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Melatonin Reduces Prostate Cancer Cell Growth Leading to Neuroendocrine Differentiation via a Receptor and PKA Independent Mechanism

Rosa M. Sainz, Juan C. Mayo, Dun-xian Tan, Josefa León, Lucien Manchester, and Russel J. Reiter*

Department of Cellular and Structural Biology, University of Texas Health Science Center, San Antonio, Texas

BACKGROUND. Melatonin, the main secretory product of the pineal gland, inhibits the growth of several types of cancer cells. Melatonin limits human prostate cancer cell growth by a mechanism which involves the regulation of androgen receptor function but it is not clear whether other mechanisms may also be involved.

METHODS. Time-course and dose-dependent studies were performed using androgen-dependent (LNCaP) and independent (PC3) prostate cancer cells. Cell number, cell viability, and cell cycle progression were studied. Neuroendocrine differentiation of these cells was evaluated by studying morphological and biochemical markers. Finally, molecular mechanisms including the participation of melatonin membrane receptors, intracellular cAMP levels, and the PKA signal transduction pathway were also analyzed.

RESULTS. Melatonin treatment dramatically reduced the number of prostate cancer cells and stopped cell cycle progression in both LNCaP and PC3 cells. In addition, it induced cellular differentiation as indicated by obvious morphological changes and neuroendocrine biochemical parameters. The role of melatonin in cellular proliferation and differentiation of prostate cancer cells is not mediated by its membrane receptors nor related to PKA activation.

CONCLUSIONS. The treatment of prostate cancer cells with pharmacological concentrations of melatonin influences not only androgen-sensitive but also androgen-insensitive epithelial prostate cancer cells. Cell differentiation promoted by melatonin is not mediated by PKA activation although it increases, in a transitory manner, intracellular cAMP levels. Melatonin markedly influences the proliferative status of prostate cancer cells. These effects should be evaluated thoroughly since melatonin levels are diminished in aged individuals when prostate cancer typically occurs. *Prostate 63: 29–43, 2005.* © 2004 Wiley-Liss, Inc.

KEY WORDS: **melatonin; neuroendocrine differentiation; prostate; cancer; proliferation**

INTRODUCTION

Prostate cancer is the most common malignancy among males in the United States [1]. Moreover, it is the most common cause of cancer-related death among men in northern Europe and the second most common in the United States [2]. Prostate cancer is characterized by the most rapid increase in incidence of all malignancies in older men. Since prostate is an organ dependent on androgens for its normal development and function, prostate cancer patients respond initially to hormonal ablative therapies. However, within 1–3 years of treatment the disease generally recurs

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*Correspondence to: Russel J. Reiter, Department of Cellular and Structural Biology, Mail Code 7762, University of Texas Health Science Center, San Antonio, 7703 Floyd Curl Drive, San Antonio, TX. E-mail: reiter@uthscsa.edu

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and these recurrent tumors no longer require androgens for growth [3]. Furthermore, they tend to be highly resistant to conventional cytotoxic agents [4]. Hormone-refractory prostate cancer cells develop resistance to radiotherapeutic and chemotherapeutic treatments [5,6] by a mechanism that it is not fully understood but might involve a disruption of the intracellular pathways that lead to apoptosis [7] and the expression of antiapoptotic proteins [8]. This tumor progression to the androgen-refractory stage is a complex phenomenon.

Clinical incidence of prostate cancer is low in Asians and the highest in African-Americans and Scandinavians. In Japan and other Asian countries the incidence of latent small or non-infiltrating prostate cancer is the same but the mortality rate is much lower than in occidental countries. However, this mortality increases in those individuals that move to America indicating the possibility of exogenous factors in the control of the progression of the disease [9]. Traditional Asian diets are low in animal proteins and fat but rich in fiber and phytoestrogens with antioxidant activity. Since conventional treatments are not successful in the control of prostate cancer progression, prevention has focused on the possible role of nutrition. Researchers have been attentive to antioxidant and non-antioxidant properties of various dietary substances. Vitamins, carotenoids, phytoestrogens, and polyphenols have been the focus of several studies. Not only have these antioxidants reduced cell proliferation in the prostate but, also, some of them increased the efficiency and efficacy of conventional treatments (for review see [10]). Their interference with reactive oxygen species (ROS) which cause macromolecular damage and play an important role in tumor development and aging [11] might be the main reason.

Melatonin is a potent antioxidant mainly synthesized by the pineal gland of all vertebrates. Its primary production during the dark phase of the light-dark cycle makes melatonin a major player in the coordination of circadian rhythms and seasonal breeding in photoperiodic species [12]. Antioxidant properties of melatonin have been demonstrated in many in vivo and in vitro systems [13]. Melatonin has been considered to be associated with age-related diseases because there is a persistent reduction in circulating melatonin throughout life due to the gradual failure of the melatonin synthesizing machinery in the pineal gland [14]. Melatonin levels in old individuals are a fraction of those in young but, interestingly, blood melatonin levels are better preserved in healthy elderly than in those individuals who are frail. Since the early 80s, a link between melatonin and neoplasic growth has been obvious [15]. Serum melatonin levels are lower in prostate cancer patients than in matched-aged controls [16]. Membrane melatonin receptors have been found in benign prostate epithelial cells in which the indole reduces cell growth and viability [17]. Likewise, melatonin reduces cell growth of androgen-sensitive LNCaP cells [18] but its role in hormone-independent prostate cancer cells, i.e., PC3 cells, is more controversial and may depend on cellular plating density [19]. The antiproliferative role of melatonin in prostate tumor growth was also demonstrated in vivo in athymic nude mice implanted with LNCaP cells but not with PC3 xenografts [20]. The presence of melatonin membrane receptors and its interplay with androgen receptor function [21] have been proposed as the molecular mechanisms by which melatonin restrains prostate cancer cell growth.

Several controversies have emerged as to the role of melatonin in prostate cancer cell growth. Some authors believe that melatonin's antiproliferative properties are restricted to those cells which are androgen sensitive and contain melatonin membrane receptors, while others contend its antiproliferative role exists in both androgen sensitive or insensitive cells with or without melatonin membrane receptors [22]. In the current study, by using pharmacological concentrations as well as melatonin receptor antagonists of the indole, we attempted to avoid melatonin actions that are mediated by membrane receptors. We found that melatonin not only participate in the control of prostate cancer cell proliferation for both androgen sensitive and insensitive cancer cells, but also the indole induces the differentiation of the cells by a mechanism not mediated by its membrane receptors or involving PKA.

MATERIALS AND METHODS

Cell Culture and Reagents

Human androgen-sensitive LNCaP prostate cell line was obtained from American Type Culture Collection (Rockville, MD). Cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic cocktail (containing penicillin, streptomycin, amphotericin B, and fungizone). Human androgen-insensitive PC3 cells were also obtained from American Type Culture Collection. Cells were grown in a mixture of DMEM/F12 (1:1) supplemented with 10% FBS, 15 mM Hepes, and 1% antibiotic-antimycotic cocktail. All cell culture reagents were purchased from Gibco-BRL, Rockville, MD. Cells were grown at 37°C in a humidified 5% CO_2 environment. The medium was changed every 2 days and cultures were split twice a week. Cells were subcultured before each experiment, seeded at a density of 25,000 cell/ml and allowed to attach overnight before treatment. Ultra pure grade melatonin was a gift from Helsinn Chemical (Biasca, Switzerland). Melatonin was always freshly prepared

in a DMSO stock solution 1,000× and diluted to a desired concentration directly in the culture medium. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolim bromide (MTT), propidium iodide (PI), Bt_2cAMP, isobutylmethylxanthine (IBMX), H89 (*N*-[2-((*p*-bromocinamyl)amino)ethyl]-5-isoquinoline-sulfonamide), and Pertussis toxin (Px) from *Bordetella pertusis* were obtained from Sigma Chemical Co. (St. Louis, Mo). Polyvinyl alcohol mounting medium with DABCO was purchased from Fluka Riedel-deHaen (Milwaukee, WI). The melatonin agonist *N*-acetyl-5-hydroxytryptamine (*N*-acetylserotonin) was purchased from Sigma Chemical Co. Luzindole and S20928 were used as melatonin antagonists. Luzindole was purchased from TOCRIS (Bristol, UK), dissolved in DMSO at a concentration 25 mM and stored in small aliquots at −20°C until used. S20928 was a kind gift of Dr. David E. Blask (Bassett Research Institute, Cooperstown, NY). It was also prepared as a stock solution (100 mM in DMSO) and stored in small aliquots at −20°C. Appropriate concentrations of both reagents were obtained by diluting stocks solutions in complete media. The rest of reagents were purchased from Sigma Chemical Co.

