Melatonin decreases cell proliferation, impairs myogenic differentiation and triggers apoptotic cell death in rhabdomyosarcoma cell lines

SILVIA CODENOTTI1, MICHELA BATTISTELLI2, SABRINA BURATTINI2, SARA SALUCCI2, ELISABETTA FALCIERI2, RITA REZZANI3, FIORELLA FAGGI1, MARINA COLOMBI1, EUGENIO MONTI1 and ALESSANDRO FANZANI1

1Department of Molecular and Translational Medicine, University of Brescia, Viale Europa 11, I-25123 Brescia; 2Department of Earth, Life and Environmental Sciences (DiSTeVA), University Carlo Bo, I-61029 Urbino; 3Department of Clinical and Experimental Sciences, University of Brescia, Viale Europa 11, I-25123 Brescia, Italy

Received January 14, 2015; Accepted March 30, 2015

DOI: 10.3892/or.2015.3987

Abstract. Melatonin is a small indole produced by the pineal gland and other tissues, and has numerous functions that aid in the maintenance of the whole body homeostasis, ranging from the regulation of circadian rhythms and sleep to protection from oxidative stress. Melatonin has also been reported to counteract cell growth and chemoresistance in different types of cancer. In the present study, we investigated the effects of exogenous melatonin administration on different human cell lines and primary mouse tumor cultures of rhabdomyosarcoma (RMS), the most frequent soft tissue sarcoma affecting childhood. The results showed that melatonin significantly affected the behavior of RMS cells, leading to inhibition of cell proliferation and impairment of myogenic differentiation followed by increased apoptotic cell death, as observed by immunoblotting analysis of apoptosis-related markers including Bax, Bcl-2 and caspase-3. Similar findings were observed using a combination of microscopy techniques, including scanning/transmission electron and confocal microscopy. Furthermore, melatonin in combination with doxorubicin or cisplatin, two compounds commonly used for the treatment of solid tumors, increased the sensitivity of RMS cells to apoptosis. These data indicated that melatonin may be effective in counteracting RMS tumor growth and chemoresistance.

Introduction

Soft tissue sarcomas are tumors arising from mesenchymal cell precursors that are committed towards the morphogenesis of soft tissues such as fat, muscle and deep skin tissues. Rhabdomyosarcoma (RMS) is considered a myogenic tumor and is classified as the most frequent sarcoma affecting children and adolescents (1). The current classification defines five different histotypes, with embryonal (eRMS) and alveolar (aRMS) subsets being the most frequently observed in children <5 years and in adolescents, respectively (2,3). The eRMS variant is the most treatable and most common subtype representing ~80% of RMS, while aRMS is more aggressive and characterized by a poorer prognosis. The genetic alterations characterizing eRMS commonly involve the loss of heterozygosis on chromosome region 11p15.5 (4), gain of chromosomes (5,6) and mutations on genes involved with growth factor signaling pathways (7-15). This leads to uncontrolled cell growth and the interruption of proper myogenic differentiation. Conversely, the aRMS subset is commonly characterized by the expression of Pax3-forkhead box O1 (FOXO1), a fused transcription factor derived from the chromosomal translocation t(2;13)(q35;q14), which juxtaposes the DNA-binding domain of Pax3 to the potent transactivation domain of FOXO1 (16). In the absence of the original Pax3 transactivation domain, the chimeric protein drives in a constitutive manner the transcription of numerous genes involved in muscle embryogenesis, such as c-MET and FGFR4, essentially maintaining the muscle precursors in a long-lasting proliferative state and thereby facilitating tumor initiation, aggressiveness and metastatic ability (16-19).

Melatonin is a small molecule derived from tryptophan metabolism and secreted by the pineal gland during periods of darkness (20,21). Melatonin is involved in the regulation of seasonal and circadian rhythms, effects that are mediated by melatonin binding to specific receptors, such as MT1 and MT2, which in turn trigger the downstream activation of CLOCK and BMAL1 factors driving a complex transcriptional program (22,23). Besides regulating the

Correspondence to: Dr Alessandro Fanzani, Department of Molecular and Translational Medicine, University of Brescia, Viale Europa 11, I-25123 Brescia, Italy
E-mail: alessandro.fanzani@unibs.it

Abbreviations: FGFR4, fibroblast growth factor receptor 4; FOXO1, forkhead box O1; Myf6, myogenic factor 6; Pax3 or -7, paired box 3 or -7

Key words: apoptosis, chemoresistance, melatonin, rhabdomyosarcoma
rhythm adaptations, melatonin exhibits additional functions, including the promotion of cell survival, neuroprotection and cardioprotection likely due to antioxidant properties (24-27). Notably, previous findings have shown a role for melatonin in preventing tumor initiation and progression (28-34). Specifically, melatonin was reported to inhibit cell proliferation and induce apoptosis in osteosarcoma (35), B-lymphoma (36) and colorectal cancer cells (37), as well as to decrease the weight of tumor masses in breast and prostate cancer (38-40). Based on those reports, the present study was conducted to examine the effects of melatonin on RMS cell lines in vitro. For this purpose, we employed human RMS cell lines as well as primary mouse tumor cultures established from transgenic mice (41) to evaluate the effects of melatonin on cell viability, proliferation and differentiation. In vitro assays and morphological analysis using electronic and confocal microscopy were performed.

