**Melatonin and Prostate Cancer Cell Proliferation: Interplay With Castration, Epidermal Growth Factor, and Androgen Sensitivity**

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**BACKGROUND.** Potential modulatory effects of melatonin on the proliferation of androgen-sensitive LNCaP and androgen-insensitive PC-3 and DU 145 prostate cancer cells were reported recently. In this study, we investigated the effects of combined melatonin and castration on LNCaP tumor growth in vivo, the interactions between melatonin and epidermal growth factor (EGF) on LNCaP cell proliferation, and melatonin actions on the proliferation of PC-3 and DU 145 cells.

**METHODS.** Tumor development and growth in castrated nude mice inoculated with LNCaP cells or in intact animals inoculated with DU 145 cells, with or without daily melatonin treatment, were monitored by observation and caliper measurement. MT1 receptor expression in native or transfected prostate cancer cell lines was examined by immunocytochemistry or 2-125Iodomelatonin binding. Cyclin D1 expression in LNCaP cells was assessed by Western blotting, and cell proliferation was measured by thymidine incorporation and/or cell count.

**RESULTS.** Melatonin treatment was associated with further decreases in LNCaP tumor incidence and growth rate in castrated nude mice. Melatonin and 2-iodomelatonin (a melatonin receptor agonist) attenuated EGF-stimulated increases in LNCaP cell proliferation and cyclin D1 levels. Melatonin had no effect on the proliferation or growth of MT1 receptor-expressing DU 145 cells, and of PC-3 cells in which MT1 receptor protein was undetectable. The proliferation of transfected PC-3 cells expressing MT1 receptor was unaffected by 2-iodomelatonin.

**CONCLUSION.** Together with previous data, the present results indicate synergistic action of melatonin and castration in inhibiting the growth of androgen-sensitive LNCaP tumor. Androgen-sensitive prostate cancer cell proliferation may be modulated by opposite changes in cyclin D1 levels induced by activated MT1 and EGF receptors. In androgen-insensitive prostate cancer cells, MT1 receptor-mediated signal transduction may become defective not only through changes in membrane receptor protein expression and/or functions, but also by means of alterations in downstream postreceptor signaling events. Prostate 52: 106–122, 2002.

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**KEY WORDS:** 2-iodomelatonin; MT1 receptor; LNCaP; PC-3; DU 145; EGF; cyclin D1

**INTRODUCTION**

Prostate cancer is the most commonly diagnosed noncutaneous neoplasia among males in the developed world. In many western countries, it is the second most frequent cause of male cancer death. Its growing impact on the morbidity and mortality of the elderly population is transforming it into a disease of public health importance in the 21st century [1]. Although treatment options that are potentially
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curative are available for the management of early localized disease whose detection is facilitated by prostate specific antigen testing, palliative hormonal therapy in the form of medical or surgical castration forms the mainstay of treatment for patients with advanced prostate cancer [2]. Given that approximately 80% of the castrated patients will suffer from a relapse of the disease in 2 years, with progression of the tumor from a hormone-dependent stage to a hormone-independent stage [3,4], it is not surprising that the search for novel therapeutic approaches for the management of advanced prostate cancer is a top-priority research goal for both academia and industry.

Of the various experimental prostate cancer models that are available [5], hormone (androgen)-sensitive and hormone (androgen)-insensitive metastatic human prostate cancer cell lines are widely used for the evaluation of pharmacological agents with therapeutic potential for the disease in the laboratory. By using a similar strategy, we [6] and others [7,8] have shown recently that melatonin, a pineal gland indoleamine hormone, inhibits the growth of the androgen-independent but androgen-sensitive (responsive) human LNCaP prostate cancer cells in culture. We have also obtained evidence that the antiproliferative action of melatonin is mediated in part by means of MT1 receptor activation and partly by means of attenuation of dihydrotestosterone-induced calcium influx [6]. Importantly, our group has further validated the in vitro antiproliferative action of the pineal hormone in nude mice xenograft model for LNCaP cells [9]. Taken together with multiple lines of accumulated evidence which showed an association of decreased pineal melatonin synthesis and nocturnal serum melatonin with the development of human prostate cancer [10], as well as an inhibitory action of the pineal hormone on the growth of the prostate gland in rodents [11,12] and of benign prostatic epithelial cells in humans [13], the above data indicate that melatonin may be an important endogenous negative mitogenic hormonal signal implicated in the regulation of human prostate growth. Moreover, the pineal indoleamine small molecule holds promise for further development as a novel hormonal agent for the treatment of prostate cancer.

To exploit the therapeutic potential of melatonin in the management of advanced prostate cancer, it is crucial to determine whether and how melatonin, a putative negative growth regulator, will interact with other relevant positive regulators such as androgens [14] and peptide growth factors [15] in the modulation of prostate cancer growth and progression. Of note, attenuation of androgen-stimulated LNCaP cell proliferation and inhibition of LNCaP tumor growth by melatonin have been demonstrated, respectively, in vitro under a serum-free condition [6] and in vivo under an androgen-rich condition in postpubertal nude mice with intact testes [9]. Although proliferation of LNCaP cells was inhibited by melatonin under a serum-free (androgen-free) condition in vitro [6], it is yet unclear whether melatonin can exert its inhibitory action on LNCaP cancer growth in androgen-deprived condition in vivo. Apparently, this is an important practical issue that needs to be addressed because a novel combination hormonal approach, which involves the administration of melatonin to castrated patients, would have been conceived to have additional therapeutic benefit of further slowing advanced prostate cancer progression. To provide proof of the above concept, the effect of melatonin on androgen-sensitive LNCaP prostate cancer growth in castrated male athymic nude mice was investigated in the present study. In addition, potential interaction and the attendant mechanism between melatonin and epidermal growth factor (EGF), a key peptide growth factor implicated in prostate cancer growth regulation, in the modulation of proliferation of LNCaP cells, which express melatonin MT1 [6] but not MT2 receptor [8] subtype, were also studied.

As the evolution of prostate cancer cells to an androgen-independent/insensitive state, by means of activation of various adaptation mechanisms in response to the selection pressure of castration-induced androgen deprivation, seems inevitable, it is obviously important to define the functional role and usefulness of melatonin in the modulation of hormone-insensitive prostate cancer cell proliferation. Interestingly, whereas our previous study has failed to demonstrate MT1 receptor expression in PC-3 cells as well as an antiproliferative action of melatonin on PC-3 tumor growth in nude mice [9], others have reported melatonin-induced inhibition of PC-3 and DU 145 cell proliferation in vitro, which were associated with the expression of low-affinity melatonin binding sites [16] and melatonin MT1 receptor [17], respectively. To clarify the potential role of MT1 receptor and melatonin in the modulation of hormone-insensitive prostate cancer cell proliferation, the effects of melatonin and/or 2-iodomelatonin (a melatonin receptor agonist) on the proliferation of PC-3 cells stably transfected with cloned MT1 receptor cDNA and of DU 145 cells were examined, together with their MT1 receptor expression status, in the present investigation.

