Antitumour activity of melatonin in a mouse model of human prostate cancer: relationship with hypoxia signalling

Abstract: Melatonin is known to exert antitumour activity in several types of human cancers, but the underlying mechanisms as well as the efficacy of different doses of melatonin are not well defined. Here, we test the hypothesis whether melatonin in the nanomolar range is effective in exerting antitumour activity in vivo and examine the correlation with the hypoxia signalling mechanism, which may be a major molecular mechanism by which melatonin antagonizes cancer. To test this hypothesis, LNCaP human prostate cancer cells were xenografted into seven-wk-old Foxn1nu/nu male mice that were treated with melatonin (18 i.p. injections of 1 mg/kg in 41 days). Saline-treated mice served as control. We found that the melatonin levels in plasma and xenografted tissue were 4× and 60× higher, respectively, than in control samples. Melatonin tended to restore the redox imbalance by increasing expression of Nrf2. As part of the phenotypic response to these perturbations, xenograft microvessel density was less in melatonin-treated animals, indicative of lower angiogenesis, and the xenograft growth rate was slower (P < 0.0001). These changes were accompanied by a reduced expression of Ki67, elevated expression of HIF-1α and increased phosphorylation of Akt in melatonin than saline-treated mice. We conclude that the beneficial effect of melatonin in reducing cancer growth in vivo was evident at melatonin plasma levels as low as 4 nm and was associated with decreased angiogenesis. Higher HIF-1α expression in xenograft tissue indicates that the antitumour effect cannot be due to a postulated antihypoxic effect, but may stem from lower angiogenesis potential.

Introduction

A circadian-regulated indolamine secreted by the pineal gland, that is, the natural compound melatonin (N-acetyl-5-methoxy tryptamine), is characterized by a wide functional diversity associated with cell protection and survival [1, 2]. Additionally, melatonin has antitumour activity against several types of cancers including androgen-dependent prostate tumours [3], most probably through the MT1 receptor [4]. Several mechanisms have been proposed to explain the effects of melatonin in prostate cancer, including stimulation of apoptosis either by activation of c-JUN N-terminal kinase (JNK) and p-38 [5] and cell cycle inhibition by down-regulation of the transcriptional activity by androgens binding to their cognate receptors [6]. Another candidate mechanism underlying the antitumour effect of melatonin involves hypoxia signalling and the hypoxia-inducible factor-1α (HIF-1α). Indeed, it has been proposed that melatonin-mediated HIF-1α inhibition suppresses tumour angiogenesis. This feature, in addition to others involved in antioxidant defence and in the modulation of several proteins participating to various cell pathways, may result in tumour inhibition.

Hypoxia is believed to play a pivotal role in the development of most solid tumours [7] as depressed O₂ level in the core of solid tumours is believed to induce greater resistance of tumour cells to a hostile environment; this translates into repression of apoptosis, faster cell turnover and growth [8]. Indeed, when the degree of hypoxia is increased by breathing an O₂-poor atmosphere, the growth rate of prostate LNCaP xenografts nearly doubles [9]. Because HIF-1α, an O₂ sensor that activates hundreds of downstream genes in response to hypoxia, is a central hub of cell adaptation to lowered O₂ levels [10], it is intuitive that it might mediate cell responses enabling survival in O₂-poor microenvironments.

In support of this hypothesis, in LNCaP cells exposed to mimetic hypoxia, melatonin down-regulates HIF-1α [11]. Furthermore, in the HCT116 human colon cancer cell line, melatonin destabilizes HIF-1α secondary to its antioxidant activity through a mechanism that involves the dephosphorylation of p70S6K and its target RPS6 [12]. Such evidence, however, stems essentially from in vitro studies performed in the presence of millimolar levels of melatonin in the incubation medium. This may represent a critical issue for translation of the results into humans because such levels of melatonin may be attainable with difficulty in vivo. Reports addressing the antitumour effect of melatonin in vivo often have been obtained in situations where plasma melatonin levels are two-to-three orders of magnitude lower than those found in the incubation medium.
magnitude lower than those utilized in cell cultures. In the current study, we test the antitumour potential of nanomolar levels of melatonin in an established in vivo model of human prostate cancer suitable to address the complex interaction between melatonin and HIF-1α signalling.