Materials and methods

Reagents. Reagents were purchased from Sigma-Aldrich (Milan, Italy), unless otherwise stated. Cell culture materials were purchased from Jet-Biofil (Carlo Erba Reagents-Dasit Group, Cornaredo, Italy).

Human cell lines and primary mouse tumor cultures. Human RD (eRMS) and RH30 (aRMS) cells were purchased from the European Collection of Cell Cultures (ECACC; Salisbury, UK). The primary tumor mouse cultures, U57610 (eRMS) and U23674 (aRMS), were established from transgenic mice (41). In particular, eRMS mouse models were generated by crossing p53- or Ptch1-deficient conditional mice with Myf6-Cre mice to achieve the deletion of p53 with or without the presence of chemotherapeutic drugs such as doxorubicin (0.15 ng/ml) (D1515) and cisplatin (2 µg/ml) (P4394), which were previously diluted in H2O and DMSO, respectively.

Cell proliferation assay. eRMS and aRMS cells were seeded in 24-well plates at a density of 10x10^3 and 15x10^3, respectively. After 24, 48 and 72 h of melatonin treatment, the cells were harvested, fixed in paraformaldehyde (F8775) and stained for 10 min with crystal violet (C0075) solution [0.2% in phosphate-buffered saline (PBS) (D8537) with 20% methanol (32213)]. The samples were then collected in 600 µl of SDS (74255) solution (1% in PBS) and absorbance of the total homogenates, as measured by reading the plate at 540 nm emission wavelengths, was proportional to the amount of viable and proliferating cells that incorporated the crystal violet. In addition, the cell proliferation was expressed as the growth rate, which was calculated using Microsoft Excel 2010 software. The results were representative of at least three independent experiments.

Cell viability assay. A neutral red assay was employed to determine the percentage of viable cells that incorporated the neutral red dye in lysosomes, as initially described by Borenfreund and Puerner (42), a protocol subsequently modified by Repetto et al (43). Briefly, the cells were seeded in 96-well plates at a density of 1.5x10^4. After 24, 48 and 72 h of melatonin treatment, the cells were incubated for 2 h with neutral red dye (40 µg/ml) (N7005) dissolved in DMEM with 5% FBS. After washing the cells with PBS, 150 µl of neutral red destaining solution [50% ethanol (02860), 49% deionized water, and 1% glacial acetic acid (I00015N; BDH Laboratory Supplies, Dawsonville, GA, USA)] was added, followed by gentle agitation for 10 min, until complete dissolution was achieved. Absorbance was then measured by reading the plate at 540 nm emission wavelengths. The results were analyzed using Microsoft Excel 2010 software and presented as the percentage of control values. Images of cell viability assays showed representative results of at least three independent experiments.

Immunoblotting analysis. Protein homogenates were obtained by harvesting cells in a cold RIPA lysis buffer, comprising 20 mM Tris-HCl (pH 7.6) (T1503), 1% Nonidet P40 (N40), 0.5% sodium deoxycholate (D6750), 0.1% SDS (74255), 50 mM NaCl (S7653) and a cocktail of protease inhibitors (1836153; Roche, Milan, Italy) plus phosphatase inhibitors [1 mM Na3V04 (56508) and 4 mM NaF (S7920)]. The protein concentration was calculated by a Bradford reagent (B6916) assay and an equal amount of protein samples was separated by SDS-PAGE under reducing conditions and transferred to polyvinylidene fluoride membranes (P2938). Incubation with specific primary antibodies was followed by peroxidase-conjugated secondary antibodies (goat polyclonal anti-mouse IgG-HRP sc-2005; from Santa Cruz Biotechnology, Inc., Dallas, TX, USA; donkey polyclonal anti-rabbit IgG No. 31458; from Thermo Scientific, Erembodegem, Belgium) and the resulting immune complexes were visualized using the enhanced chemiluminescence reagent (STS-E 500; GeneSpin, Milan, Italy). Immune-reactive bands were quantified using densitometry analyses (Software Gel-Pro Analyzer, version 4).

Antibodies. The antibodies were purchased from Santa Cruz Biotechnology, Inc., unless otherwise stated. The primary antibodies used were: mouse monoclonal anti-myosin heavy...
chain, (sc-32732; 1:1,000 dilution); mouse monoclonal anti-caveolin-3 (610420; 1:1,000 dilution; BD, Buccinasco, Italy); rabbit polyclonal anti-caspase-3 (h-277) (sc-7148; 1:500 dilution); rabbit polyclonal anti-Bax (sc-526; 1:500 dilution); rabbit polyclonal anti-Bcl-2 (sc-492; 1:500 dilution); and mouse monoclonal anti-α-tubulin (T5168; 1:10,000 dilution; Sigma-Aldrich).