MATERIALS AND METHODS

Chemicals, Cells, and Animals

Chemicals for cell culture were purchased from Gibco BRL Chemical Co. (Grand Island, NY). Melatonin, 2-iodomelatonin (a melatonin receptor...
agonist), and EGF were ordered from Sigma Chemical Co. (St. Louis, MO). Purified polyclonal rabbit anti-MT1 receptor serum was a gift from Professor G.M. Brown (University of Toronto, Canada). MT1 receptor cDNA clone (AA-1) was kindly supplied by Professor S.M. Reppert (Harvard University). 2-[125I]iodomelatonin (2,200 Ci/mmol) and [methyl-3H]thymidine (82 Ci/mmol) were purchased from NEN Life Science Products, Inc. (Boston, MA) and Amersham Pharmacia Biotech (Aylesbury, UK), respectively. The human prostate carcinoma cell lines LNCaP.FGC (CRL-1740), PC-3 (CRL-1435), and DU 145 (HTB-81) were obtained from American Type Culture Collection (Rockville, MD, USA) and propagated in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with L-glutamine and 10% fetal bovine serum (FBS) at 37°C and 5% CO2. Male BALB/c athymic nude mice (6 weeks old; weight: 16 ± 2 g) were provided by the Laboratory Animal Unit of The University of Hong Kong. The animals were adapted to a controlled lighting schedule of 12 hr light and 12 hr dark (LD 12:12; lights on 3:00AM to 3:00PM) for 2 weeks before experimental manipulation.

**Effects of Melatonin on Prostate Tumor Growth in Athymic Nude Mice Inoculated with Human Prostate Cancer Cells**

Protocols on animal experimentation were approved by the Committee on the Use of Live Animals for Teaching and Research (CULATR) of The University of Hong Kong. For studies on LNCaP cells, male BALB/c athymic nude mice were inoculated subcutaneously with LNCaP prostate cancer cells (0.5 ml; 1 × 106 cells/site) in the dorsal flank region. LNCaP cells were suspended in RPMI 1640 medium containing 10% FBS and mixed with equal volume of Matrigel basement membrane matrix (Collaborative Biomedical Products/Becton Dickinson, Bedford, MA) [18] before they were inoculated into the mice. Ten days after LNCaP cell transplantation, the mice were castrated and given daily saline or melatonin injection. In parallel, mice of a separate group, inoculated with LNCaP cells 10 days before as described above, were sham operated and given daily saline injection. Saline or melatonin (4 μg/g body weight) was administered intraperitoneally (0.2 ml/day) 1 hr before room lighting was switched off. The time of tumor appearance (when tumor became noticeable) was recorded. The volume of the tumor developed in the mice was assessed at 5-day intervals postinoculation of tumor cells. The linear dimensions of the tumor were measured with electronic caliper, and tumor volume was calculated by the formula \( V = \frac{1}{2} \pi r^2h = \frac{1}{2} \pi \left(\frac{w}{2}\right)^2h \), which was derived from a formula for calculating the volume of a hemi-ellipsoid, the geometric figure most nearly approximating the shape of tumors [19]. The animals were observed for 60 days after tumor cell inoculation. For studies on DU 145 cells, male BALB/c athymic nude mice were similarly inoculated subcutaneously with DU 145 prostate cancer cells (0.5 ml; 1 × 106 cells/site) in the dorsal flank region. The prostate cancer cells were suspended in RPMI 1640 medium containing 10% FBS before they were inoculated into the mice. Mice were given daily intraperitoneal saline or melatonin injection initiated 10 days before tumor cell transplantation. Saline or melatonin (4 μg/g body weight) was injected (0.2 ml/day) 1 hr before room lighting was switched off. Volume of the tumor developed in nude mice was measured at 5-day intervals postinoculation of tumor cells. The animals were observed for 60 days after tumor cell inoculation.

**Immunohisto(cyto)chemical Analysis of Melatonin MT1 Receptor in Xenograft Tumors and Prostate Cancer Cell Lines**

The explanted tumor tissues from animals were immediately frozen at −70°C and cut into 12-μm cryostat sections. Prostate cancer cells in exponential growth phase were seeded onto coverslips and incubated in RPMI 1640 medium supplemented with 10% FBS at 37°C. The tissues and cells were washed three times, 5 min each, with 0.03% Triton-X-100 in phosphate-buffered saline (PBS) (138 mM NaCl, 2.7 mM KCl, 1.8 mM KH2PO4 and 10 mM Na2HPO4, pH 7.4) before fixation in 4% paraformaldehyde in PBS for 30 min. After fixation, the samples were washed three times, 10 min each, with Triton X-PBS to permeabilize the cell membrane and treated with 0.3% H2O2/methanol for 20 min to quench the endogenous peroxidase. Samples were then washed three times, 5 min each, with Triton X-PBS and incubated with 5% normal goat serum in Triton X-PBS for 30 min. Purified polyclonal rabbit anti-MT1 receptor serum (anti-MT1 receptor subtype-specific peptide IgG [anti-MT1 IgG TIL3]) (5 mg/l) was then added with or without 0.1 mg/1 synthetic TIL3 peptide (cDNA-encoded amino acid residues 226-238 of the third intracellular loop of human MT1 melatonin receptor: KPKLKPQDFRNFV) [one letter amino acid code] that had been used to generate the receptor subtype-specific antiserum [20] and the samples were incubated overnight at 4°C. After washing with Triton X-PBS, samples were incubated with diluted (1:500 in Triton X-PBS) biotinylated goat anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA) for 30 min. The samples were then washed again with Triton X-PBS and were incubated with diluted (1:500 in Triton X-PBS) biotinylated goat anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA) for 30 min. The samples were then washed again with Triton X-PBS and were incubated with diluted (1:500 in Triton X-PBS) biotinylated goat anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA) for 30 min.
X-PBS) peroxidase-conjugated streptavidin for 30 min. Finally, after washing with Triton X-PBS, samples were exposed to 0.025% 3,3’-diaminobenzidine (DAB)/0.01% H₂O₂ in Tris-HCl buffer (pH 7.4) for optimal signal development. Nuclei of the cancer cells were counterstained with hematoxylin (BDH Laboratory, England) for 5 sec before dehydrated in ethanol (70%, 80%, 90%, 95%, and 100%) for 5 min each and then in xylene for 2 × 5 min.