MTT Reduction Assay

Cells were seeded in 96-well plates at a density of 2,500 cells per well and allowed to attach before the addition of the drugs. Doses of melatonin used in each experiment are indicated in ''Results'' section. All the experimental and control groups were cultured in the very same conditions. Thus, all melatonin groups were adjusted to the same percentage of DMSO and even when the treatments did not required the presence of DMSO for solution, we added the maximal concentration of DMSO used to dissolve melatonin into the culture to avoid solvent interference with the results. After treatments, 10 μl MTT (stock solution 5 mg/ml of phenol red free Dulbecco's PBS) was added to each well. Four hours later, 100 μl lysis buffer (20% SDS in 50% formamide at pH 4.7) was added and cells were lysed at 37°C overnight. Thereafter, samples were measured in an automatic microplate reader Perkin-Elmer7000 BioAssay Reader at 570 nm. Media was estimated from the results of seven samples in each experimental group. Experiments were performed at least three times.

Cell Growth and Viability Assays

Cell growth was estimated by counting cells at different time points after treatment. Once cells were attached, treatments were performed as appropriate. Cells were harvested after trypsinization, collected by centrifugation for 5 min at 200*g* and washed several times in ice-cold Dulbecco's phosphate buffer (DPBS) (pH = 7.4). Then, cells were either stained with 200 μl of 0.04% trypan blue solution to evaluate cell viability or fixed in 1 ml of 4% paraformaldehyde to count cell number. Four samples per group were counted in each experiment and the experiments were performed at least four times.

Cell Cycle Analysis by Flow Cytometry

Cells were seeded in a 100 mm dishes and allowed to attach overnight before treatments were performed. After treatment, cells were harvested by trypsinization and collected by centrifugation for 5 min at 200*g*. After washing twice in ice-cold DPBS without Ca^{2+} and Mg^{2+} supplemented with 1 g/L glucose, cells were stained with 500 μl PI staining solution (100 μg/ml PI, 1,000 kU RNase-DNase free in PBS plus glucose) for 30 min at room temperature in darkness. DNA content was analyzed in at least 20,000 nuclei by using a Becton-Dickinson Immunocytometry Systems FACStar Plus flow cytometer, equipped with an argon-ion laser. Experiments were performed at least three times and three samples per group were analyzed in each one.

Morphological Studies

Cells were grown on small coverslips (NUNC, Rochester, NY) in 100 mm tissue culture dishes. After leaving the cells to attach overnight, treatments were performed. Cells were cultured at the times indicated in ''Results'' section and then cell-attached coverslips were carefully rinsed with ice-cold DPBS (pH 7.4) and fixed in 4% paraformaldehyde in 50 mM phosphate buffer (pH 7.4) at 4°C overnight. The following day, cells were washed once and mounted on a slide. Cells were observed under an Olympus IX-70 microscope, and recorded using an ORCA100 CCD camera (Hamamatsu, East Syracuse, NY).

The neuroendocrine phenotype was defined by cells with neuritic processes at least two times the length of the cell body. The percentage of neuroendocrine cells (% NE cells) was quantified. The morphology of 50–100 cells was examined in at least five randomly selected microscopic fields per group. Results were expressed as the mean ± SEM of four independent experiments.

Immunoblotting

Cells were seeded in 100 mm dishes, and after treatment, they were washed in ice-cold DPBS and incubated in lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% SDS, 1% Igepal C, 0.5% sodium deoxycholate, 10 μg/ml leupeptin, 2 μg/ml aprotinin, and 1 μg/ml pepstatin) on ice for 20 min. Lysates were clarified by centrifugation at 10,000*g* for 10 min at 4°C and the

concentration of proteins was estimated using Bradford reagent from Sigma. Fifty micrograms of protein was loaded into 7.5% SDS–polyacrylamide gels. Proteins were transferred to nitrocellulose filters and blots were probed with anti-neuron specific enolase (NSE) mouse monoclonal antibodies. After stripping, membranes were reacted with anti-actin pan Ab-5 mouse monoclonal antibodies (1:1,000) from Lab Vision Corporation (Fremont, CA) overnight at 4°C. Secondary antibodies were visualized by enhanced chemiluminescence using HRP-conjugated anti-mouse secondary antibodies (Amersham Pharmacia, Buckinghamshire, UK). Band intensity was estimated using the Scion Image Beta 4.02 for Windows™ analysis software, downloaded at the web site address http://www.scioncorp.com.

cAMP Measurement

LNCaP cells were seeded in 24-well plates at 100,000 cells per well and treated as indicated. Then, cultures were extracted for 1 hr at 37°C in 0.1 N HCl. After acetylation, intracellular cAMP was assayed by direct enzyme immunoassay following the specifications of the manufacturer (Cayman Chemical, Ann Arbor, MI).

Statistical Analysis

Data are presented as mean ± SEM of at least three different experiments using four samples per group unless otherwise indicated. A one way ANOVA was performed to compare different groups, followed by a Student–Newman–Keuls *t*-test. Statistical significance was considered when $P < 0.05$.

RESULTS

Melatonin Effects on Cell Growth and Viability

In order to evaluate the antiproliferative role of melatonin on prostate cancer cells, two-widely used human prostate cancer cell lines, LNCaP and PC3, were employed. These cell lines differ in several parameters including androgen responsiveness and melatonin membrane receptors presence, which make them excellent models to evaluate the antiproliferative role of melatonin and the possible mechanisms involved. First we studied, by using MTT reduction assay and cell counting, the number of cells present in the culture after melatonin treatment. As shown in Figure 1A, MTT reduction was significant lower after culturing LNCaP cells with melatonin for 4 and 6 days. Doses of melatonin higher than 0.5 mM decreased cellular growth more than 50% after 6 days of culture. The graph shows a clear dose and time-dependent effect. Similarly, several doses of melatonin decreased MTT reduction in PC3 cells (Fig. 1B). In this model, melatonin seemed to be less effective, since concentrations greater than 0.5 mM were necessary to obtain a statistically significant effect. Cell counting also confirmed these data. Thus, after 2 days of culture, significant fewer LNCaP cells were found in those groups treated with melatonin than in those cultured only in the presence of the vehicle. Also, melatonin reduced the number of PC3 cells (Fig. 1D) but differences were less obvious than in LNCaP cells.

MTT reduction assay is considered both a cell growth and a cytotoxicity assay. Even though we observed no evidence of cytotoxicity, this was proven by using the vital dye trypan blue. In Table I, we show that 1 mM melatonin decreased the number of LNCaP cells up to a $59.42 \pm 1.91\%$ versus control group without changing the percentage of viable cells, since cell viability was the same for melatonin cultured cells and cells cultured in the presence of the vehicle alone. Melatonin also did not cause any change in PC3 cell viability (data not shown). Given these findings, we considered MTT assay as an adequate test to estimate cell growth in our system.

As shown in Figure 2, melatonin decreased cell proliferation in both LNCaP and PC3 prostate cancer cells by arresting cell cycle progression at the G_0/G_1 stage. When the concentration of melatonin was increased, the percentage of cells in S-phase diminished dramatically. Cells were analyzed 6 days after incubation with melatonin or with the vehicle. As shown previously, LNCaP cells seemed to be more sensitive to melatonin. Thus, 0.5 mM melatonin reduced total S-phase cells from 31.91% in controls to 21.12%, 1 mM to 17.85%, and 2 mM to 8.61%. The highest dose of melatonin used decreased the number of cells in S phase by more than 73% without changing the G_2/G_1 ratio. In addition, only 17.57% of the PC3 cells were found at S-phase when harvested. However, the presence of melatonin also reduced the total S-phase percentage in a dose-dependent manner; 0.5, 1, and 2 mM melatonin lowered the number of total cells in S-phase to 16.01, 12.61, and 7.40%, respectively.