Materials and methods

Mice

Seven-week-old Foxn1nu/nu male mice (code 069; Harlan Laboratories, Indianapolis, IN, USA; n = 19) were used in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (Institute for Laboratory Animal Research. 2011. Guide for the care and use of laboratory animals (8th Edition): Washington (DC): National Academy Press, www.nap.edu). The Ethical Committee of the University of Milan approved the experimental protocol (All. 5verb. 16.03.2010). Mice had free access to sterilized water and 60Co γ-irradiated laboratory diet until 24 hr before sacrifice. A 12/12 hr light/dark cycle was maintained. All operations were performed under sterile laminar flow hoods.

Xenografts

LNCaP cells American Type Culture Collection (ATCC) were maintained in RPMI-1640 medium (Euroclone SpA, Life Science Division, Pero, Italy) supplemented with 10% (v/v) heat-inactivated foetal bovine serum plus l-glutamine and cultured in 5% CO2. On day zero, LNCaP cells (80–90% confluence) were resuspended (1:1) in Matrigel (Beckton Dickinson Italia SpA, Bucinasco, Italy) and inoculated (3 x 106/0.1 mL) in each flank of the mice.

Groups and treatments

Mice were divided into two groups, saline-treated (n = 6) and melatonin-treated (n = 13). Each treatment consisted in 18 i.p. injections (0.1 mL) of either saline or melatonin for 41 days (3 injections/wk, at 9.00–10.00 a.m.). We selected this protocol as a compromise between the need to mimic the real situation occurring in human subjects under long-term melatonin treatment and the need to minimize the stress induced in animals. The delivered melatonin dose per injection was 1 mg/kg or 0.03 mg/mouse/0.1 mL (assuming 30 g body weight). Due to poor melatonin water solubility, the solution was prepared by dissolving 6 mg melatonin in 1 mL EtOH plus 19 mL sterile saline, followed by filtration through 0.2-μm-pore-size filter and storage under sterile conditions at 4°C until use. Saline contained 5% EtOH as the solution with melatonin. The same solution was used for the entire treatment.

In vivo measurements

Body weight and tumour volume were monitored 3 times/wk. The volume of the xenografts was measured as length-width-height 0.5236 using a digital caliper.

Tissue collection

At day 41, 72 hr after the last administration, mice were anesthetized with sodium thiopental (10 mg/100 g body weight) plus heparin (500 units) and euthanized by cervical dislocation. After thoracotomy, an aliquot of blood was withdrawn by intracardiac puncture from the left ventricle into a heparinized syringe. The blood sample was immediately centrifuged (4400 g for 7 min at 4°C), and plasma was recovered and stored at −20°C. Xenografts were dissected from the surrounding skin, weighed and divided into two aliquots to be either immersed in liquid N2 and frozen at −80°C for subsequent analysis, or placed in a formalin solution for morphological studies.

Melatonin assay

Melatonin was assayed in blood plasma and tissue by a competitive enzyme immunoassay (Melatonin ELISA REF RE54021; IBL, Hamburg, Germany) that includes sample pre-purification by solid-phase extraction (SPE) on C18 RP columns provided by the manufacturer. Aliquots (0.5 mL) of samples, controls or calibrators were purified by SPE following the manufacturer’s instructions, dried under nitrogen and stored at −20°C for up to 48 hr. Plasma samples from melatonin-treated group suspected to contain concentrations higher than the highest standard (300 pg/mL) were diluted 1: 50 (v/v) with diluted assay buffer prior to the extraction step. Dried extracts were reconstituted with 0.15 mL of bidistilled water and 0.05 mL transferred in duplicate into the microtitre plate. After processing as described by the manufacturer’s instructions, the microplate was read at 405 nm. By considering B = OD standard, B0 = OD blank, Logit B/B0 = LN [(B/B0)/(B/B0−1)], the concentration of plasma melatonin in pg/mL (i.e. ng/L) was calculated by plotting logit B/B0 on γ-axis versus LN of melatonin concentration (LN pg/mL) on x-axis. In case of diluted sample, the final value was multiplied with the corresponding dilution factor. Samples showing concentrations above the highest standard were re-assayed after appropriate dilution. The sensitivity of the assay was 1.6 pg/mL. Both intra- and interassay coefficients of variation were <20%.

