

Cross Talk Between Melatonin and TGF β I in Human Benign Prostate Epithelial Cells

Avi Rimler,¹ Haim Matzkin,² and Nava Zisapel^{1*}

¹Department of Neurobiochemistry, George S. Wise Faculty of Life Sciences,
Tel Aviv University, Tel Aviv, Israel

²Department of Urology, Tel Aviv Medical Center, Tel Aviv, Israel

BACKGROUND. Epithelial cells from the human benign prostate express melatonin receptors which effect transient suppression of DNA synthesis and sustained attenuation of growth. The role of transforming growth factor- β 1 (TGF β 1), which is produced in prostate epithelial cells and inhibits their growth, was examined in the action of melatonin.

METHODS. The effects of melatonin and TGF β 1 and their combination on ³H-thymidine incorporation were assessed. The possibility that melatonin effected TGF β 1 release from cells was studied.

RESULTS. Incubation of the cells with TGF β 1 resulted in a time- and dose-dependent inhibition of ³H-thymidine incorporation into cells. Melatonin (10–500 pM) inhibited ³H-thymidine incorporation, and its effects were attenuated at higher (1–10 nM) concentrations. In the presence of submaximal doses of TGF β 1, the inhibitory effect of melatonin was maintained over the entire concentration range tested (10 pM–10 nM). The inhibition of ³H-thymidine incorporation by TGF β 1 was more pronounced in the absence of dihydrotestosterone (DHT) than in its presence, and melatonin had no further effect. Melatonin enhanced the release of proteins from cells, among them proteins recognized by specific TGF β 1 antisera. The TGF β 1-neutralizing antisera prevented the inhibitory action of melatonin on ³H-thymidine incorporation into cells.

CONCLUSIONS. These data indicate a role for TGF β 1 in the melatonin-mediated attenuation of benign prostate epithelial cell growth. *Prostate* 40:211–217, 1999. © 1999 Wiley-Liss, Inc.

KEY WORDS: melatonin; prostate; androgen; TGF β 1; human

INTRODUCTION

Melatonin, a neurohormone produced and secreted nocturnally from the pineal gland, inhibits pubertal development in rats and possibly humans [1,2]. Benign prostatic hyperplasia (BPH) is a disease of elderly men characterized histologically by the continuous growth of nonmalignant prostatic nodules and clinically by obstruction of the urethral lumen [3]. We recently found high-affinity melatonin receptors in BPH tissue [4]. These sites (apparent half-saturation at 120 pM) were primarily associated with the microsomal fraction of prostate epithelial cells. In cell cultures of benign prostate epithelial cells, melatonin at physiological concentrations suppressed cyclic guanosine 3', 5' monophosphate (cGMP) augmented cyclic adenosine 3', 5' monophosphate (cAMP) and inhibited DNA and protein synthesis [5,6]. The effects of

melatonin on thymidine incorporation were transient, due to a protein-kinase C-mediated inactivation of the receptors [5,7]. Despite the transient nature of the response, melatonin effected a sustained inhibition of cell growth [5], suggesting involvement of a long-acting mediator.

Transforming growth factor- β (TGF β) family members have been detected in the normal prostate [8]. TGF β 1 (a 25-kDa homodimer, which is part of the TGF β family) immunoreactivity was identified in the secretory epithelial cells in both normal and malignant human prostates [9,10]. In the presence of androgens,

*Correspondence to: Professor Nava Zisapel, Ph.D., Department of Neurobiochemistry, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel.

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the interplay between the stimulatory and inhibitory peptide growth factors maintains prostate cell homeostasis [11]. TGF β 1 appears to inhibit normal epithelial cell proliferation [11] and induce apoptosis in prostate epithelial cell in culture in the absence of androgens and epidermal growth factor [12]. BPH is accompanied by increased expression of TGF β 1 and its receptors in the epithelial cells [11,13,14].

Our previous studies on murine melanoma cells indicated that melatonin can modulate the constitutive secretion of proteins from cells [15–17]. In addition, melatonin has been shown to affect a variety of growth factors, including TGF β 1, in the hormone-responsive human breast cancer MCF-7 cell line [18]. The present studies were aimed at exploring the role of TGF β 1 in the attenuation of prostate epithelial cell growth by melatonin. This was pursued by studying: 1) whether melatonin modulated the effects of TGF β 1 on cell ^3H -thymidine incorporation and vice versa; 2) whether melatonin effected the release of TGF β 1 from cells; and 3) whether the inhibitory action of melatonin on ^3H -thymidine incorporation could be neutralized by anti-TGF β 1 antibodies. The results indicated a cross talk between melatonin and TGF β 1 in the regulation of benign prostate epithelial cell growth.