Morphological Studies of Cell Differentiation

Surprisingly, cells cultured with melatonin for 6 days showed a clear morphological change with respect to those cultured without melatonin. LNCaP cells emitted long prolongations when melatonin was present in the culture media. Also, cell bodies were more rounded and smaller. These morphological changes are similar to changes described in the literature when LNCaP cells undergo differentiation toward a neuroendocrine

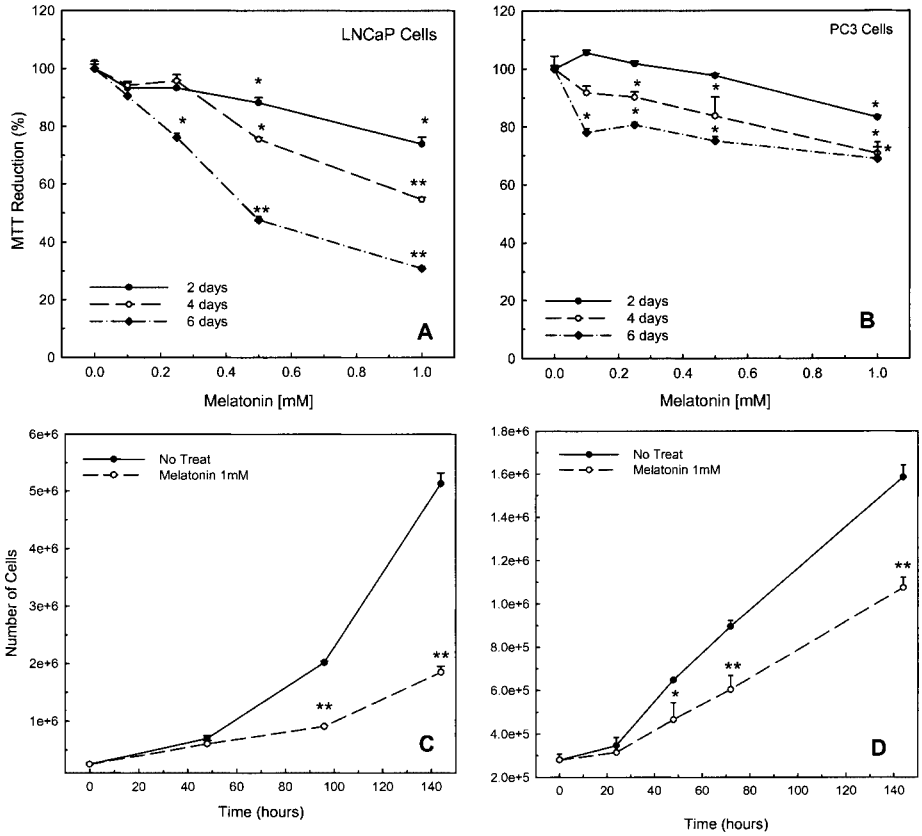

Fig. 1. Melatonin inhibition of LNCaP and PC3 cell proliferation. Cells were seeded in 96 -well plates at a density of 2,500 cells per well. After 2, 4, and 6 days, 10 μl of 5 mg/ml MTT was added to each well. Absorbance was determined 4 hr later for LNCaP (**A**) and PC3 (**B**) cells; $*P < 0.01$ versus CON, $**P < 0.001$ versus CON. In addition, LNCaP cells (**C**) and PC3 cells (**D**) were seeded at a density of 25,000 cells per ml in 35 mm dishes and counted after 2, 4, and 6 days of culture in presence or absence of 1 mM melatonin. Treatments were replenished every 2 days; $*P < 0.01$ versus no treat, $**P < 0.001$ versus no treat.

phenotype [23,24]. Figure 3 illustrates how LNCaP cells treated with 1 mM melatonin for 6 days (Fig. 3B) displayed a clear neuroendocrine morphology as compared to the control cells (Fig. 3A). Some of the cells showed a bipolar morphology while other cells were multipolar as shown in detail in Figure 3C. Similarly, PC3 cells responded to 1mM melatonin by changing their morphological appearance. After melatonin treatment, cells acquired an elongated morphology and sometimes they had long processes (Fig. 3E,F) which contrasted with the control cells (Fig. 3D).

TABLE I. Percentage of Cell Viability and Cell Number After MelatoninTreatment

	Number of cells (% vs. CON)	Cell viability (%)
Control	100 ± 5.01	89.15 ± 0.43
10 nM melatonin	89.27 ± 5.13	89.63 ± 0.95
10 μM melatonin	87.96 ± 2.78	91.46 ± 0.82
1 mM melatonin	59.42 ± 1.91*	92.03 ± 2.65

Cells were cultured with melatonin for 6 days. Then, cells were harvested and stained with trypan blue. Data are represented as the percentage of cells versus control. The number of cells in control group was considered 100%. $*P < 0.01$ versus rest of groups.

Neuronal specific enolase (NSE), a widely used neuroendocrine marker, was employed to identify the nature of cell differentiation, i.e., epithelial or neuroendocrine. As expected by the morphological studies, melatonin increased protein levels of NSE in both LNCaP and PC3 although its effect on LNCaP cells was more dramatic (Fig. 3G).

Neuroendocrine cells were quantified by counting those which had processes two times the length of the cell body. Five fields were selected at random for each experimental group and at least 50–100 cells were examined. Cells (LNCaP and PC3) with long processes cultured with 1 mM melatonin for 6 days were counted. As shown in Table II, both cell lines contained an increased number of neuroendocrine cells after culturing in the presence of melatonin. The percentage of LNCaP cells with long processes increased from