For the determination of melatonin concentration in tumour tissue, a described procedure [13] was followed, with minor modifications. Frozen tumour biopsies were quickly cut with a lancet on an ice block, weighed (5–20 mg) and homogenized in a fixed volume (500 μL) of PBS [1X] using a Politron Ultra-Turrax for 1 min; during this procedure, the tubes were kept in ice. After centrifugation at 18,000 g for 5 min, the supernatants were diluted (1:15 for saline and 1:100 for melatonin-treated group) and 0.5 mL loaded on SPE column and processed as described for plasma samples. Results were expressed as ng/g tissue.

Redox imbalance

To determine the level of oxidative stress in the plasma, we measured the capacity of in vivo formed hydroperoxides (ROMs) to generate in vitro alkoxyl (R-O•) and...
peroxyl (R-OO⁻) radicals in the presence of iron released from plasma by an acidic buffer by the d-ROMS test (cod. MC001, DIACRON Labs S.r.l., Grosseto, Italy). The analytical procedure described for human samples [14] on microtitre plate in end-point mode was adapted for determination in the plasma of mice. Water, calibrator or plasma sample (1 μL) were added into the wells, followed by 2 μL of chromogenic mixture (aromatic alkyl-amine) and 200 μL of acetate buffer pH 4.8. After delicately mixing, the plate was incubated at 37°C for 90 min and the optical densities read at λ = 540 nm in an automatic microplate reader. The lyophilized calibrator was stated to contain 320 CARR U. As 1 CARR U is equivalent to 0.08 mg/μL H₂O₂, the calibrator concentration was calculated to be 256 mg/L H₂O₂, that is, 7.53 mM. The results of d-ROMS test were expressed according to the following formula:

\[
\text{[Sample] (mM H}_2\text{O}_2) = \frac{\text{Abs sample/Abs calibrator} \times [\text{calibrator} (\text{mM H}_2\text{O}_2)}}{	ext{calibrator}}.
\]

The sensitivity of the d-ROMS test was 0.26 mM H₂O₂, and the method was linear up to 267 mM. Intra- and interassay CV’s were <5%.

**Plasma antioxidant capacity**

The plasma antioxidant capacity was assayed using a colorimetric assay kit (Total Antioxidant Capacity Assay kit Catalog #K274-100; BioVision, Inc., Mountain View, CA, USA) that measures Cu²⁺ reduction to Cu⁺ by the antioxidant factors in the sample by coupling with a colorimetric probe. For calibration, 1 mM Trolox in DMSO: water was used. Each microtitre plate was filled with either 0.1 mL calibrators (0, 4, 8, 16 or 20 nmol Trolox) or 0.1 mL diluted plasma (1:2000 v:v, equivalent to 0.25 μL). Then, 0.1 mL freshly prepared Cu²⁺ working solution was added into each well and the mixture incubated at room temperature for 1.5 hr. The optical density was determined at λ = 540 nm in a microplate reader. The standard Trolox (nmol/well) versus optical density curve was used to obtain the sample antioxidant capacity expressed as nmol Trolox equivalent/L plasma (i.e. mM Trolox equivalents): [(sample OD-blank OD)/(slope of standard curve in nmol)]/undiluted plasma volume (μL) added to the wells.

**Morphology**

Paraffin-embedded specimens were serially sectioned at 7 μm thickness. These sections were deparaffinized, rehydrated and stained with haematoxylin–eosin. A minimum of five fields from five sections for each experimental group was observed with an optical light microscope (Olympus, Hamburg, Germany) at a final magnification of 400× by two observers blinded to the treatment. Images were analysed using Image Pro Plus software (Image Pro PlusTM 4.5.1 Version, Milan, Italy). For the evaluation of microvessel diameter, digital images of xenograft sections were captured using a light microscope at 200× magnification. For each group, five fields from five different haematoxylin–eosin-stained sections per xenograft were examined.

The microvessel diameter (μm) was determined in each field using image analysis software (Image Pro PlusTM 4.5.1 Version).

**Western blot**

Extracts were prepared for each biopsy at 4°C. Frozen tissue was homogenized in a glass potter in a 1:3 ratio (w:v) with 10 mM HEPES, 1.5 mM MgCl₂, 0.5 mM DTT, 0.2 mM PMSF, 10 mM KCl and Protease Inhibitor Cocktail (Complete Protease Inhibitor Cocktail Tables, EDTA-free, Roche Diagnostics GmbH, Mannheim, Germany), pH 7.9, centrifuged for 20 min at 14,000 rpm, and the pellet was resuspended and centrifuged again for 10 min at 20,600 g. The extract was obtained by pooling the supernatant fractions from both centrifugations.