MATERIALS AND METHODS

Materials

RPMI-1640 medium (RPMI), RPMI-1640 medium without phenol red (RPMI-pr), newborn calf serum (NBS), charcoal-stripped NBS (cNBS), glutamine, insulin, transferrin, selenium, penicillin, streptomycin, and amphotericin B were obtained from Biological Industries (Beit Haemek Israel). Melatonin, dihydrotestosterone (DHT), and human recombinant epidermal growth factor (EGF) were obtained from Sigma Chemical Co. (St. Louis, MO). Methyl- ^3H -thymidine was obtained from DuPont-New England Nuclear (Boston, MA) and ^{35}S -methionine/cysteine from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Antitransforming growth factor-1, human, natural (aTGF β 1) and transforming growth factor- β 1, human, natural (TGF β 1) were obtained from Promega Corporation (Madison, WI).

Epithelial Cell Cultures

Epithelial cells were cultured as described [5] and grown at 37°C in growth medium (RPMI containing 10% NBS, 10 ng/ml EGF, 5 ng/ml insulin, 5 ng/ml transferrin, 5 ng/ml selenium, 50 units/ml penicillin, 50 μg /ml streptomycin, and 250 ng/ml amphotericin B, with or without 10 ng/ml DHT) in a humidified atmosphere with 5% CO_2 . Before each experiment,

cells were harvested by trypsin, adjusted to a density of 10^6 cells/ml in charcoal-stripped medium (RPMI-pr supplemented with 2 mM L-glutamine, containing 10 ng/ml EGF, 5 ng/ml insulin, 5 ng/ml transferrin, 5 ng/ml selenium, 50 units/ml penicillin, 50 μg /ml streptomycin, and 10% cNBS) with or without 10 ng/ml DHT, and replated in 24-well multiplates. After 24 hr, the medium was replaced with charcoal-stripped medium containing 2% cNBS, with or without 10 nM DHT, and cell were then used in the experiments.

Thymidine Incorporation

^3H -thymidine incorporation assays were performed as described [5], with minor modifications. Briefly, cells attached to plates were incubated in charcoal-stripped medium containing 2% cNBS with or without 10 nM DHT, with TGF β 1 (0.02–20 ng/ml), melatonin (10^{-11} – 10^{-7} M), their combination, or an equivalent volume of vehicle (0.01% ethanol), for 12–48 hr at 37°C. In some experiments the cells were incubated with vehicle, melatonin (10^{-9} M), aTGF β 1 (1:5,000 dilution, which neutralizes 50% bioactivity of 2 ng/ml TGF β 1), or both for 24 hr at 37°C. ^3H -thymidine (60 Ci/mmol, 1 Ci/well) was then added, and incubation resumed for 4 hr. Media were discarded, the cells were washed (2×1 ml) with ice-cold phosphate-buffered saline (PBS), and trichloroacetic acid (10% TCA 4×1 ml) was then added. The insoluble materials were collected, washed in ethanol (2×1 ml), and dissolved in 0.5 ml of 0.4 N NaOH. The amount of radioactivity was determined by scintillation spectrometry. Protein content was determined in aliquots of the solubilized material [19], and data of all experiments were normalized to protein content.

Metabolic Labeling and Secretion Studies

At 8 hr after plating, the medium was replaced with methionine-free charcoal-stripped medium containing 2% cNBS with 10 nM DHT, and the cells were incubated for 12 hr with 4 μCi /ml ^{35}S -methionine/cysteine. The metabolically labeled cells were washed once with charcoal-stripped medium containing 2% cNBS with 10 nM DHT medium, and then chased for 24 hr at 37°C in the same medium in the presence or absence of melatonin (10^{-12} – 10^{-7} M dissolved in 0.001% ethanol) or vehicle. Media were collected and centrifuged ($10^4\text{g} \cdot \text{min}$). The supernatants containing the secreted [^{35}S]-labeled polypeptides were solubilized in 1% sodium dodecyl sulfate (SDS) and subjected to polyacrylamide gel electrophoresis and autoradiography. The pellets (containing detached cells) were combined with the cells in the plates and solubilized in 1% SDS. Protein content was determined in aliquots of the solubilized cell samples. The [^{35}S]-