**Immunohistochemical Analysis of MT₁ Receptor, EGF, and EGF Receptor in Human Prostate Cancer Tissue**

Experimental protocols on human prostate tissues obtained by transurethral resection of the prostate (TURP) from prostate cancer patients under the clinical care of the Department of Surgery, Queen Mary Hospital, Hong Kong, were approved by the Ethics Committee, Faculty of Medicine, The University of Hong Kong. For this study, a sample of prostate cancer tissue (histopathological combined Gleason score 7) was obtained by TURP, with informed consent, from a 76-year-old patient. Prostatectomy was performed for the relief of his bladder outflow obstruction. This patient had not received prior hormonal therapy, and his prostate cancer was treated with surgical castration. The human prostate specimen was processed for immunohistochemical analysis following the procedures described above. After incubation with goat serum, 5 mg/l purified polyclonal rabbit anti-MT₁ receptor serum was added with or without 0.1 mg/l synthetic TIL3 peptide. In parallel, some sections were incubated with diluted (1:500 in Triton X-PBS) mouse anti-epidermal growth factor receptor monoclonal antibody (528) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or diluted (1:500 in Triton X-PBS) rabbit antibody (528) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or diluted (1:500 in Triton X-PBS) mouse monoclonal anti–α-tubulin (N356) (Amersham Pharmacia Biotech UK limited, Buckinghamshire, England) at 4°C overnight. After the end of the incubation, membranes were washed with TBS-T twice for 10 min each, before incubation with diluted (1:1000 in TBS-T) horseradish peroxidase-linked goat anti-rabbit IgG or anti-mouse IgG (ZYMED Laboratories, San Francisco, CA) for 1.5 hr at room temperature. After incubation, membranes were rinsed with TBS-T four times for 10 min each. The blots were then developed by using enhanced chemiluminescence (ECL) following the protocol recommended by the manufacturer (Amersham Pharmacia Biotech, Buckinghamshire, UK). Films of the developed blots were scanned with imaging densitometer and quantitated by using Molecular Analyst software (Bio-Rad Laboratories, Hercules, CA). Densitometric scanings of three separate Western blots, normalized against α-tubulin, were determined.

**Thymidine Incorporation Assay**

LNCaP, PC-3, DU 145, and PC-3 cells stably transfected with MT₁ receptor cDNA (PC3-MT₁-a and PC3-MT₁-b cells) in the exponential growth phase (2 × 10⁵/ml) were seeded into each well of six-well cell culture plates and incubated in RPMI 1640 medium supplemented with 10% FBS at 37°C for the first 24 hr, before the cells were cultured in serum-free RPMI 1640 medium. The cells were treated separately with vehicle, melatonin (1 × 10⁻⁹ M or 5 × 10⁻⁷ M), 2-iodomelatonin (1 × 10⁻⁹ M or 5 × 10⁻⁷ M), EGF (1 × 10⁻¹⁰ M to 1 × 10⁻⁸ M), melatonin (5 × 10⁻¹² M to 5 × 10⁻⁷ M) plus EGF (1 × 10⁻⁸ M), or 2-iodomelatonin (5 × 10⁻¹² M to 5 × 10⁻⁷ M) plus EGF (10⁻⁸ M) in serum-free medium for 48 hr. The cells were harvested in lysis buffer (150 mM NaCl, 1% NP-40, 50 mM Tris-HCl [pH 8], 1 mg/l aprotinin, 1 mg/l leupeptin, 1 mg/l pepstatin, and 1 mM phenylmethylsulfonyl fluoride (PMSF)) at 4°C. Lysates in sample buffer (0.2% SDS, 10% glycerol, 0.06 M Tris-HCl [pH 6.8], 100 mM DTT, and 0.01% bromophenol blue) were heated at 95°C for 5 min and centrifuged at 10,000g for 10 min to remove cell debris. Cell protein extracts (50 μg/lane) in sample buffer were resolved on 10% SDS-polyacrylamide gel and electroblotted onto nitrocellulose paper. The nitrocellulose membranes were blocked with 5% dry milk powder in TBS-T (20 mM Tris, 137 mmol/l NaCl, 0.2% Tween-20, and 0.01% sodium azide, pH 7.6) for 1 hr at room temperature. The nitrocellulose membranes were then incubated with diluted (1:500 in TBS-T) rabbit polyclonal anti-cyclin D1 antibody (H-295) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or diluted (1:1,000 in TBS-T) mouse monoclonal anti–α-tubulin (N356) (Amersham Pharmacia Biotech UK limited, Buckinghamshire, England) at 4°C overnight. After the end of the incubation, membranes were washed with TBS-T twice for 10 min each, before incubation with diluted (1:1000 in TBS-T) horseradish peroxidase-linked goat anti-rabbit IgG or anti-mouse IgG (ZYMED Laboratories, San Francisco, CA) for 1.5 hr at room temperature. After incubation, membranes were rinsed with TBS-T four times for 10 min each. The blots were then developed by using enhanced chemiluminescence (ECL) following the protocol recommended by the manufacturer (Amersham Pharmacia Biotech, Buckinghamshire, UK). Films of the developed blots were scanned with imaging densitometer and quantitated by using Molecular Analyst software (Bio-Rad Laboratories, Hercules, CA). Densitometric scanings of three separate Western blots, normalized against α-tubulin, were determined.
to each well and further incubated for 8 hr. The cells were harvested and processed according to previously described procedures [6] at the end of the incubation period. Cell viability was also determined by trypan blue staining before and after treatment with vehicle, 10^{-8} M EGF, 5 \times 10^{-7} M melatonin, 5 \times 10^{-7} M 2-iodomelatonin, 5 \times 10^{-7} M melatonin plus 10^{-8} M EGF, or 5 \times 10^{-7} M 2-iodomelatonin plus 10^{-8} M EGF.

Cell Count

Prostate cancer cells (2 \times 10^5/ml for LNCaP and 1 \times 10^5/ml for PC-3, DU 145, PC3-MT1-a, and PC3-MT1-b) were seeded into 25-cm\(^2\) cell culture flasks and incubated in RPMI 1640 medium supplemented with 10% FBS at 37°C for the first 24 hr. The cells were then treated separately with vehicle, melatonin (1 \times 10^{-9} M and 5 \times 10^{-7} M) or 2-iodomelatonin (5 \times 10^{-7} M) for 7 days. Culture medium added with vehicle, melatonin, or 2-iodomelatonon was replaced every 2 days. The cells were counted by hemocytometer at the end of vehicle or indoleamine treatment. Cell viability was also determined by trypan blue staining before and after treatment with vehicle, 5 \times 10^{-7} M melatonin or 5 \times 10^{-7} M 2-iodomelatonin.