The protein concentration was measured by the Coomassie Plus Protein Assay reagent kit (#1856210; Thermo Scientific, Rockford, IL, USA), and 70 μg protein was loaded per lane and separated by SDS-PAGE (6% acrylamide gels). Then proteins were blotted onto a nitrocellulose membrane (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) and blocked with 5% nonfat dry milk in TRIS-buffere saline containing 0.1% Tween (1 hr, room temperature). Membranes incubation overnight at 4°C with the primary antibody was followed by incubation with horseradish peroxidase-conjugated secondary antibody (1 hr, room temperature). The used primary antibodies and dilutions were: rabbit polyclonal anti-HIF-1α (sc-10790 Santa Cruz Biotechnology, Inc., Dallas, TX, USA, 1:300), rabbit polyclonal anti-VEGF165 (PC315 Calbiochem; Merck KGAA, Darmstadt, Germany, 1:400), rabbit monoclonal anti-actin (A2066 Sigma Aldrich, St Louis, MI, USA 1:2000), rabbit polyclonal anti-phospho-Akt-Ser473 (#9271 Cell Signaling Technology, Inc., Danvers, MA, USA, 1:1000), rabbit polyclonal anti-Akt (#9272 Cell Signaling Technology, 1:1000), rabbit polyclonal anti-Nrf2 (sc-722 Santa Cruz Biotechnology, 1:250). The secondary antibodies were hors eradish peroxidase-conjugated anti-rabbit IgG (11-035-003 Jackson Immuno Research, West Grove, PA, USA, 1:10000), Chemiluminescence was detected by incubating the membrane with LiteAblot Chemiluminescent substrate (Lite Ablot, EuroClone, EMPO10004) followed by x-ray film exposure (Kodak X-Omat Blue XB-1 Film; Eastman Kodak Company, Rochester, NY, USA). The resulting image was acquired, and blot intensity quantified by Gel Doc (Bio-Rad quantitation software Quantity One).

**Ki67, CD31 immunohistochemistry**

For the immunohistochemical study, sections were deparaffinized and rehydrated by routine protocol and immersed in 3% hydrogen peroxide in methanol for 30 min to remove endogenous peroxidase. Sections were incubated for 1 hr, slide by slide, at room temperature in normal serum (X0907, Dako Cytomation, Glostrup, Denmark, diluted 1:5). Samples were then incubated overnight with primary antibodies anti-CD31 (550274; BD Pharmingen, San Diego, CA, USA, diluted 1:50 in Tris buffer,
or the nonparametric Mann–Whitney increased approximately 60-fold (48 in the melatonin-treated mice. In xenograft tissue, melatonin levels in both plasma and xenografts. To assess the response of xenografts to the redox imbalance, about twice the level with respect to controls without tumours (data not shown) and the ends of the whiskers represent the minimum and maximum of all of the data. *P < 0.001 (nonparametric Mann–Whitney U-test).

Statistics

Depending on the normality test for Gaussian distribution, data are expressed either as mean ± S.E.M. or as box plots indicating the 25th percentile, the median and the 75th percentile, with whiskers indicating the max and min values. To assess the significance of the between-treatment differences, we performed either the Student’s t-test or the nonparametric Mann–Whitney U-test, respectively.

Results

All mice survived treatments without any evident adverse effects. Melatonin-treated mice did not suffer the body weight reduction observed in saline-treated mice. At the end of the observation period, the body weight in melatonin-treated mice increased by 1.3 ± 0.7 g versus a decrease of 1.6 ± 1.2 g in saline-treated mice (P < 0.0001, Fig. 1).

All mice received bilateral LNCaP cells xenografts, and 12 of 12 and 20 of 26 tumours became visible in SAL and melatonin, respectively. The success rate (100 and 77%, respectively) is without statistical significance (chi-square test P = NS). The time course of xenograft volume to body weight ratio in Fig. 1 shows a marked antitumour effect in melatonin mice as early as t = 17 days after xenograft.