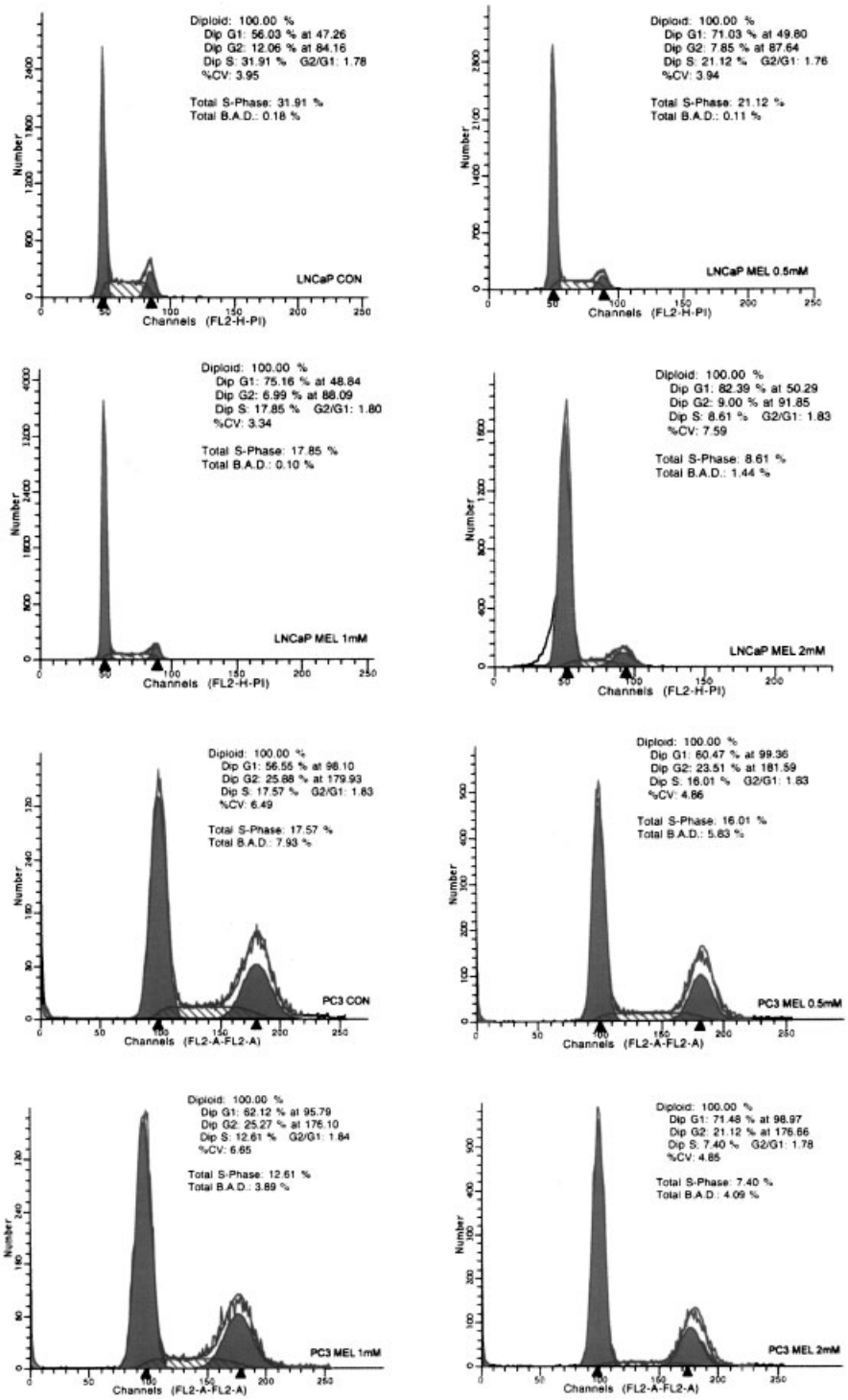

Fig. 2. Flow cytometry analysis of cell cycle. After 6 days of melatonin treatment, cells were harvested and the DNA was stained with PI. Twenty thousand nuclei were analyzed in each sample. The experiment was repeated three times and a representative experiment is shown.

$5.85 \pm 0.87\%$ in the controls to 34 ± 2.24 in cells treated with melatonin. Similarly, the number of PC3 cells with morphological characteristics of neuroendocrine cells increased from 4.4 ± 1.32 to 18.0 ± 1.51. As mentioned before, melatonin was again more effective on LNCaP cells than it was on PC3 cells.

Role of Melatonin Agonists and Antagonists on Prostate Cancer Cell Proliferation and Differentiation

Previous studies have demonstrated that the role of physiological doses of melatonin in the control of cell

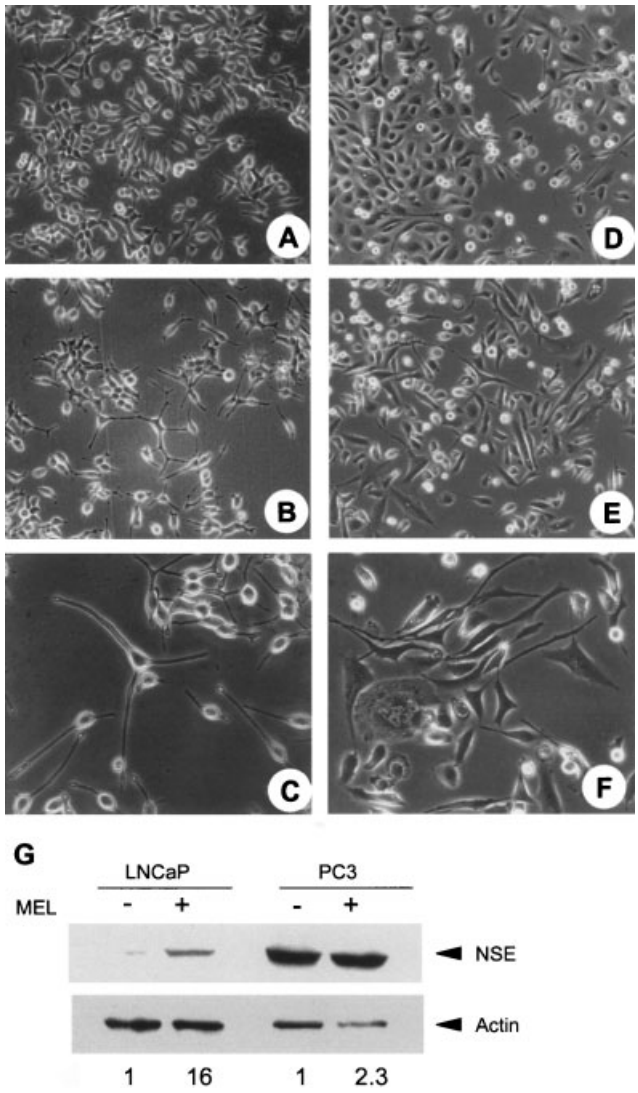

Fig. 3. Microscopy study of cell morphology and Western-blot of NSE. Cells were seeded at a density of 25,000 cells/ml and cultured with or without 1 mM melatonin for 6 days. **A**: LNCaP control, (**B**,**C**) LNCaP plus melatonin, (**D**) PC3 control, (**E**, **F**) PC3 plus melatonin. In addition, cells were seeded at a density of 25,000 cells/ml, cultured with or without 1 mM melatonin and proteins were extracted. Fifty micrograms of total protein was loaded in a 10% PAGE gel. Proteins were blotted into nitrocellulose filters and reacted with NSE and actin monoclonal antibodies (**G**).

proliferation is mediated by its transmembrane receptors, through a mechanism which implicates adenylate cyclase inhibition and a reduction of intracellular cAMP levels. Melatonin concentrations used in the present study were much greater than those used when studying its interaction with receptors. However, in order to determine whether melatonin membrane receptors do or do not play a role in proliferation and differentiation of prostate cancer cells, several antagonists or agonists were used. S20928, luzindole, and

TABLE II. Percentage of Neuroendocrine Cells After Melatonin Treatment

	LNCap cells	PC3 cells
Control	5.85 ± 0.87	4.4 ± 1.32
1 mM melatonin	34.0 ± 2.24*	18.0 ± 1.51*

Cells were cultured with or without 1 mM melatonin for 6 days. Then, cells were harvested, fixed, and photographed. Five randomly-selected fields were counted and 50–100 cells were examined. Data are presented as the percentage of cells with processes at least two times the length of their cell body. *$P < 0.01$ versus control.

N-acetylserotonin compete for melatonin binding sites displacing melatonin in a concentration-response manner [25–27]. The competitive melatonin receptor blockers, luzindole and S20928, antagonize both the melatonin-induced inhibition of forskolin-stimulated cAMP formation in Chinese hamster ovary (CHO) cells expressing human melatonin receptors and the melatonin-induced potentiation of norepinephrine-mediated vasoconstriction in rat caudal artery [28].

Thus, we observed that the presence of the antagonist S20928 in the culture was incapable of preventing melatonin's antiproliferative action. S20928 was added for at least 1 hr prior to the addition of melatonin. S20928 did not inhibit melatonin's ability to reduce cell growth of either LNCaP (Fig. 4A) or PC3 cells (Fig. 4B). Moreover, higher concentrations of S20928 were toxic to the cells and could not be used (data not shown). Likewise, luzindole, a commercially available and widely used melatonin antagonist, was not functional in reducing melatonin's antiproliferative capacity in LNCaP (Fig. 4C) or PC3 cells (Fig. 4D). We used a melatonin agonist, *N*-acetylserotonin, to confirm that melatonin receptors do not participate in its antiproliferative actions. Identical doses of melatonin or *N*-acetylserotonin were added to cell cultures for 6 days and cellular growth was estimated. As shown above, doses of melatonin higher than 0.5 mM were very effective in inhibiting prostate cancer cell proliferation but *N*-acetylserotonin had no effect (Fig. 4E,F). As before, melatonin was more effective in diminishing LNCaP than PC3 proliferation.

Melatonin membrane receptors are linked to a Pertussis toxin (Px) sensitive G-inhibitory protein. Thus, melatonin receptor mediated pathways are blocked by using Px. We used Px before the addition of melatonin and then evaluated cell growth in LNCaP cells after 6 days of culture. Px did not prevent melatonin's antiproliferative role on LNCaP cells at the pharmacological doses used (Fig. 5). Also, none of these drugs altered the effects of melatonin on cellular differentiation (data not shown).