Construction of Expression Plasmid pcDNA3-MT\(_1\)

MT\(_1\) receptor cDNA was amplified from clone AA-1 by polymerase chain reaction (PCR) with sense primer 5'-acaagcttATGCAGGGCAACCGCAGC-3' (GenBank accession no. U14109; nucleotides 33-50) and anti-sense primer 5'-ctctcgaatCTTTAACGGAGTCCAC-3' (GenBank accession no. U14109; nucleotides 1071-1085) designed from published human MT\(_1\) receptor sequence [21]. The 5' ends of the sense and anti-sense amplimers were tagged with the restriction enzyme sequences of HindIII and XhoI, respectively, to facilitate subsequent subcloning of amplified MT\(_1\) receptor cDNA, which contained the full open reading frame (GenBank accession no. U14109; nucleotides 1071-1085), into the expression vector pcDNA3. The PCR (50 \mu l) reaction mixture contained 1 \times High Fidelity PCR Buffer (60 mM Tris-SO\(_4\) [pH 8.9] and 18 mM ammonium sulfate; GIBCO BRL Chemical Co., Grand Island, NY), 0.2 mM of each dNTP, 2 mM MgSO\(_4\) (GIBCO BRL Chemical Co.), 0.2 \mu M of each primer, 50 ng MT\(_1\) receptor cDNA (clone AA-1) and 2.5 units PLATINUM Taq DNA polymerase (GIBCO BRL Chemical Co.). The 30 cycles of PCR amplification were preceded by a heat denaturing step at 94°C for 10 min. The specific PCR product of correct predicted size was then digested with HindIII and XhoI before gel electrophoresis and purification by QIAquick\textsuperscript{TM} Gel Extraction Kit (QIAGEN GmbH, Germany). The digested and purified MT\(_1\) receptor cDNA was ligated with HindIII- and XhoI-digested pcDNA3 vector DNA by using Rapid DNA Ligation Kit (Roche Molecular Biochemicals, Mannheim, Germany) before transformation into competent DH5\textsuperscript{TM} bacterial cells (GIBCO BRL Chemical Co.). The subcloned MT\(_1\) receptor cDNA in pcDNA3 (pcDNA3-MT\(_1\)) was sequenced in both directions and its nucleotide sequence was confirmed to be identical to that deposited in the database.

Stable Transfection of PC-3 Cells with pcDNA3-MT\(_1\) and pcDNA3 Plasmids

Plasmid pcDNA3-MT\(_1\) and pcDNA3 (control) DNAs were purified with QIAGEN Plasmid Mini Kit and transfected into PC-3 cells by using SuperFect\textsuperscript{TM} Transfection Reagent according to the protocol recommended by the manufacturer (QIAGEN GmbH, Germany). Briefly, PC-3 cells (2 \times 10^5/ml) were seeded onto 60-mm dishes and incubated at 37°C and 5% CO\(_2\) for 24 hr until the cells reached 50% confluence. Purified pcDNA3-MT\(_1\) or pcDNA3 DNA (5 \mu g) was dissolved in TE buffer (10 mM Tris-HCl [pH 7.4] and 1 mM ethylenediamine tetraacetic acid [EDTA]) and diluted with serum-free RPMI 1640 medium to a total volume of 150 \mu l. SuperFect\textsuperscript{TM} transfection reagent (20 \mu l) was added to the DNA solution and mixed by gentle vortexing for 10 sec. The mixture was then incubated at room temperature for 5–10 min to allow complex formation. At the same time, medium was aspirated from the dish and the cells were washed with 4 ml of PBS. RPMI 1640 medium supplemented with 10% FBS (1 ml) was added to the transfection complexes. After mixing, the mixture was added to the cells immediately and the cells were incubated at 37°C and 5% CO\(_2\) for 3 hr. The medium was then aspirated, and the cells were washed three times with 4 ml of PBS each. Serum-containing fresh medium was then added and the cells were incubated for another 48 hr. The cells were subsequently passaged at 1:12 into RPMI 1640 medium supplemented with 10% FBS and 0.4 mg/ml geneticin (G-418 sulfate) (GIBCO BRL Chemical Co.), plated, and cultured until G-418-resistant clones appeared.

2-[\textsuperscript{125}I]Iodomelatonin Binding Assay

Twelve clones of transfected PC-3 cells were isolated and grown in RPMI 1640 medium supplemented with 10% FBS and 0.4 mg/ml geneticin in 25-cm\(^2\) culture flasks. After the cells reached
confluence, they were washed three times with PBS. The cells were harvested in 0.05 M Tris-HCl buffer with proteinase inhibitors (1 mM DTT, 1 mM EDTA, 1 mg/ml aprotinin, 1 mM bezamidine-HCl, and 0.1 mM PMSF, pH 7.4). The collected cells were pelleted, frozen in liquid nitrogen, and stored at −70 °C for future 2-[125I]iodomelatonin binding study. On the day of assay, frozen cell pellets were thawed, homogenized in binding buffer (0.05 M Tris-HCl buffer with 0.1 M DTT, 0.04% soyabean trypsin inhibitor, 0.4% bacitracin, 3 mM MgCl₂ and 2 mg/ml bovine serum albumin, pH 7.4), and prepared for binding assay as previously described [22]. Briefly, membranes were incubated with 179 pM or 9-442 pM 2-[125I]iodomelatonin for 2 hr at 37 °C for 1-point or saturation studies, respectively. Nonspecific binding was determined as binding in the presence of 1 μM melatonin.

**Data and Statistical Analyses**

Numerical results were presented as mean ± SE, and two group comparisons were analyzed by Student’s t-test. The level of significance for all statistical analyses was set at P < 0.05. For in vivo studies, any association of LNCaP tumor incidence with different experimental manipulations was determined by chi-squared (χ²) test. The rate of LNCaP tumor growth in each animal was estimated by the ratio of the last recorded tumor volume to the number of days during which the animals inoculated with tumor cells were monitored. The mean tumor growth rate for each group of animals was the average of the estimated growth rates for individual tumors within the group. Data shown are mean ± SE.