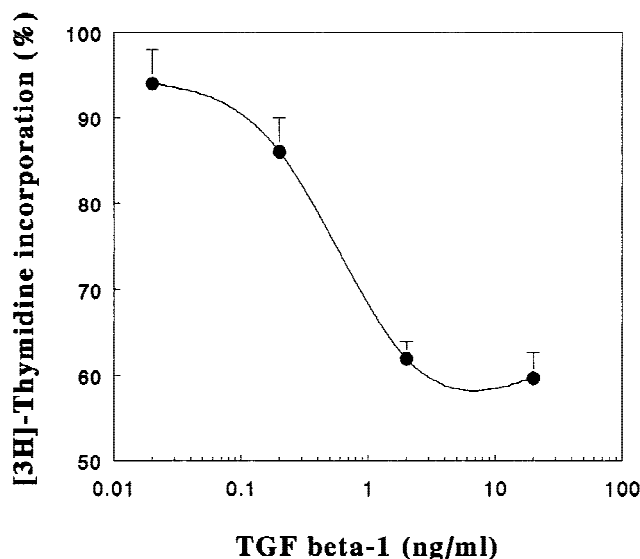


Fig. 1. Effects of TGF β 1 on 3 H-thymidine incorporation into prostate epithelial cells. Cells were incubated with TGF β 1 (0–20 ng/ml) or vehicle for 24 hr in the presence of DHT. The incorporation of 3 H-thymidine was then assessed. Results (mean \pm SD, $n = 8$) are expressed as percent of the incorporation in control cells incubated with vehicle (100% = 3,600 dpm/ μ g protein).

labeled polypeptides secreted from the cells (normalized to equivalent amounts of cell protein) were loaded on polyacrylamide gels and analyzed by electrophoresis and autoradiography as described [15], or subjected to immunoblot analyses with aTGF β 1. Immunoblots were performed by incubating the nitrocellulose sheets with aTGF β 1 antibody (1:5,000 dilution) with or without 2% NBS. The bound primary antibody was identified by means of a secondary antibody linked to horseradish peroxidase and detected by ECL (Amersham, Buckinghamshire, UK).

Statistical Analyses

Results were compared by analysis of variance (ANOVA) followed by Student-Newman-Keul's test for multiple comparisons, with significance at $P < 0.05$ [20].

RESULTS

The effects of TGF β 1 (24-hr treatment) in the presence of DHT on 3 H-thymidine incorporation in prostate epithelial cells are shown in Figure 1. TGF β 1 inhibited thymidine incorporation into the epithelial cells in a dose-dependent manner: 3 H-thymidine incorporation decreased to $85 \pm 6\%$ at 0.2 ng/ml ($P < 0.05$) and to $63 \pm 5\%$ at 2 ng/ml TGF β 1 ($P < 0.01$) of respective values found in control cells without TGF β 1.

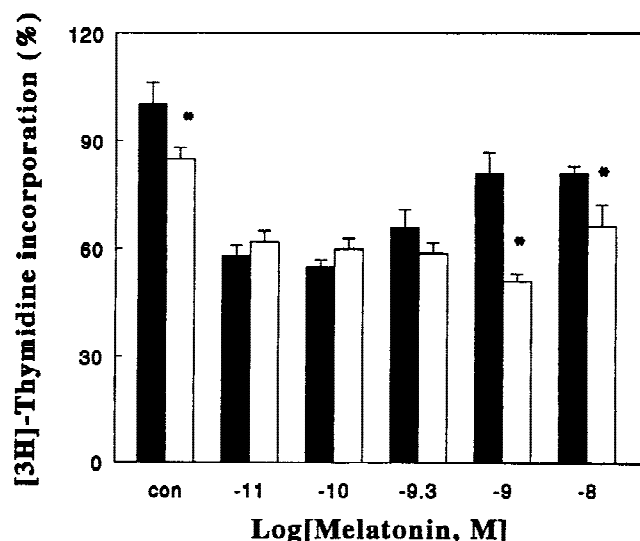


Fig. 2. Effects of melatonin in the absence (solid bars) or presence (open bars) of TGF β 1 on 3 H-thymidine incorporation into prostate epithelial cells. Cells were incubated in the presence of DHT with melatonin, with or without TGF β 1 (2 ng/ml), for 24 hr. The incorporation of 3 H-thymidine was then assessed. Results ($n = 8$) are expressed as percent of the incorporation in control (con) cells incubated in the absence of TGF β 1. * $P < 0.05$ in the presence compared to the absence of TGF β 1 (100% = 4,500 dpm/ μ g protein).