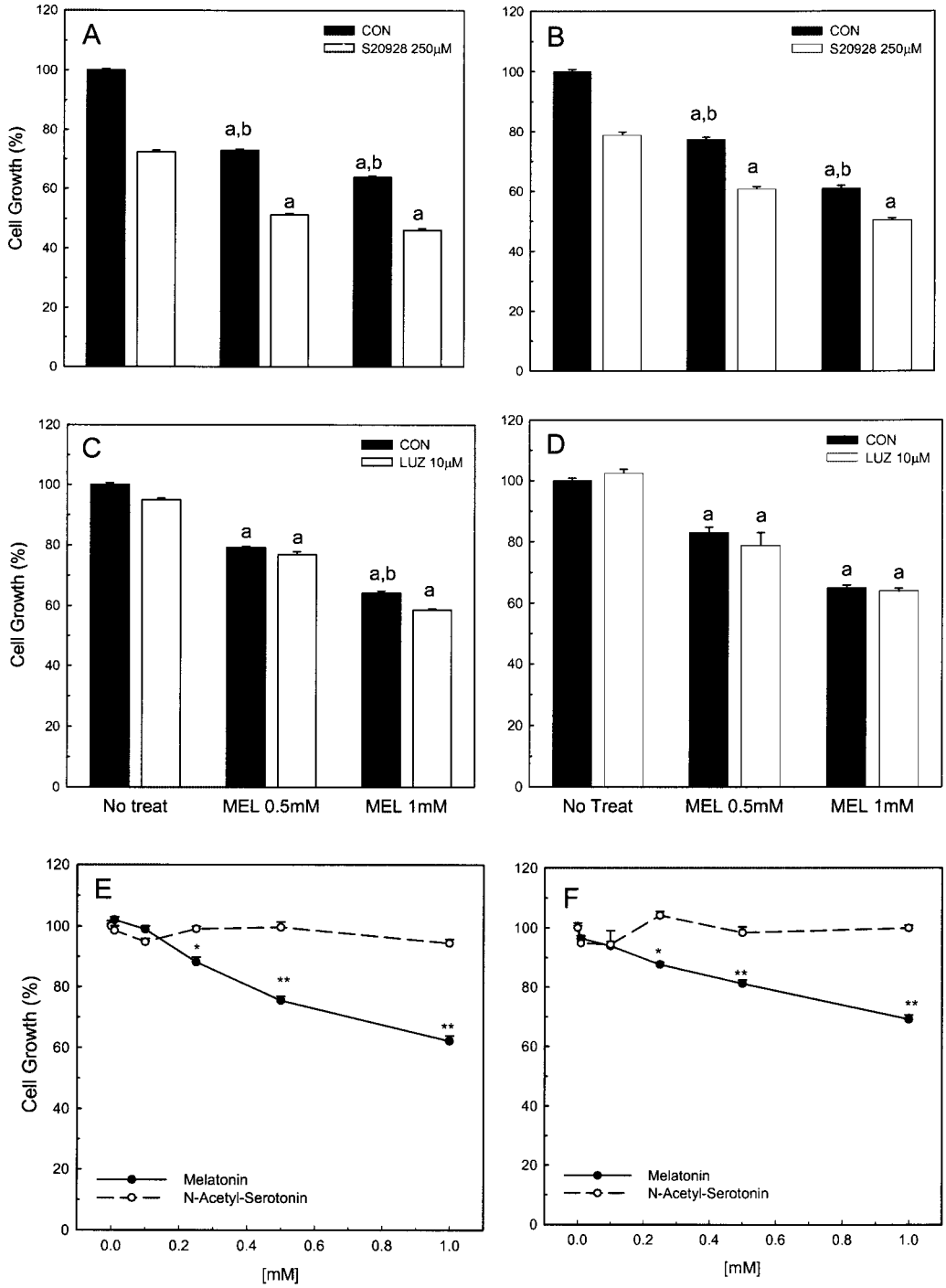

Fig. 4. Effects of melatonin agonist and antagonists on prostate cancer cell proliferation. LNCaP cells (**A**) and PC3 (**B**) cells were cultured with or without 250 μM S20928 for 1 hr. Then, 0.5 and 1 mM melatonin was added and cells were cultured for 6 days. After that, 10 μl of 5 mg/ml MTT solution were added and absorbance was determined 4 hr later. Likewise, cells were treated with 10 μM luzindole for 1 hr and then, with or without 0.5 and 1 mM melatonin for 6 days. Treatments were replenished every 2 days. MTT reduction was analyzed by reading absorbance at 570 nm of LNCaP (**C**) and PC3 (**D**) cells. **a**: $P < 0.01$ versus no treat; **b**: $P < 0.01$ versus S20928 group or luzindole group. LNCaP (**E**) and PC3 (**F**) cells were cultured with several concentrations of *N*-acetylserotonin or melatonin for 6 days. Cellular growth was estimated by MTT reduction as mentioned; $^{*}P < 0.01$ versus CON, $^{**}P < 0.001$ versus CON. Experiments were repeated three times.

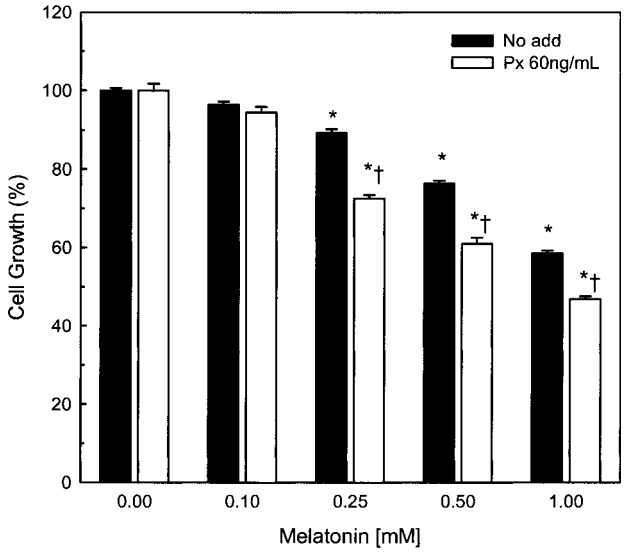

Fig. 5. Effect of melatonin on prostate cancer cell proliferation in presence or absence of Pertussis toxin (Px). LNCaP cells were cultured with Px for 1 hr. Then, melatonin was added at different concentrations. Cell growth was estimated by adding 10 μl MTT 5 mg/ml. Seven samples per group were analyzed; $*P < 0.01$ versus no melatonin treatment, $\dagger P < 0.01$ versus respective melatonin groups. The experiment was repeated three times.

cAMP Studies

Prostate cancer cell differentiation is promoted by several agents including those which increase intracellular cAMP concentrations [23]. Although membrane melatonin receptors are linked to an inhibitory G-protein which decreases intracellular cAMP levels after activation [29], some examples in which melatonin treatment increases cAMP levels have also been described [30]. For this reason, the participation of cAMP in the role of melatonin in prostate neuroendocrine differentiation was studied. First, we observed that the concentrations of Bt_2cAMP necessary to promote neurite like processes in LNCaP cells after 6 days of culture are similar to melatonin. Figure 6 shows that melatonin (B) and Bt_2cAMP (C) promoted cellular differentiation. In addition, NSE expression was also increased by the presence of either drug as shown in Figure 6D. Similar effects of melatonin and Bt_2cAMP were also found in reducing cellular growth of LNCaP and PC3 prostate cancer cells (Fig. 7A). As shown before, melatonin's effect on LNCaP is more potent than on PC3 cells, but the antiproliferative effect of Bt_2cAMP seemed to be similar in both cell types.

The phosphodiesterase inhibitor, IBMX, was used to verify that the morphological changes observed in response to Bt_2cAMP on prostate cancer cells were due to increased cAMP levels and not to nonspecific effects of the dibutyryl cAMP [31]. By using IBMX the degradation of cAMP is prevented, and as a result, the effect of drugs which work by increasing intracellular cAMP levels is enhanced. To evaluate whether melatonin actions on prostate cancer cells would be improved by substances which preserve cAMP levels, IBMX was employed in combination with melatonin. Proliferation and NSE expression were evaluated in the presence or absence of IBMX when cells were cultured with or without melatonin. The results obtained demonstrate that melatonin's antiproliferative action on LNCaP cell growth was enhanced by the phosphodiesterase inhibitor IBMX (Fig. 7B). No significant potentiation in terms of NSE expression was observed. This may be a result of the increase of the basal protein levels caused by IBMX alone (Fig. 6D).