**RESULTS**

**Effects of Castration and Melatonin on LNCaP Tumor Growth and MT₁ Receptor Expression in Developed Xenograft Tumors**

Whereas 83% (15 of 18) of the sham and saline-treated mice inoculated with LNCaP cells developed tumors, tumor development was noticed in only 55% (16 of 29 mice) of castrated and saline-treated mice inoculated with tumor cells. Castration was associated with a significant (P < 0.05) decrease in the incidence of LNCaP tumor development (Table I). Among the castrated animals, the percentage of melatonin-treated mice (32%, 9 of 28) that developed LNCaP tumor was lower than that of saline-treated group (55%, 16 of 29). Melatonin treatment was associated with a significant (P < 0.05) decrease in the incidence of LNCaP tumor development in castrated animals (Table I). The average time of tumor appearance (when tumor became noticeable) for the sham plus saline, castration plus saline, castration plus melatonin groups was, respectively, 22.33 days, 30.19 days, and 33.11 days after tumor cell inoculation (Table I). The prolongation of the time of tumor appearance induced by castration or melatonin treatment was paralleled by corresponding decreases in the mean tumor growth rates approximated to 7.40 mm³/day, 2.33 mm³/day, 1.94 mm³/day for the sham plus saline, castration plus saline, castration plus melatonin groups, respectively (Fig. 1; Table I). Melatonin MT₁ receptor protein was expressed in LNCaP tumors developed in all three groups of mice (Fig. 2).

**TABLE I. Effects of Castration and Melatonin (MLT) on LNCaP Tumor Growth in Nude Mice**

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of animals developed tumors/no. of animals inoculated (%)</th>
<th>Time of tumor appearance (days postinoculation of tumor cells)a</th>
<th>Tumor growth rate (mm³/day)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham + saline</td>
<td>15/18 (83%)</td>
<td>22.33 ± 2.86</td>
<td>7.40 ± 1.81</td>
</tr>
<tr>
<td>Castration + saline</td>
<td>16/29 (55%)*</td>
<td>30.19 ± 4.04</td>
<td>2.33 ± 0.64</td>
</tr>
<tr>
<td>Castration + MLT</td>
<td>9/28 (32%)**</td>
<td>33.11 ± 4.42</td>
<td>1.94 ± 0.41</td>
</tr>
</tbody>
</table>

*aP < 0.05 compared with sham + saline group (χ² test).

**P < 0.05 compared with castration + saline group (χ² test).

When the tumor became noticeable. Data shown are mean ± SE.

The rate of LNCaP tumor growth in each animal was estimated by the ratio of the last recorded tumor volume to the number of days during which the animals inoculated with tumor cells were monitored. The mean tumor growth rate for each group of animals was the average of the estimated growth rates for individual tumors within the group. Data shown are mean ± SE.
increase in \[\text{[H]}\text{thymidine incorporation into LNCaP cells stimulated by 10}^{-9} \text{ or 2-iodomelatonin in combination with EGF.}

Volunteer changes of LNCaP xenograft tumors developed in castrated nude mice given daily saline or melatonin (MLT) injection. (Fig. 1)

Expression of MT1 Receptor, EGF, and EGF Receptor in Human Prostate Cancer Tissue

MT1 receptor protein expression was detected in invasive adenocarcinoma cells by using purified anti-MT1 receptor serum by immunohistochemistry (Fig. 3A). The anti-MT1 receptor immunoreactivity was blocked by TIL3 peptide (Fig. 3B). Positive staining of the neoplastic cells with anti-EGF (Fig. 3C) and anti-EGF receptor (anti-EGFR) antibodies (Fig. 3D) was also demonstrated in the same specimen of human prostate cancer tissue (histopathological combined Gleason score 7).

Effects of EGF Alone and in Combination with Melatonin/2-Iodomelatonin on Thymidine Incorporation into LNCaP Cells

Concentration-dependent stimulatory effect on \[\text{[H]}\text{thymidine incorporation into LNCaP cells was observed with EGF treatment. Significant (P < 0.01) increases in \[\text{[H]}\text{thymidine incorporation into LNCaP cells were noted when the cells were incubated with increasing concentrations (1} \times 10^{-10} \text{ M to 1} \times 10^{-8} \text{ M) of EGF. Up to 38.9% (P < 0.01) and 90.0% (P < 0.001) increases in \[\text{[H]}\text{thymidine incorporation was observed when the cells were treated with 1} \times 10^{-8} \text{ M and 1} \times 10^{-9} \text{ M EGF, respectively.}

The effects of increasing concentrations of melatonin or 2-iodomelatonin in combination with 10^{-8} \text{ M EGF on \[\text{[H]}\text{thymidine incorporation into LNCaP cells are shown in Figure 4. Melatonin (5} \times 10^{-12} \text{ M to 5} \times 10^{-7} \text{ M) significantly (P < 0.05) attenuated the increase in \[\text{[H]}\text{thymidine incorporation into LNCaP cells stimulated by 10^{-8} M EGF (Fig. 4A). At 5} \times 10^{-7} \text{ M melatonin, \[\text{[H]}\text{thymidine incorporation into EGF-stimulated LNCaP cells was attenuated 26.0\% (P < 0.05) compared with vehicle-treated control. Similarly, 2-iodomelatonin (5} \times 10^{-12} \text{ M to 5} \times 10^{-7} \text{ M) reduced the EGF-induced increase in \[\text{[H]}\text{thymidine incorporation into LNCaP cells in a concentration-dependent manner (Fig. 4B). Compared with control, up to 23.2\% (P < 0.05) attenuation in \[\text{[H]}\text{thymidine incorporation into LNCaP cells was observed at 5} \times 10^{-7} \text{ M 2-iodomelatonin. Furthermore, there was no difference in LNCaP cell viability with EGF, EGF plus melatonin, or EGF plus 2-iodomelatonin treatment under serum-free condition (data not shown).}

Effects of Melatonin/2-Iodomelatonin, EGF, and Their Combinations on Cyclin D1 Levels in LNCaP Cells

Figure 5 depicts the immunoblots of LNCaP cell lysates probed with anti-cyclin D1 antibody after the tumor cells had been incubated separately with vehicle, 5 \times 10^{-7} \text{ M melatonin or 2-iodomelatonin, 10^{-8} M EGF and their combinations. Compared with vehicle-treated controls, steady-state levels of cyclin D1 protein were decreased in melatonin-treated (Fig. 5A,C) and 2-iodomelatonin-treated (Fig. 5B,D) LNCaP cells by 20.7\% (P < 0.05) and 24.1\% (P < 0.05), respectively. The inhibitory effect of melatonin (Fig. 5A,C) or 2-iodomelatonin (Fig. 5B,D) on LNCaP cyclin D1 was also observed under EGF stimulation. Melatonin and 2-iodomelatonin induced 20.6\% (P < 0.05) and 21.1\% (P < 0.01) decreases in cyclin D1 levels compared with EGF-treated cells.