The selected melatonin dose (18 i.p. injections of 0.03 mg/mouse, i.e. 0.13 μmol/mouse) corresponds to a total administration of 0.54 mg melatonin (i.e. 2.33 μmol/mouse) during the total treatment period. When assayed 72 hr after the last injection (Fig. 2), and despite the short half-life of melatonin in plasma [15], melatonin values were fourfold higher (243 ± 28 versus 897 ± 115 ng/g plasma (i.e. pg/mL), or 1.05 ± 0.12 versus 3.87 ± 0.49 nm/L plasma) in the melatonin-treated mice. In xenograft tissue, melatonin increased approximately 60-fold (48 ± 8 versus 1790 ± 600 ng/g tissue, or 0.21 ± 0.03 versus 7.72 ± 2.58

nmol/g tissue) with respect to saline-treated mice. Thus, the selected melatonin treatment resulted in marked elevation of melatonin levels in both plasma and xenografts.

To evaluate the redox imbalance in plasma, we measured hydroperoxide-generated alkoxyl (R-ÖO•) and peroxyl (R-OÖ•) radicals. Saline-treated mice displayed a significant redox imbalance, about twice the level with respect to controls without tumours (data not shown) and in the melatonin-treated mice (Fig. 3). The antioxidant capacity, assessed as Trolox equivalents, was not affected by melatonin, indicating a direct antiradical scavenging effect by melatonin.

To assess the response of xenografts to the redox imbalance, the nuclear factor (erythroid-derived 2)-like 2 (Nrf2)
protein, a transcription factor that triggers the cell antioxidant response, was measured. The lower panel in Fig. 3 shows that the expression level of Nrf2, assayed by Western blot, was increased in melatonin-treated mice.

Haematoxylin–eosin staining of xenograft tissue showed that in both groups, tissue had a lobular organization, with tumour cells often interspaced with normal parenchymal cells (Fig. 4A, left panel). Areas of necrosis within the tissue were also observed, but without appreciable differences between the groups. Xenografts in the melatonin group were characterized by nests of tumour cells circumscribed by normal parenchymal cells.

Haematoxylin–eosin staining was used for quantitative evaluation of microvessels. Fig. 4A (right panel) shows that the average microvessel diameter was markedly less in melatonin than saline xenografts, indicative of relatively immature microvessel network. To further address this issue, we measured the platelet-endothelial cell adhesion molecule (PECAM-1), also known as a cluster of differentiation 31 (CD31), by immunohistochemistry (Fig. 4B, left panel). Because CD31 is an endothelial cell marker [16], we quantitated the depressed anti-CD31 positivity observed in tumours of melatonin mice by determining the percentage of CD31-positive cells, which were markedly less in melatonin than saline xenografts (Fig. 4C, right panel).

Finally, to assess whether melatonin affects the signalling pathways leading to cell proliferation and the activation of the cell cycle, we focused on the expression of Ki67, a nuclear protein associated with cellular proliferation (Fig. 4C, left panel). Ki67 was quantitated as per cent Ki67 cell positivity. Fig. 4C (right panel) shows that Ki67 expression was markedly less in melatonin than saline xenografts, indicating depressed cell proliferation in the tumours of the melatonin-treated mice.

To assess the impact of the hypoxia signalling pathway, we measured the expression of HIF-1α by Western blot (Fig. 5). The results show that HIF-1α expression was increased in the xenografts of melatonin-treated mice compared to saline-injected control. As melatonin xenografts exhibited less neovascularization with respect to saline, but at the same time also greater HIF-1α expression, we measured the expression of two key proteins that are known to mediate angiogenesis, VEGF and its receptor VEGF-R2. The expression of both proteins is higher in melatonin than saline xenografts. Finally, to assess whether melatonin affects the signalling leading to the control of apoptotic processes, we measured by Western blot the expression of Akt, also known as protein kinase B, a Ser/Thr-specific protein kinase and its phosphorylated isoform that plays a key role in the control of apoptosis and cell proliferation. Fig. 5 shows that Akt phosphorylation at Ser473 was markedly increased in the xenografts as a result of melatonin treatment.

**Discussion**

The described experimental model of LNCaP xenografts in immuno-suppressed mice was suitable to investigate the long-term effects of melatonin, as its i.p. administration increased melatonin levels in plasma and in xenograft tissue. The tumour growth rate and vascularization were markedly reduced in melatonin-treated mice versus those treated with saline. At a molecular level, melatonin increased xenograft tissue expression of HIF-1α and Nrf2 while decreasing the expression of Ki67 and increasing Akt phosphorylation.