Having found that cAMP may be essential for melatonin's effect, we studied intracellular cAMP levels after melatonin treatment in LNCaP cells. Melatonin promoted cAMP accumulation soon after treatment. Forskolin was used as a positive control. The rise in cAMP occurred rapidly and then, the values quickly returned to control intracellular levels (Fig. 7C). Since melatonin augmented cAMP levels, the participation of PKA on LNCaP cell proliferation and differentiation induced by melatonin was evaluated. It was previously reported that nuclear translocation of the catalytic subunit of PKA causes alterations in LNCaP morphology consistent with neuroendocrine differentiation [32]. As shown in Figure 7D, when using the PKA specific inhibitor H89, melatonin effect on cell proliferation of either LNCaP or PC3 cells was not prevented. Melatonin reduced LNCaP proliferation to $47.04 \pm 0.34\%$ and PC3 proliferation to $64.31 \pm 2.13\%$, while the presence of 5 μM H89 only modified slightly this effect (40.60 ± 0.82 and $69.21 \pm 1.8\%$, respectively). In addition, H89 failed to inhibit melatonin's ability to promote neuroendocrine differentiation (Fig. 8).

Effect of Pharmacological Doses of Melatonin in Testosterone-Induced Prostate Cell Growth

As mentioned above, melatonin inhibits androgen-stimulated cell growth of prostate cancer cells [18]. In this study, pharmacological doses of the indole modulated not only androgen-sensitive prostate cancer cells proliferation but also the growth of androgen-independent cells. We also tested whether pharmacological doses of melatonin could interact in androgen-stimulated cellular growth. To determine this, LNCaP cells were cultured with or without androgens in absence or presence of melatonin. Initially, cells were synchronized to interrupt the cell cycle; they were then grown in media depleted of steroids and cell division was subsequently stimulated by the addition of androgens. As shown in Figure 9, cells grew much more rapidly when testosterone was present in the culture media.

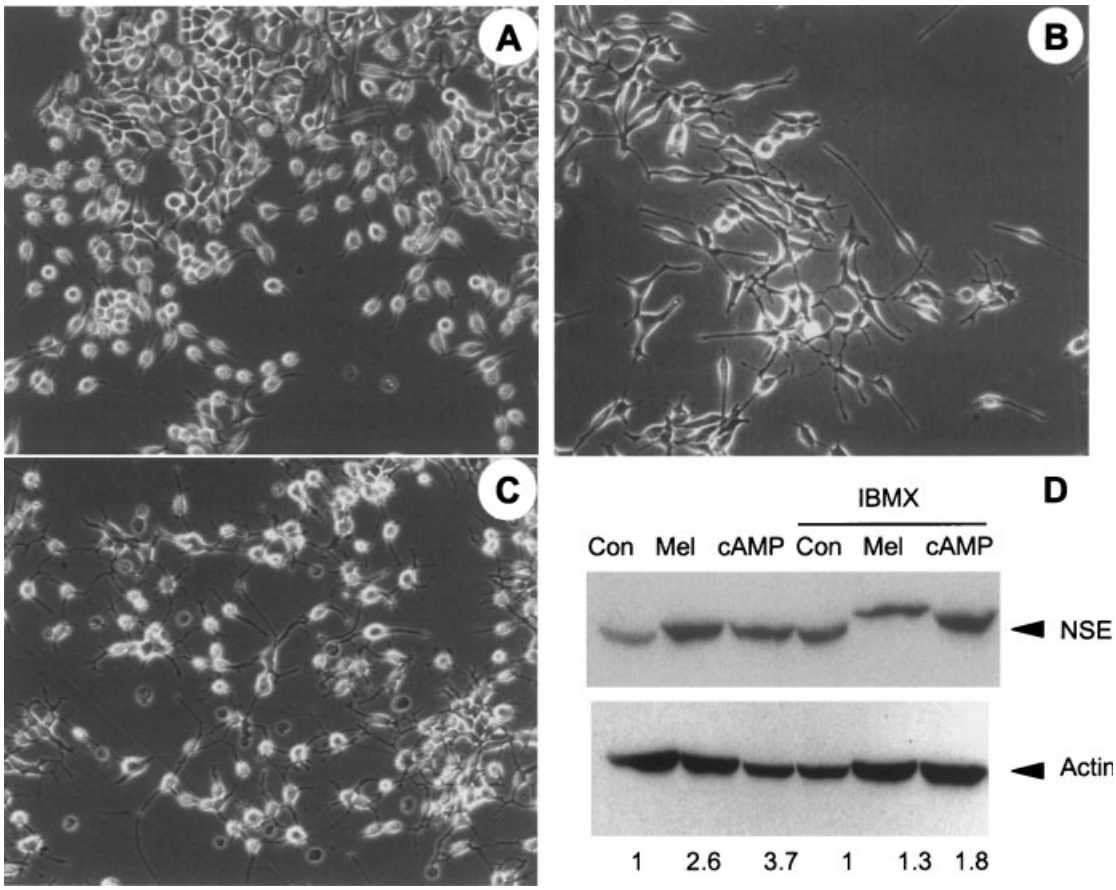

Fig. 6. Microscopy analysis of LNCaP cells morphology after melatonin or Bt_2cAMP treatment. Cells were seeded and cultured for 6 days; after that, cells were fixed and photographed. **A**: LNCaP controls, (**B**) LNCaP plus I mM melatonin, (**C**) LNCaP plus I mM Bt_2cAMP. Also, cells were cultured under the same conditions with or without IBMX 0.5 mM. Total proteins were extracted and loaded in a 10% PAGE gel. After blotting, they were reacted with NSE and actin monoclonal antibodies (**D**).

Significant differences were found after only 2 days of culture. Interestingly, melatonin diminished significantly cellular growth even in the presence of androgens in the culture media.

DISCUSSION

Neuroendocrine cells are a normal component of the prostate and they play an important role in gland development and function. Secretory products such us chromogranine and serotonin are important autocrine or paracrine regulatory agents in normal prostate growth or differentiation [1]. Also, neuroendocrine cells might also have a function in prostate cancer development or progression [33,34]. In prostate carcinomas, especially under androgen-ablation therapy, the neuroendocrine cell population increases by several-fold with scattered clusters among other malignant cells. Androgen deprivation of the prostate results initially in apoptosis of the secretory epithelial cells and reduces their number by 90%. However, not all the cells die and an androgen-independent population emerges and predominates. This progression seems to involve adaptive upregulation of genes that assist cells to survive and grow independent of the presence of androgen [1]. Thus, clinical studies have shown that neuroendocrine cells, which are a minor population of the prostate epithelium, accumulate in hormone refractory tumors and the levels of neuroendocrine markers increase in patients with a poor prognosis [35,36]. Even though there is a significant increase in neuroendocrine cells after androgen deprivation and their presence is related to a poor prognosis, the actual prognostic value of even extensive neuroendocrine differentiation is still debated [37,38]. These cells are non-dividing, post-mitotic cells [39] but they may contribute to the focal growth of cancer cells by providing paracrine stimuli for proliferation of the surrounding carcinoma cells [40].