Effects of Melatonin/2-Iodomelatonin on Proliferation of Androgen-Sensitive and Androgen-Insensitive Human Prostate Cancer Cells

The effects of melatonin and/or 2-iodomelatonin on \[\text{[H]}\text{thymidine incorporation into androgen-sensitive LNCaP cells, androgen-insensitive PC-3 and DU 145 cells, and the effects of the indoleamine on the cell counts of these three human prostate cancer cell lines are described in Table II. For PC-3 and DU 145 cells, no significant changes in cellular \[\text{[H]}\text{thymidine incorporation and cell count were detected in response to melatonin or 2-iodomelatonin treatment. In contrast, melatonin (1} \times 10^{-9} \text{ M and 5} \times 10^{-7} \text{ M) decreased LNCaP cellular \[\text{[H]}\text{thymidine incorporation (P < 0.05) and cell count (P < 0.01). Similarly, 2-iodomelatonin (1} \times 10^{-9} \text{ M and 5} \times 10^{-7} \text{ M) decreased \[\text{[H]}\text{thymidine incorporation into LNCaP cells (P < 0.01). No significant differences in cell viability were detected for all three prostate cancer cell lines in response to vehicle, melatonin, or 2-iodomelatonin treatments (data not shown).}
Expression of MT₁ Receptor in Human Prostate Cancer Cell Lines

LNCaP and DU 145 cells were positively stained by immunocytochemistry by using purified anti-MT₁ receptor serum (Fig. 6A,C) and the reactivities were blocked by TIL3 peptide (Fig. 6B,D). In contrast, MT₁ receptor protein could not be detected in PC-3 cells (Fig. 6E).

Effect of Melatonin on DU 145 Tumor Growth

When daily saline or melatonin treatment was initiated 10 days before DU 145 tumor cell inoculation,
80% of the nude mice (4 of 5) in each group developed tumors. No significant differences in tumor volumes were recorded between melatonin-treated animals and saline-treated animals (data not shown).

**MT₁ Receptor Expression in PC-3 Cells Stably Transfected with MT₁ Receptor cDNA**

Twelve independent clonal lines of PC-3 cells stably transfected with MT₁ receptor cDNA were isolated. Screening by 2-[¹²⁵I]iodomelatonin binding revealed six clonal cell lines that exhibited significant specific binding (data not shown). Two clonal cell lines with the highest specific binding for 2-[¹²⁵I]iodomelatonin were designated as PC3-MT₁-a and PC3-MT₁-b, respectively. MT₁ receptor expression in PC3-MT₁-a and PC3-MT₁-b transfected cells was characterized by saturation 2-[¹²⁵I]iodomelatonin binding assays and by immunocytochemistry using purified anti-MT₁ receptor serum.

2-[¹²⁵I]iodomelatonin binding to the membranes of PC3-MT₁-a and PC3-MT₁-b transfected cells increased with increasing concentrations of 2-[¹²⁵I]iodomelatonin and approached saturation at approximately 200 pM. 2-[¹²⁵I]iodomelatonin binding to the membranes of both transfected cell lines was saturable and of high affinity. For PC3-MT₁-a cells, the dissociation constant (K_d) and maximum number of binding sites (B_max) analyzed from the saturation binding data were 31.98 pM and 99.78 fmol/mg protein, respectively (Fig. 7A,C). For PC3-MT₁-b cells, the dissociation constant (K_d) and maximum number of binding sites
On the other hand, PC-3 cells stably transfected with plasmid vector pcDNA3 (PC3-vector) showed no immunoreactivity with purified anti-MT1 receptor serum (Fig. 8E).

### Effects of 2-Iodomelatonin on Proliferation of Transfected PC-3 Cells Expressing MT1 Receptor

The effects of 2-iodomelatonin on [\(^{3}\)H]thymidine incorporation into PC3-MT1-a and PC3-MT1-b cells are shown in Table II. No significant changes in [\(^{3}\)H]thymidine incorporation into both clonal lines of transfected cells were noted when the cells were treated with 5 × 10⁻⁷ M 2-iodomelatonin for 48 hr. Table II also depicts the effects of 2-iodomelatonin on the cell counts of PC3-MT1-a and PC3-MT1-b cells. 2-Iodomelatonin induced no significant changes in the cell counts of the two clonal transfected cell lines. In addition, viabilities of PC3-MT1-a and PC3-MT1-b cells were unaffected by 2-iodomelatonin (data not shown).

### DISCUSSION

In the nude mice xenograft model of the present study, androgen deprivation induced by castration was shown to be associated with a 28% decrease in the incidence in androgen-sensitive LNCaP tumor development as well as a decrease in the mean growth rate of developed tumors in castrated mice compared with sham-operated controls (Table I). This finding reflects the loss of androgenic stimulation of LNCaP cell growth in castrated nude mice, which agrees with the findings of the initial report on the in vivo characterization of this hormone-sensitive human prostate cancer cell line [23]. Apparently, LNCaP tumor growth in castrated nude mice, as measured by the incidence of development and estimated by the averaged growth rates of developed tumors, would be good indicators for assessing any potential additive or synergistic effect of the antiproliferative action of melatonin (MLT) on androgen-sensitive prostate cancer growth under androgen-deprived conditions in vivo. In our study, a 23% decrease in the incidence of LNCaP tumor development in castrated mice was associated with timed daily injections of melatonin (Table I). Furthermore, the mean growth rate of LNCaP tumors that did develop in castrated mice was lower in the group that received melatonin than in the group that received saline (Table I; Fig. 1).

In conjunction with our previous observations of melatonin-induced inhibition of LNCaP cell proliferation both in vitro under a serum-free (androgen-free) condition [6] and in vivo under an androgen-rich condition in postpubertal nude mice with intact testis [9], the present data indicate that the direct antiproliferative action of melatonin can also be validated...
in vivo under castration-induced androgen-deprived condition. It is noteworthy that, while under the androgen-rich condition in nude mice with intact testis, timed daily i.p. administration of melatonin initiated 10 days after tumor cell inoculation resulted in slowing of the LNCaP tumor growth, no significant decrease in the incidence of LNCaP tumor development was observed [9]. In contrast, the same regimen of melatonin treatment resulted in a significant decrease in the incidence of LNCaP tumor development, associated with a decrease in the growth rate, under castration-induced androgen-deprived condition in the present investigation (Table I; Fig. 1). Together with the fact that the loss of androgenic stimulation by castration already induced decreases in the incidence and growth of LNCaP prostate tumor, the further drop in LNCaP tumor incidence in castrated mice due to antiproliferation by melatonin would argue in favor of an overall potential synergistic action of melatonin and castration in inhibiting androgen-sensitive LNCaP tumor growth in vivo.