The observation that melatonin treatment increases by 60-fold the xenograft tissue levels versus a fourfold rise in plasma shows that melatonin concentrates in the tumour. Xenografted LNCaP cells appear to accumulate melatonin in their intracellular milieu. This finding indicates that melatonin enters LNCaP cells via facilitated or active diffusion through the cell membrane, in agreement with other findings [17]. As the rate of melatonin uptake is different in various cell types [18], it would not be surprising if melatonin antiproliferative properties vary in different tissues.

The association of melatonin with antitumour activity is in agreement with several in vitro and in vivo studies. For example, LNCaP cell proliferation is inhibited by 10 nM melatonin [19], whereas 50 nM and 1 mM melatonin, respectively, is required to reduce cell growth [20] and viability [21]. Melatonin may act by binding to G-protein-coupled receptors that activate K⁺ ATP and K⁺ Ca channels [22, 23], attenuate sex steroid-induced calcium influx (at a concentration of 5 nM [22]) and inhibit SIRT1, a NAD⁺-dependent histone deacetylase [at a concentration of 1 mM
[24, 25]). In vivo studies show that 4 mg/kg/day i.p. melatonin inhibits LNCaP xenografts when treatment is initiated 10 days before tumour inoculation, whereas treatments initiated 10 days after inoculation seem ineffective [26]. Furthermore, melatonin and the loss of androgens due to castration synergize to decrease LNCaP xenografts growth [27]. In an allograft model of BALB/c-derived renal adenocarcinoma cell line (RENCA) xenografts, 20 mg/kg/day melatonin for a week suppressed the growth in sprout xenografts [28]. None of these studies, however, addressed the actual plasma melatonin level.

Melatonin and its metabolites are well known as antioxidant [29–31]. The underlying mechanisms may, however, encompass not only direct scavenging of a variety of ROS, but also the modulation of the redox imbalance [32]. These mechanisms may be critical in assessing melatonin’s antitumour activity because melatonin has been shown to regulate cytoskeletal organization and to affect microfilament rearrangements of stress fibres, which implies protection of cytoskeletal organization from ROS [33]. ROS are also involved in O2-sensing processes because they may activate HIF-1α independently of the lack of O2.

In the model used here, melatonin treatment increased cytoplasmic Nrf2 expression, as previously observed [34–37]. Although its role in cancer development is still debated [38], Nrf2 is believed to function as a tumour suppressor because of its ability to detoxify the intracellular environment [39]. In fact, whereas oncosuppressive compounds activate Nrf2, oncoproteins degrade it [40]. It should be noted, however, that other studies show that Nrf2 accelerates proliferation of certain types of tumour cells, because Nrf2 also allows cancer cells to escape from

Fig. 4. Representative microphotographs illustrating the effects of melatonin (MLT) with respect to saline (SAL) treatments on tissue morphology stained with haematoxylin–eosin (A), CD31 immunoreactivity (B) and Ki67 immunoreactivity (C). The bar represents 50 µm. The arrows in panel A (MLT) indicate parenchymal cells. The box plots on the right side of the figure show (from top to bottom) the average microvessel diameter, CD31 positivity and Ki67 positivity as described in the text. Data are expressed as mean ± S.E.M., *P < 0.01 at the Student’s t-test.
Changes in VEGF-R2 expression [44]. Of interest, however, melatonin clearly down-regulated Ki-67 as in the present study.

Formation of a mature capillary network is known to require a sustained VEGF and/or VEGF-R2 overexpression during an extended period of time, but we presently ignore whether the proteins pattern observed 41 days after xenograft is consistent over the preceding weeks. Furthermore, the role of the VEGF/VEGF-R2 axis in cancer development is not yet understood, and several observations reflect its controversial role in cancer progression. For example, it has been shown that the presence of certain growth factors can induce differential splicing of the VEGF gene that expresses pro- or anti-angiogenic isoforms of the VEGF protein [45]. Future work on this issue would assess the anti-angiogenic property of melatonin by time-resolved analyses of VEGF, VEGF-R2 and Ki67.

Whatever the cause for reduced angiogenesis in melatonin xenografts, this might lead to greater hypoxia in the tumour core microenvironment, which increases HIF-1α expression in xenografts of melatonin-treated mice.