The effects of melatonin (10^{-11} – 10^{-7} M; 24-hr incubations) in the presence of DHT and in the absence and presence of TGF β 1 (2 ng/ml) on 3 H-thymidine incorporation into prostate epithelial cells are shown in Figure 2. Maximal inhibition by melatonin of 3 H-thymidine incorporation was observed at 0.1 nM. At higher (≥ 0.5 nM) concentrations, the inhibitory activity of melatonin on 3 H-thymidine incorporation was less pronounced. The effects of melatonin (≤ 0.5 nM) and TGF β 1 (2 ng/ml) were nonadditive. However, in the presence of TGF β 1, the inhibitory action of melatonin was not attenuated at higher (≥ 0.5 nM) concentrations, and 3 H-thymidine incorporation in the presence of melatonin (0.1 and 10 nM) was significantly lower in the presence than in the absence of TGF β 1 (Fig. 2).

The effects of melatonin (10^{-11} – 10^{-7} M; 24-hr incubations) in the absence of DHT and in the absence and presence of TGF β 1 (2 ng/ml; 24 hr) on 3 H-thymidine incorporation into prostate epithelial cells are shown in Figure 3. In the absence of DHT, the inhibition by TGF β 1 of 3 H-thymidine incorporation (46%; Fig. 3, "con") was more pronounced than that found in the presence of DHT (14%; Fig. 2, "con"). No further suppression of 3 H-thymidine incorporation over that effected by TGF β 1 was found with melatonin in the absence of DHT, regardless of the concentration used (Fig. 3).

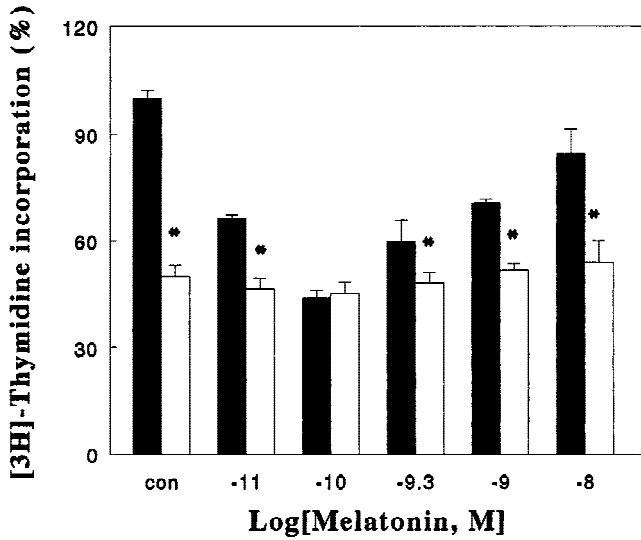


Fig. 3. Effects of melatonin in the absence (solid bars) or presence (open bars) of TGFβ1 on ³H-thymidine incorporation into prostate epithelial cells. Cells were incubated in the absence of DHT with melatonin, with or without TGFβ1 (2 ng/ml), for 24 hr. The incorporation of ³H-thymidine was then assessed. Results (*n* = 8) are expressed as percent of the incorporation in control (con) cells incubated in the absence of TGFβ1 (100% = 1,800 dpm/μg protein). **P* < 0.05 in the presence compared to the absence of TGFβ1.