In addition to androgens, other mitogenic regulators such as peptide growth factors, including EGF and related peptide such as TGF-α which share the same receptor (EGFR), are well recognized to play pivotal roles in the progression of prostate cancer by their autocrine/paracrine modes of growth-stimulatory actions on malignant prostate epithelial cells [15]. It can be inferred from earlier separate reports on melatonin MT₁ receptor [6] and EGF/EGFR...
expression [24] in human prostate cancer tissues that potential interactions between melatonin and EGF signaling in the modulation of prostate cancer pathophysiology in a subgroup of advanced androgen-sensitive prostate cancer patients probably exist. To support this prediction, MT1 receptor, EGF, and EGFR expression were examined and, indeed, found to be expressed in the malignant cells of the same specimen of human prostate cancer tissue (Fig. 3), which was androgen-sensitive, as indicated by a negative history of previous hormonal treatment of the patient before surgery and a significant decrease in the patient's serum prostate specific antigen after castration. Because only one prostate cancer sample has been studied, further investigations on a larger number of cancer specimens are needed to estimate the potential size of this subgroup of advanced androgen-sensitive prostate cancer patients.

Given that EGF [25] and EGFR [26] are expressed in LNCaP cells that also express melatonin MT1 [6] but not MT2 receptor [8] subtype, this cell line would appear to be a suitable and relevant experimental model to investigate whether and how melatonin/2-iodomelatonin may interact with EGF in the regulation of androgen-sensitive prostate cancer cell proliferation. Melatonin was found to attenuate the proliferation of EGF-stimulated LNCaP cells concentration-dependently (Fig. 4A). Similarly, 2-iodomelatonin, a melatonin receptor agonist, reduced the EGF-induced increase in [3H]thymidine incorporation into LNCaP cells in a concentration-dependent manner (Fig. 4B). Compared with the growth-inhibitory actions of other compounds such as vitamin D [27] on LNCaP cells, the reduction of EGF-stimulated thymidine incorporation by melatonin, although significant, is relatively low.

In light of previous report which demonstrated that EGF can stimulate LNCaP cell proliferation by up-regulating cyclin D1 [28], a cell cycle protein known to be important for G1/S phase transition, cyclin D1 expression in EGF-stimulated LNCaP cells in response to melatonin or 2-iodomelatonin was also examined in the present investigation. Steady-state LNCaP cyclin D1 level was found to be inhibited by melatonin or 2-iodomelatonin (5 × 10^{-7} M) alone (Fig. 5). This finding is compatible with the inhibition of cell proliferation and induction of G1/S progression delay demonstrated recently in cell count and flow cytometric analyses of melatonin-treated LNCaP cells [8]. In addition, either indoleamine could reduce EGF-stimulated increase in cyclin D1 (Fig. 5). The above findings suggest that significant interactions between MLT/MT1 receptor- and EGF/EGFR-mediated signaling in the modulation of androgen-sensitive prostate cancer cell proliferation probably exist. Part of these interactions may be mediated by means of opposite changes in cyclin D1 levels, with attendant secondary effects on G1/S cell cycle progression, induced by activated EGFR and MT1 receptor. Apparently, such interaction between MLT/MT1 receptor- and EGF/EGFR-mediated signaling in androgen-sensitive prostate cancer would be particularly important under androgen-deprived conditions, when the modulated cyclin D1 levels will integrate with changes in other positive and negative cell cycle regulators, such as cdk2, cdk4, and p16, that would have occurred with loss of androgen stimulation [29].

### TABLE II. Effects of Melatonin (MLT) or 2-Iodomelatonin (IodoMLT) on [3H]Thymidine Incorporation and Cell Count of LNCaP, PC-3, DU 145, PC3-MT1-a, and PC3-MT1-b Cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LNCaP</th>
<th>PC-3</th>
<th>DU 145</th>
<th>PC3-MT1-a</th>
<th>PC3-MT1-b</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3H]thymidine incorporation (dpm/mg protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>5,386 ± 128</td>
<td>6,211 ± 257</td>
<td>7,586 ± 191</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>MLT (1 × 10^{-9} M)</td>
<td>5,070 ± 93*</td>
<td>6,157 ± 267</td>
<td>7,440 ± 121</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>MLT (5 × 10^{-7} M)</td>
<td>4,695 ± 135***</td>
<td>6,280 ± 150</td>
<td>7,939 ± 271</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Vehicle</td>
<td>4,858 ± 41</td>
<td>5,984 ± 192</td>
<td>6,174 ± 199</td>
<td>9,471 ± 221</td>
<td>8,844 ± 289</td>
</tr>
<tr>
<td>IodoMLT (1 × 10^{-9} M)</td>
<td>4,271 ± 216**</td>
<td>5,966 ± 268</td>
<td>6,134 ± 230</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>IodoMLT (5 × 10^{-7} M)</td>
<td>4,235 ± 43**</td>
<td>6,182 ± 284</td>
<td>5,876 ± 303</td>
<td>9,555 ± 237</td>
<td>9,319 ± 184</td>
</tr>
<tr>
<td>Cell count (× 10^6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>2.98 ± 0.11</td>
<td>1.58 ± 0.10</td>
<td>5.32 ± 0.14</td>
<td>3.82 ± 0.15</td>
<td>2.17 ± 0.15</td>
</tr>
<tr>
<td>MLT (1 × 10^{-9} M)</td>
<td>2.56 ± 0.07**</td>
<td>1.69 ± 0.08</td>
<td>5.28 ± 0.23</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>MLT (5 × 10^{-7} M)</td>
<td>2.20 ± 0.09***</td>
<td>1.61 ± 0.09</td>
<td>5.35 ± 0.19</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>IodoMLT (5 × 10^{-7} M)</td>
<td>—</td>
<td>—</td>
<td>4.03 ± 0.12</td>
<td>2.32 ± 0.11</td>
<td>—</td>
</tr>
</tbody>
</table>

*Data shown are mean ± SE (n = 6 for [3H]thymidine incorporation; n = 8 for cell count).

*P < 0.05 compared with vehicle.

