causing a decrease in cell attachment and cell motility probably by interacting with mechanisms of MCF-7 cell invasion mediated by 17β-estradiol.

Motility and attachment are two key cellular functions for the process of tumor metastasis. The metastatic spread of tumors is dependent on the motility and invasiveness of the cells as well as on their adhesive properties toward the extracellular matrix. The inhibition of one or more of these cellular functions may lead to the decreased metastatic potential of the tumor. Tumor cell motility and invasion are adhesion-dependent phenomena related to the presence of cell surface adhesion molecules for both cell-cell and cell-matrix interactions. A down-regulation or a loss of expression of these cell
surface adhesion molecules correlates with an increase in the invasiveness of tumor cells as well as with poor cell differentiation and bad prognosis of the tumor process (21–24, 38–40). The expression of E-cadherin, a calcium-dependent membrane protein responsible for cell-cell contact, has been inversely correlated with in vitro invasion and tumor cell differentiation (23, 40). Here we found an increase in cell surface E-cadherin expression after melatonin treatment. In addition, the expression of the β1 integrin (a subunit of integrins, which are receptors that regulate interaction between cells and the extracellular matrix) has been correlated with cell differentiation and is generally down-regulated in breast cancer, especially in its invasive components (21, 41). An important finding in our study was that melatonin increases β1 integrin expression in MCF-7 cells.

Fig. 9 depicts the evolution of human breast cancer cells from poorly to highly invasive cells in terms of the acquisition or loss of markers that represent the differentiation status of the cell. The progression traverses a spectrum of invasiveness from the poorly invasive cells (estrogen receptor-positive cells) to the highly invasive cells that are poorly differentiated cells, which lose estrogen receptors and E-cadherin expression, have a lower expression of integrins, and acquire vimentin expression. Our results suggest that melatonin shifts MCF-7 human breast cancer cells to a lower invasive status, increasing β1 integrin subunit expression and E-cadherin expression and promoting the differentiation of tumor cells, as has been demonstrated previously by morphological and morphometric studies of our group (42).

It has been shown that treating mice bearing established MCF-7 tumors with estrogens and antiestrogens modulates tumor growth in vivo. Estrogen deprivation or antiestrogen treatment inhibits tumor growth (18, 43). Our results suggest that the in vitro anti-invasive effect of melatonin could also be correlated with an in vivo decrease in the tumorigenicity of MCF-7 cells induced by melatonin. However, because of the high mortality of nude mice and the consequently low number of animals that survived until the end of the experiment, we still could not demonstrate this hypothesis. In this regard, Das Gupta and Terz (44) demonstrated that the ablation of the pineal gland in Syrian hamsters increases the growth and spread of malignant melanoma. A recent study suggests that melatonin may amplify the therapeutic efficacy of tamoxifen in women with metastatic breast cancer and induce objective tumor regression in patients who have not responded to previous therapy with tamoxifen alone (45). The anti-invasive actions of melatonin could play an important role in stopping the progression of the disease in these patients. Finally, we can conclude that our study points out the importance of the anti-invasive actions of melatonin as a part of the oncostatic action of melatonin. Melatonin could delay cancer progression not only via inhibition of the proliferation of tumor cells, but also as a direct antagonist of metastatic cell functions.

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*Cancer Res* 1998;58:4383-4390.