The interaction between TGFβ1 and melatonin was further investigated under conditions wherein their activities were apparently additive (i.e., in the presence of DHT and at 1 nM melatonin and 2 ng/ml TGFβ1; Fig. 2). The time dependencies of the effects of melatonin and TGFβ1 on ³H-thymidine incorporation are shown in Figure 4. Melatonin (1 nM) effected a slight ($11 \pm 4\%$; *P* < 0.05) inhibition of ³H-thymidine incorporation at 24 hr, but this inhibition diminished at 48 hr ($98 \pm 5\%$ of control values; Fig. 4). The inhibitory effects of TGFβ1 (2 ng/ml) on ³H-thymidine incorporation became more pronounced at longer incubation times. In cells treated with TGFβ1 for 24 and 48 hr, ³H-thymidine incorporation decreased to $72 \pm 5\%$ (*P* < 0.01) and $55 \pm 4\%$ (*P* < 0.001), respectively, of control values (i.e., in cells incubated with vehicle for the same period of time). In cells treated with TGFβ1 and melatonin, ³H-thymidine incorporation decreased with time, leveling off ($53 \pm 4\%$; *P* < 0.001) at 24 hr (Fig. 4).

The effects of melatonin on protein secretion from the cells are shown in Figure 5. In cells incubated for 24 hr with melatonin (0.1 and 1 nM), secretion of [³⁵S]-labeled proteins ($6,420 \pm 670$ and $5,750 \pm 360$ decompositions per minute (dpm) per culture dish containing equivalent amounts of cell protein, respectively)

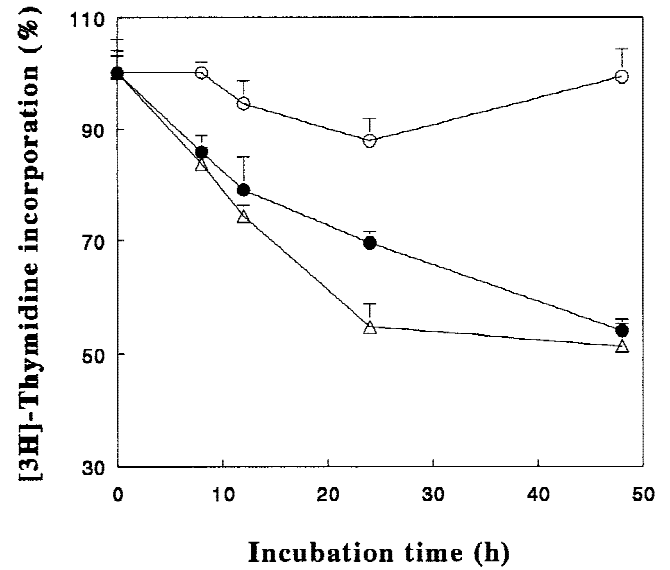


Fig. 4. Effects of incubation time of prostate epithelial cells with melatonin, TGFβ1, and their combination on ³H-thymidine incorporation. Cells were incubated in the presence of DHT and melatonin (1 nM; o), TGFβ1 (2 ng/ml; ●), and their combination (Δ) for 8–48 hr. The incorporation of ³H-thymidine was assessed. Results (*n* = 8) are expressed as percent of the incorporation in control cells incubated for the same period of time with vehicle (100% = 3,800 dpm/μg protein).

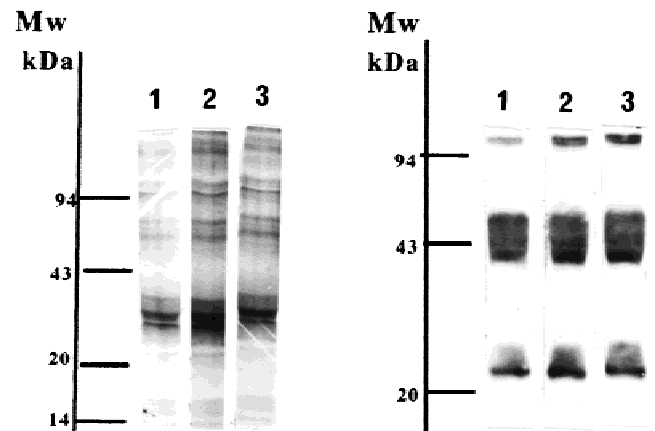


Fig. 5. Effects of melatonin on protein secretion. **Left:** Cells were metabolically labeled with ³⁵S-methionine-cysteine and chased in the presence of DHT and in the absence (lane 1) or presence of 0.1 nM (lane 2) and 1 nM (lane 3) melatonin for 24 hr. The ³⁵S-labeled proteins released into the media were separated by gel electrophoresis and subjected to autoradiography. **Right:** Sister cultures were treated similarly, but without radioactive amino acids. The released proteins were separated by gel electrophoresis and subjected to immunoblotting with aTGFβ1. Results are of a representative experiment (out of two repetitions). The apparent molecular weight values of protein markers run on the same gels are depicted.