Unlike other neuroendocrine cells, prostate neuroendocrine cells seem not to derive from the neural crest; rather, they appear to arise from epithelial stem

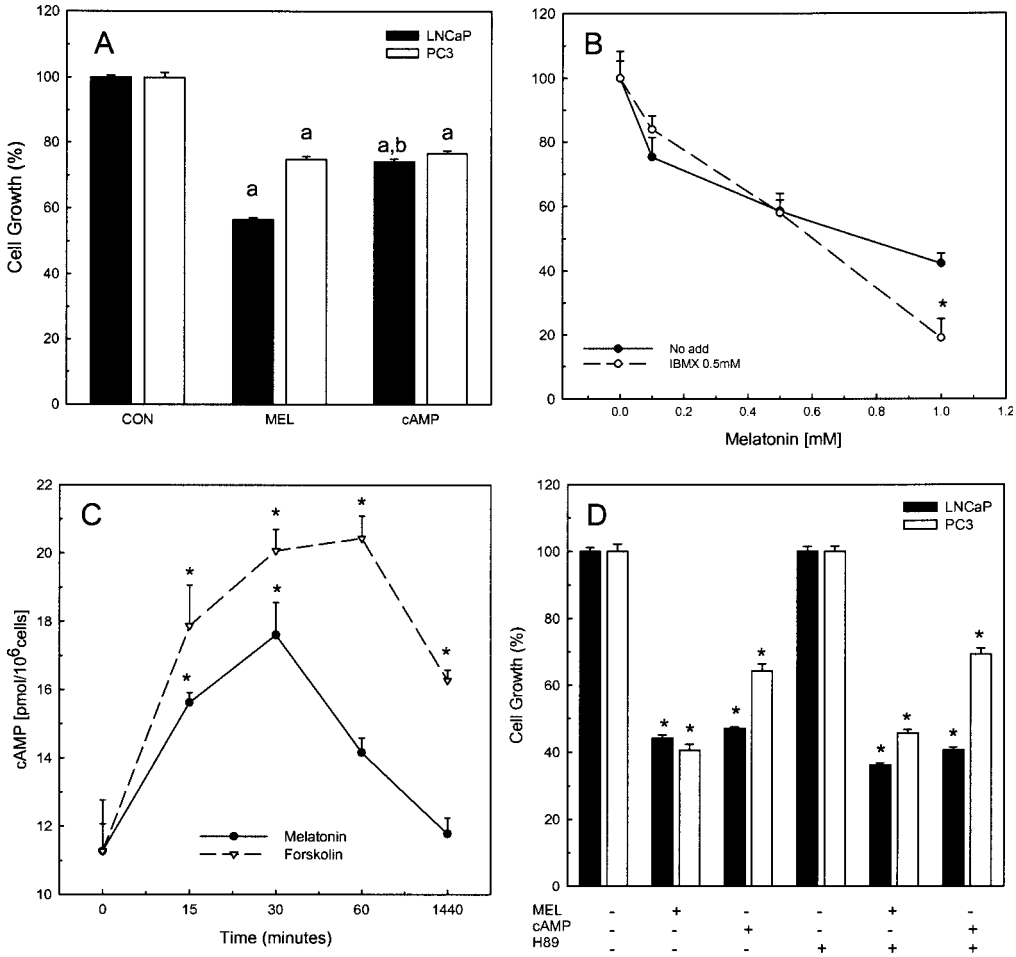

Fig. 7. Melatonin and Bt_2cAMP effect on cellular proliferation. Cellular growth was studied in LNCaP and PC3 cells cultured with melatonin or Bt_2cAMP for 6 days (**A**); **a**, $P < 0.01$ versus CON; **b**, $P < 0.01$ versus MEL. Cells were cultured with or without 0.5 mM IBMX in the presence or absence of 1 mM melatonin and cellular growth was calculated (**B**); *$P < 0.01$ versus 1 mM melatonin group. Total concentration of cAMP was calculated after culturing LNCaP cells for 15 min, 30 min, 1 and 24 hr with 1 mM melatonin or 10 μM forskolin. Cells were lysed with 0.1 N HCl and after acetylation, cAMP was estimated by immunoassay (**C**); *$P < 0.01$ versus control group. Finally, prostate cancer cell growth was studied when cells were cultured with or without 5 μM H89. After 1 hr of preincubation, melatonin and Bt_2cAMP were added. Cellular growth was calculated by MTT reduction as mentioned before (**D**); *$P < 0.01$ versus no treatment and H89 groups.

cells within the prostate given that they express several markers of prostate epithelial cells including prostate-specific antigen and androgen receptors [41]. Because of this, it is the current belief that neuroendocrine differentiation is a dynamic process and epithelial cells can differentiate to neuroendocrine cells depending on their extracellular milieu. Several agents have been shown to induce differentiation of epithelial prostate cancer cells in culture. Thus, androgen-responsive LNCaP cells acquire neuroendocrine characteristics in response to increased intracellular cAMP levels [23,31], long term androgen ablation [42], or stimulation with cytokines interleukin-1β and 6 [43]. Here, we describe, for the first time, that pharmacological concentrations of melatonin in culture also promote morphological and biochemical parameters of neuroendocrine differentiation of prostate cancer cells. Melatonin reduces cell proliferation and interrupts the cell cycle in both androgen-sensitive and insensitive prostate cancer cells in a dose-dependent manner. Melatonin's ability to inhibit growth of prostate cancer cells has been shown previously [18,20]; however, its exclusive role in androgen dependent cellular growth is controversial. Gilad et al. [19] claimed that melatonin works exclusively as an antiproliferative agent in PC3 cells when they are plated at low density; PC3 is a human steroid-independent prostate cancer cell line. However, melatonin increases proliferation when cells are grown under high density plating conditions. This effect of melatonin correlates with the ability of the indole to

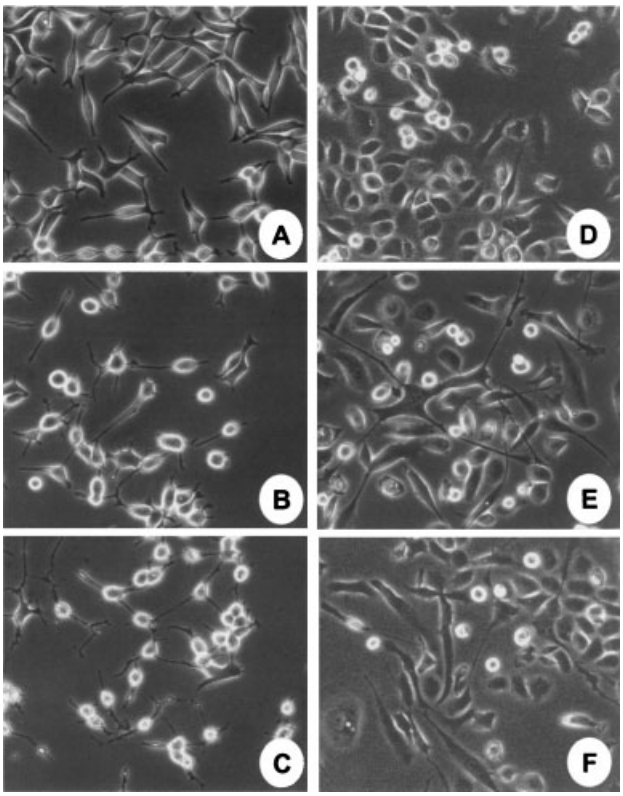

Fig. 8. Microscopy study of LNCaP cell differentiation after melatonin and melatonin plus H89 treatments. Cells were seeded at a density of 25,000 cells/ml of complete media. Cells were differentiated in the presence of 1 mM melatonin or 1 mM melatonin plus 5 μM H89. LNCaP cells control (**A**), LNCaP cells plus melatonin (**B**), LNCaP cells plus H89 and melatonin (**C**), PC3 control (**D**), PC3 cells plus melatonin (**E**), PC3 cells plus H89 and melatonin (**F**).