**P < 0.01 compared with vehicle.

***P < 0.001 compared with vehicle.
in slowing malignant cell cycle G1/S progression. This mechanism may also provide a plausible explanation for the observed synergistic inhibitory effect of melatonin on androgen-sensitive LNCaP tumor growth in castrated mice described above.

With respect to the potential role of MLT/MT1 receptor in the modulation of hormone-insensitive prostate cancer cell proliferation, melatonin was found to have no effects on the proliferation (as measured by cell count and thymidine incorporation) of PC-3 and DU 145 cells in the present study (Table II), whereas a direct antiproliferative effect of the indoleamine on hormone-sensitive LNCaP cells, which express MT1 receptor protein, was demonstrated both in this study.
(Table II) and in previous report [6]. Interestingly, our results are different from others who reported inhibitory actions of melatonin on PC-3 [16] and DU 145 [17] cell proliferation in vitro. Although the above discrepancies in research findings can readily be explained by differences in the details of experimental designs and methods, it is noteworthy that the lack of melatonin-induced inhibition on PC-3 and DU 145 cell proliferation in vitro reported in this investigation is in concordance with the lack of any effects of melatonin on the growth of DU 145 and PC-3 tumor in nude mice demonstrated in this (data not shown) and previous [9] studies. Furthermore, MT1 receptor protein expression was detected in DU 145 cells but not in PC-3 cells (Fig. 6). These results were compatible with previous findings on the expression of MT1 receptor gene in DU 145 cells [17] and the absence of MT1 receptor protein expression in PC-3 xenograft tumors [9]. Interestingly, despite the expression of MT1 receptor gene and protein in DU 145 cells, 2-[125I]iodomelatonin binding to DU 145 cell membranes could not be demonstrated [17]. Given that MT1 receptor is responsible for the transduction of the antiproliferative signal of melatonin [6,9] and for mediating potential interactions of the pineal indoleamine with EGF/EGFR signaling as indicated above for androgen-sensitive LNCaP cells, it is possible that defective MT1 receptor protein expression and/or functions in androgen-insensitive PC-3 and DU 145 cells may account for the failure of antiproliferative signal transduction at the cell surface for melatonin.

To explore the role of MT1 receptor protein expression on the proliferative responses of androgen-insensitive prostate cancer cells to the pineal

Fig. 7. Saturation 2-[125I]iodomelatonin binding of PC3-MT1-a (A,C) and PC3-MT1-b (B,D) cells. Representative saturation isotherms of 2-[125I]iodomelatonin binding to membrane preparations of PC3-MT1-a (A) and PC3-MT1-b (B) cells. The Scatchard plots of the data. PC3-MT1-a cells: $K_d = 31.98 \text{ pM}$, $B_{\text{max}} = 99.78 \text{ fmol/mg protein}$ ($r = 0.990$) (C); PC3-MT1-b cells: $K_d = 39.69 \text{ pM}$, $B_{\text{max}} = 432.86 \text{ fmol/mg protein}$ ($r = 0.994$) (D).
indoleamine, PC-3 cells were stably transfected with MT₁ receptor subtype cDNA and the effects of 2-iodomelatonin, a melatonin receptor agonist, on the proliferation of MT₁-transfected PC-3 cells were determined. Surprisingly, no changes in thymidine incorporation into the two clonal MT₁ receptor cDNA-transfected PC-3 cell lines PC3-MT₁-a and PC3-MT₁-b (Table II), which expressed structural and functional MT₁ receptor as indicated, respectively, by immunocytochemistry (Fig. 8) and by high-affinity specific $^{2-}[^{125}]i$iodomelatonin binding (Fig. 7), in response to 2-iodomelatonin were recorded. The transfection data,
taken together with previous and present results on MT₁ receptor protein expression and functions reported for PC-3 [9] and DU 145 cells [17], suggest that melatonin/MT₁ antiproliferative signal transduction may become defective, not only through changes in membrane receptor protein expression and/or functions, but also by means of alterations in downstream postreceptor signaling events, when androgen-sensitive prostate cancer cells become androgen-insensitive.

The association of the androgen sensitivity of male prostate cancer cells with their responsiveness to the antiproliferative action of melatonin, demonstrated in previous [6,9] and present studies, is intriguing. It is paralleled by the dependency of the growth-inhibitory effects of melatonin on the functional status of estrogen/estrogen receptor signaling in female breast and endometrial cancer cells [30,31]. Given that androgen signaling is shut down in PC-3 and DU 145 cells as a result of absent androgen receptor (AR) expression [32] but is still functionally intact through mutated AR in LNCaP cells, it is likely that the functional integrity of MLT/MT₁ receptor-induced antiproliferative signaling is dependent on the activation status of androgen/AR transduction cascades in human prostate cancer. This is a possible mechanism by which androgen/AR- and MLT/MT₁ receptor-mediated signaling can interact. It would be of interest to substantiate this mechanism in future studies by determining whether the antiproliferative action of melatonin will be abolished in LNCaP cells whose AR has been inactivated by genetic manipulations. Recently, another cellular mechanism of crosstalk between melatonin and androgen signaling has also been demonstrated in LNCaP cells. Attenuation of AR transcriptional activity by melatonin was reported to be mediated by means of nuclear exclusion of AR [33].

Together with previous findings reported by this and other laboratories, the present in vitro and in vivo data on MLT/MT₁ receptor signaling may have significant implications on future clinical exploitation of the therapeutic potential of the pineal hormone melatonin in advanced prostate cancer management. By using the nude mice xenograft model of LNCaP cells, data have been obtained on the potential synergistic inhibition of androgen-sensitive tumor growth by the antiproliferative action of melatonin in combination with castration. Although caution is needed in extrapolating animal data to clinical situations, our results provide significant support for the notion of potential additional therapeutic benefits of combination hormonal therapy involving androgen deprivation and melatonin supplementation to patients suffering from advanced androgen-sensitive prostate cancer.

Whereas some of the tumor cells of relapsed prostate cancer specimens, like the androgen-insensitive PC-3 and DU 145 cells, may not express androgen receptor protein, loss of androgen receptor expression, however, is not recognized to be associated with tumor relapse from androgen ablation therapy [34–38]. As a corollary, melatonin may also be effective in the treatment of relapsed prostate cancer in which functional AR signaling is maintained after acquisition of the hormone-refractory state. Indeed, melatonin has been reported to retard the growth of some relapsed prostate tumor developed in patients who had been treated with medical castration [39]. All the above evidence indicates the need for randomized controlled trials to define the efficacies of the proposed combination castration–melatonin regimen in the management of advanced hormone-sensitive prostate cancer, and of melatonin in the treatment of relapsed tumor developed after androgen ablation therapy.

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