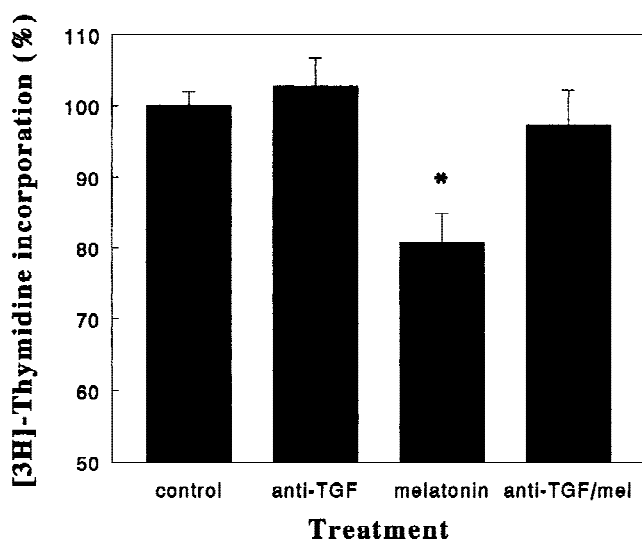


Fig. 6. Effects of aTGF β 1, melatonin, and their combination on 3 H-thymidine incorporation into prostate epithelial cells. Cells were incubated in the presence of DHT with aTGF β 1 (0.2 ng/ml), melatonin (1 nM), and their combination for 24 hr. The incorporation of 3 H-thymidine was then assessed. Results ($n = 8$) are expressed as percent of the incorporation in control cells incubated with vehicle (100% = 3,500 dpm/ μ g protein). * $P < 0.05$, compared to control.

was significantly higher than the respective amounts in control cells ($4,400 \pm 270$ dpm). The enhancement was apparently ubiquitous for all secreted proteins (Fig. 5, left). Western blotting of the secreted proteins using aTGF β 1 demonstrated the presence of aTGF β 1-identified bands with apparent molecular weight values of 25 and 50 kDa (Fig. 5, right). Both of these proteins were also present in NBS samples but at lower (approximately 50%) amounts. Preabsorption of aTGF β 1 to NBS (same amount as in cell-derived samples), prior to its use in the immunoblot, resulted in a decrease in intensity of the aTGF β 1-identified 25-kDa band in the cell samples by 50% (not shown). Densitometric scanning of the aTGF β 1-identified bands indicated that in cells treated with melatonin (0.1 and 1 nM), the intensity of these bands increased (by $39 \pm 2\%$ and $23 \pm 3\%$, respectively), compared to that in control cells without melatonin (Fig. 5, right).

The relevance of the enhanced secretion of TGF β 1 to the melatonin-mediated effects on thymidine incorporation was investigated. This was pursued by studying the ability of aTGF β 1 to neutralize the inhibitory effects of melatonin on thymidine incorporation into the cells (Fig. 6). Incorporation into the control, untreated cells slightly increased in the presence of aTGF β 1, but not significantly (Fig. 6). aTGF β 1 prevented most of the inhibitory effects of melatonin (1 nM) on 3 H-thymidine incorporation.

DISCUSSION

Results of the present study indicate interplay between melatonin and TGF β 1 in the regulation of 3 H-thymidine incorporation in prostate epithelial cells. In the presence of DHT, the suppressive activities of melatonin and TGF β 1 were nonadditive at low concentrations (≤ 0.1 nM), and additive at physiological and supraphysiological melatonin concentrations.

The diminution of the inhibitory action of melatonin, at concentrations exceeding 0.5 nM on 3 H-thymidine incorporation, may be due to the presence of more than one (unidentified) receptor subtype with lower affinity and opposite action, or to downregulation of the receptors. Diminution in the growth-inhibitory effects of melatonin at high concentrations was documented in MCF-7 breast cancer cells. In those cells, melatonin inhibited cell growth at subnanomolar concentrations, and this activity was less pronounced at higher concentrations [21]. Receptor downregulation has been suggested from animal studies in which melatonin given in the morning prevented the suppression of gonadal activity effected by melatonin given in the afternoon [22]. In rats, the density of melatonin-binding sites in the suprachiasmatic nuclei (SCN) was found to increase when animals were subjected to a 1-hr light pulse during the dark period [23]. In addition, melatonin was recently found to desensitize its own receptors in human benign prostate epithelial cells via a protein-kinase C-mediated process [7]. The fact that this attenuation is not observed in the presence of TGF β 1 suggests that the growth factor interferes with the desensitization process.