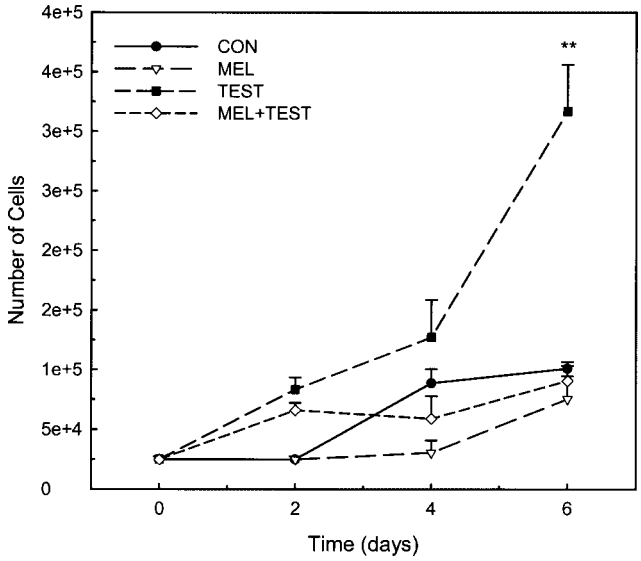

Fig. 9. Effect of melatonin in androgen-stimulated cellular growth. LNCaP cells were seeded at a density of 25,000 cells/ml. Cells were synchronized by depleting serum for 24 hr. Then, after incubating with melatonin for 3 hr proliferation was stimulated by addition of 1 nM testosterone. Cells were harvested 2, 4, or 6 days after and counted. $^{*}P < 0.01$ versus MEL + TEST group.

inhibit intracellular cAMP levels at low but augmented at high cell density. Xi et al. [20] found no effect of melatonin on the growth of PC3 xenografts in nude mice. However, the indole was extremely effective in reducing the growth of LNCaP-induced tumors in these immuno-deficient animals.

The studies described assume melatonin's actions are mediated via melatonin membrane receptors in LNCaP and with its subsequent ability to modulate androgen receptor translocation to the nucleus along with its transcriptional functions. The major difference in the work performed here relates to the concentration of melatonin used. In the earlier studies, antioxidant properties of melatonin were ignored as an important factor in modulating proliferation of the cancer cells. The physiological role of melatonin in tumor initiation or promotion is extremely interesting and the potent antioxidant and free radical scavenging activities of melatonin [13] may play a key role in several aspects of malignancy. In the current investigation, pharmacological concentrations of melatonin, which have been demonstrated to be strongly antioxidant [44–46], had a much greater effect on cellular proliferation in contrast to previous work [18]. It is possible that the negative results reported by Lupowthiz and Zisapel [18], who used longer incubation times in LNCaP cellular growth, may be a result of the rapid metabolism and depletion of melatonin from the culture medium. Of significant interest is the fact that although the antiproliferative actions of melatonin were obvious in both LNCaP and PC3 cells, the effect of melatonin was always greater in androgen-sensitive LNCaP cells than in androgen-independent PC3 cells.

A number of studies support the idea that prostate cancer growth is modulated by free radicals and antioxidants since androgens can contribute to an age-associated increase in prostate cancer by increasing oxidative stress. Even physiological concentrations of androgens induce ROS and cause a prolonged activation of redox sensitive transcription factors such us AP-1 and NFκB [47]. In addition, studies with dietary antioxidants provide encouraging data given that long-term α-tocopherol supplementation reduces prostate cancer incidence by 32% and mortality by 41% in cigarette smokers [48]. Similar results were obtained with other antioxidants including lycopene, a potent carotenoid, and selenium supplementation [49,50]. While, antioxidant mechanisms of melatonin on prostate cancer cells have been not studied in detail, the present findings strongly suggest that melatonin is not

exclusively working through its membrane receptor to limit prostate cell proliferation. The antagonists used did not block the antiproliferative actions of melatonin and the agonists of the indole were ineffective in reducing cell growth. At high concentrations the antagonists tested were found to be toxic. The compounds used herein have been widely employed in other reports. These antagonists function as competitors for melatonin binding sites and inhibit the multiple functions of the indole. Moreover, there seemed to be some stimulation of the melatonin effect, especially in the presence of S20928 and Px when the cells were cultured with the highest concentrations of melatonin. Whereas a compensatory increase in the number of melatonin receptors could possibly be an explanation for this finding, we favor the idea that the treatments influenced the biology of the cells during the long term incubations. Perhaps, treatments were slightly toxic at concentrations used. This may be enhanced by the fact that, in the plates cultured with melatonin, fewer cells were exposed to the same concentration of the drugs, and thus, the changes were more dramatic effect.

One of the most surprising findings was the potent effect of melatonin on cellular differentiation. Recently, our group described melatonin's role in the differentiation of CHO cells [44]; the mechanism in these studies related to the antioxidant properties of the indole. Such an effect has not been previous described for prostate cancer cells; however, melatonin is believed to increase neurite outgrowth in neuronal cells at physiological nighttime blood concentrations mainly mediated by its membrane receptors [51]. In our case, the morphological and biochemical characteristics of prostate cells indicated that they most likely correspond to a neuroendocrine population. After melatonin treatment, cells accumulated in G_0/G_1 stage, but rather than undergoing apoptosis such as after exposure to other antioxidants [52], the cells differentiated. This action of melatonin is similar to that of silibinin, a component of the polyphenolic flavonoid silymarin isolated from the seed of milk thistle, which also decreases cell growth via G_1 arrest and potentiates neuroendocrine differentiation of LNCaP cells [53]. Considering the potentially important role that neuroendocrine differentiation might play in prostate carcinogenesis, it is important to identify how melatonin, silibinin and the vitamin D analog 1α,24-dihydroxyvitamin D_2 [54] increase neuroendocrine differentiation of prostate epithelial cancer cells. New agents which induce neuron-like morphological changes and decrease malignancy of human prostate cancer are being studied [55].

Mechanistically, we conclude that melatonin seems not to arrest the cell cycle or induce differentiation via a receptor-mediated mechanism when it is used at high concentrations in prostate cancer cells. Two main processes have been linked to the acquisition of neuroendocrine characteristics by prostate cells; these include the accumulation of cAMP with the activation of PKA [56] or the activation of Erk mitogen-activated protein kinase [57]. Although melatonin doubled cAMP levels in LNCaP cells, its effect on cell proliferation or differentiation was not inhibited by H89, a specific inhibitor of PKA. Further studies are necessary in order to rigorously disprove the participation of PKA. However, H89 has been used in previous reports to inhibit neuroendocrine differentiation of prostate cancer cells induced by agents that increase cAMP [58]. Considering this, the nuclear translocation of the catalytic subunit of PKA induces LNCaP neuroendocrine differentiation [32]; it is thus difficult to understand why the rise of intracellular cAMP levels is not linked to an activation of PKA when differentiation is induced by melatonin. First, we have to consider the possible participation of unknown intracellular pathways. Secondly, Farini et al. [58] affirmed that a sustained accumulation of cAMP levels is necessary to maintain the neuroendocrine phenotype, but melatonin causes a transient rise of this cyclic nucleotide. Indeed, forskolin and isoproterenol, agonists of β-adrenergic receptors, rapidly induce the development of neuritic processes and increase the expression of NE markers; however, the persistent presence of these agonists is required for cellular differentiation [31]. The authors reasoned that the duration of the half life of a given intracellular transducer such as cAMP may be a critical factor in determining cell fate. Therefore, it seems possible that, although melatonin increased cAMP, it did not cause cell differentiation since the cAMP rise was only transitory.

Finally, it is shown that pharmacological concentrations of melatonin inhibit the proliferation of prostate cancer cells caused by steroids. As expected, cells treated with steroids grew faster that cells cultured in steroid-depleted media, and in both situations melatonin treatment caused a reduction in cell proliferation.

CONCLUSIONS

Here, we described for the first time the role of melatonin, the main secretory product by the pineal gland, in cell proliferation and differentiation of human prostate cancer cells. Endogenous melatonin is one of the physiological mediators that diminish during aging [12]. Because of this and based on many other observations, it has been speculated that the loss of melatonin in the aged may be associated with the increase in neurodegenerative diseases or cancer. Certainly, many other researchers have found that a reduction in physiological levels of melatonin may play an important

role in preventing cancer promotion and progression. Not only its receptor-mediated action, but additionally its scavenging and antioxidant properties may function in limiting tumor growth. Prostate cancer has a high morbidity and mortality since there is no successful therapy; cells are extremely resistant to conventional treatments and complex physiological phenomena such us neuroendocrine differentiation are not completely understood. This is the fist report showing a role for melatonin in neuroendocrine differentiation of prostate cancer cells. Melatonin has never been found to promote cancer; on the contrary all previous results suggest it may have significant utility in reducing cancer cell growth [59]. Melatonin's participation in prostate differentiation should be thoroughly investigated to further define its role in prostate cancer progression.

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