In cultured LNCaP cells, 1 μM melatonin inhibits HIF-1α expression, whereas in the presence of a hypoxia mimetic agent, 1 μM melatonin is required [11]. This inhibition was attributed to the melatonin’s antioxidant effect [12] and/or to impairment of the sphingosine kinase-1 pathway and consequent inhibition of Akt and GSK-3β phosphorylation [46]. The present study shows that melatonin increases HIF-1α expression in xenografts in vivo. Although evident in vivo versus in vitro differences may account for this paradox, the melatonin amount used in the various studies is a critical determinant. When hypoxic RENCA cells are incubated with varying melatonin doses, HIF-1α expression increases for [melatonin] ≤1 μM while declining for [melatonin] >1 μM [28]. As plasma melatonin in this study ranges from 1.7 to 6.5 nM, it is not surprising that HIF-1α expression increases in LNCaP xenografts in vivo. In qualitative agreement with the present findings, HIF-1α and VEGF expression are blocked in hypoxic HepG2 cells incubated with 1 μM melatonin, whereas lower melatonin concentrations are ineffective [47]. We are not aware of studies describing the in vivo effects of melatonin on HIF-1α, but HIF-1α is expected to peak at the beginning of hypoxia and to decline at characteristic speeds during sustained hypoxia in various cell types [48]. Although HIF-1α is over-expressed in LNCaP cells challenged with acute hypoxia [49], we ignore the time course and the rate of return to baseline levels.

It is still doubtful whether HIF-1α favours cancer progression. Whereas a HIF-1α inhibitor, digoxin, has been used for decades in the treatment of prostate cancer [50], another inhibitor, topotecan, failed to show significant anticaner effects in clinical trials. Several studies cast doubt on the effectiveness of HIF-1α inhibitors in tumour therapy and began to scrutinize the role of HIF-1α in cancer because of disappointing results [51]. Although a considerable array of in vitro studies point to the paradigm that the hypoxia-downstream adaptive patterns are mediated by HIF-1α, this pattern has not been confirmed in vivo [9]. Therefore, whereas in vitro findings converge in indicating that melatonin antitumour activity is due to an
antihypoxic role of melatonin, the present findings suggest a more complex scenario where the pivotal role is played by angiogenesis.

Akt activation is recognized as a cell survival signal in response to hypoxia [52, 53] and is associated with increased HIF-1α expression in prostate cancer cells [54]. In H4IIE hepatoma cells, Akt phosphorylation participates to the cell response to oxidative stress, while melatonin antagonizes this response [55]. Inhibition of Akt phosphorylation may be regarded as at an antitumour effect, because Akt is a common feature in advanced prostate cancers [52, 56] and predicts poor clinical outcome [40]. In the present investigation, however, melatonin increased Akt activation. Despite evident in vivo versus in vitro differences, this behaviour may be explained by low redox imbalance, which implies little effect of melatonin on Akt phosphorylation in vivo. Of interest, Akt phosphorylation is correlated with the expression of the cell proliferation antigen Ki67 in LNCaP cells [57]. Here, we show that Ki67 is negatively affected by melatonin despite increased Akt phosphorylation. Therefore, the mechanisms underlying melatonin effects on signalling are different in vivo and in vitro and deserve greater insight to be fully understood.

In this study, we did not discern a significant amount of apoptosis in neither group (data not shown) in agreement with a study demonstrating that the antitumour effect of melatonin may be mediated by inhibiting proliferation via cytokines as TNF-alpha rather than increased apoptosis [58]. This may be an unexpected finding as apoptosis and/or cell cycle arrest could be a key point in the antitumour effect of melatonin as shown in colorectal cancer cells [59]. However, although an elevated apoptotic rate has been repeatedly reported in androgen-dependent LNCaP prostate cancer cells [60], this finding was not confirmed [21], and melatonin-induced cell death in prostate cancer remains unsubstantiated [3].

In conclusion, this in vivo study confirms that i.p. treatment with melatonin fosters relevant antitumour activity even for melatonin plasma levels <5 nm. This opens new roads for alternative non-invasive ways to administer melatonin. The mechanism underlying melatonin’s antitumour activity is not linked to the melatonin antihypoxic role, but rather involves a complex scenario initiated by anti-angiogenic and antiproliferative effects linked to melatonin’s antioxidant properties. The complexity of melatonin’s effects renders this compound attractive for the development of novel therapies aimed at prevention and treatment of both indolamine-dependent and indolamine-refractory prostate cancer.

References