The nonadditive action of melatonin and TGF β 1 at low melatonin concentrations may be explained by a ceiling effect: 3 H-thymidine incorporation is reduced by melatonin alone to basal levels (55% of control values), and TGF β 1 therefore cannot further suppress it. This basal level (55% of 3 H-thymidine incorporation control values) is also compatible with the incorporation observed in cells treated with saturating concentrations of TGF β 1 (20 ng/ml), which should normally stop the cell cycle at the G1 phase. Indeed, when the suppression by melatonin was less pronounced (at >0.5 nM), 3 H-thymidine incorporation was further suppressed by TGF β 1.

A ceiling effect may also explain the results of the kinetic study. As in other systems such as the human thyroid cancer cell line NPA [24], the effect of TGF β 1 was more pronounced after 48 than at 24 hr. At 24 hr, the decrease in 3 H-thymidine incorporation in cells treated with the melatonin-TGF β 1 combination was equivalent to the sum of decrease effected by each agent alone (additive), leveling off afterwards at a level similar to that effected by TGF β 1 alone at 48 hr

(53% of control values). Moreover, a ceiling effect may also explain the lack of effect of melatonin over TGF β 1 in the absence of DHT. Apparently, in the absence of androgen, the cells became more susceptible to the inhibitory action of TGF β 1.

Based on this explanation, it may be assumed that melatonin and TGF β 1 actually affect ^3H -thymidine incorporation via independent pathways, and the apparent nonadditive effect they exert under certain conditions is because a minimal level of ^3H -thymidine incorporation has been reached. This possibility cannot be ruled out at present.

Another explanation is that melatonin enhances the release of TGF β 1 (and presumably other growth factors as well) from cells. The latter possibility agrees well with the observations that: 1) the secretion of proteins from prostate cells is enhanced by melatonin, including secretion of a protein with an apparent molecular weight compatible with that of TGF β 1; 2) the amount of a protein identified by aTGF β 1 in the extracellular medium increases in melatonin-treated cultures; and 3) aTGF β 1 abrogates the inhibitory action of melatonin on ^3H -thymidine incorporation. Taken together, these data strongly suggest that TGF β 1 is involved in the sustained effects of melatonin in epithelial cells.

The enhancement by melatonin of constitutive protein secretion from the prostate cells seems to be ubiquitous. It could thus be expected that the secretion of a number of growth factors, besides TGF β 1, would be enhanced, including some with growth-suppressing and growth-facilitating activities. In our experiments, EGF was supplemented to support cell growth in culture and might perhaps mask the effects of endogenous EGF, which might be released by melatonin as well. The observation that aTGF β 1 ablated the inhibitory action of melatonin on ^3H -thymidine incorporation implies that TGF β 1 is the major inhibitory factor secreted by prostate cells.

Could an enhancing effect by melatonin of TGF β 1 secretion account for the inhibitory action of melatonin on ^3H -thymidine incorporation? Apparently not. The small and insignificant enhancing effect of aTGF β 1 (which neutralizes endogenous TGF β 1) on ^3H -thymidine incorporation by cells suggests that some bioactive TGF β 1 is present in the culture medium. Judging from the dose-response curve to TGF β 1, the amount of TGF β 1 present in the culture medium may be estimated at 0.02 ng/ml. To achieve maximal suppression of ^3H -thymidine incorporation, the amount of TGF β 1 released by melatonin would have to be between 0.8–2 ng/ml. This means enhancement by 40–100-fold of TGF β 1 secretion. Such enhancement seems to be much greater than that anticipated from the protein secretion studies (about

3-fold). The immunoblot analysis also does not support a greater than 3–10-fold enhancement in secretion, even when considering that about half of the TGF β 1 immunostaining comes from the NBS and that it may not be bioactive. We must therefore assume that the secreted TGF β 1 acts locally, or that besides enhancing secretion of TGF β 1 from cells, melatonin sensitizes cells to TGF β 1 to a level similar to that seen upon androgen removal. The latter possibility remains to be elucidated.

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