Scanning electron microscopy (SEM). RH30 cells were cultured and treated directly on coverslips in Petri dishes. After washing with 0.1 M phosphate buffer, adherent and suspended cells were fixed with 2.5% glutaraldehyde (G5882) in 0.1 M phosphate buffer for 1 h. The suspended cells adhered to polylysine-coated coverslips. The samples were post-fixed with 1% osmium tetroxide (OsO₄) (O021; Strumenti, Roma, Italy) in 0.1 M phosphate buffer for 1 h. After alcohol dehydration, the samples were critical point dried, gold sputtered and observed using a Philips 515 scanning electron microscope (FEI, Italy) (44).

Transmission electron microscopy (TEM). RH30-treated cells were washed and fixed with 2.5% glutaraldehyde (G5882) in 0.1 M phosphate buffer for 15 min. The cells were scraped and centrifuged at 300 x g for 10 min. The pellets were fixed in 2.5% glutaraldehyde for an additional 30 min. The suspended cells were collected in Eppendorf, centrifuged and fixed for 45 min in glutaraldehyde. The samples were post-fixed in 1% OsO₄ (O021) for 1 h, alcohol dehydrated and embedded in araldite (02860) (45). Thin sections were stained with uranyl acetate and lead citrate and analyzed using a Philips CM10 transmission electron microscope (FEI).

Confocal microscopy fluorescence. Adherent cells were cultured and treated directly on coverslips in Petri dishes. The suspended cells were collected in Eppendorf, fixed in 4% paraformaldehyde (F8775) for 30 min and then plated on polylysine-coated coverslips. The cells were then fixed in 4% paraformaldehyde in PBS (pH 7.4) for 30 min and washed twice using PBS. The cells were then pre-treated with RNase A (10 µg/ml) (12091-021) in PBS for 30 min and exposed to an equal mixture of propidium iodide (PI; 1 µg/ml) (P3566) and acridine orange (AO; 1 µg/ml) (A3568) (all from Life Technologies, Monza, Italy) in PBS at room temperature in the dark for 10 min.

AO and PI are intercalating fluorochromes that emit green and red fluorescence, respectively, when they are bound to DNA. Only AO diffuses through the plasma membrane of both viable and early apoptotic cells. Viable cells exhibit a green nucleus with intact structure, while apoptotic cells exhibit a bright-green nucleus, with condensation of chromatin. PI only enters late apoptotic and necrotic cells, resulting in double staining with AO and PI (45). The samples were observed with a Leica TCS-SP5 CLSM connected to a DMI 6000 CS inverted microscope (Leica Microsystems CMS GmbH; Figure 1. Effects of melatonin administration on the proliferation and viability of RMS cell lines. Crystal violet assay was employed to assess the proliferation of human cell lines (RD and RH30) and primary mouse tumor cultures (u57810 and u23674) in the absence or presence of increasing melatonin concentrations (0.01, 0.1, 1 and 2 mM) for up to 24, 48 and 72 h. Results are representative of four independent experiments. *P<0.05; **P<0.001; ***P<0.0001. (B) Cell viability of human RD and RH30 lines, treated with the indicated doses of melatonin for 24, 48 and 72 h, was assessed using the neutral red assay. Absorbance values reflect the amount of viable cells that have incorporated the neutral red dye. Results are representative of three independent experiments. *P<0.05; **P<0.001; ***P<0.0001. RMS, rhabdomyosarcoma.
AO and PI excitation were at 488 and 500 nm, respectively, and their emission signals were detected at 617 and 525 nm, respectively).

**Statistical analysis.** The differences between the groups were analyzed by the unpaired Student's t-test and one-way ANOVA test (with Dunnett's post-hoc test), using Prism 4 software for Windows (GraphPad Software, San Diego, CA, USA). P<0.05 was considered to indicate a significant result.

**Results**

Melatonin suppresses cell proliferation and triggers apoptotic and necrotic features in RMS cells. We evaluated whether melatonin administration would influence the cell growth of the human RMS cell lines (i.e. embryonal RD and alveolar RH30) and primary mouse tumor cultures (i.e., embryonal U57810 and alveolar U23674). For this purpose, the proliferation of cells that received melatonin once was determined over a time-course of 72 h by means of crystal violet assay. Treating different lines with increasing concentrations of melatonin, ranging from 0.01 to 2 mM, led to a significant impairment of cell proliferation starting from a dose of 1 mM in comparison to vehicle-treated cells, as indicated after calculation of the growth rate (Fig. 1A). To determine whether the melatonin effects were attributable to the inhibition of cell proliferation rather than impaired cell viability, we performed the neutral red assay using the cell lines under the same experimental conditions. As shown in Fig. 1B, 72 h of exposure with a concentration of 1 or 2 mM triggered the loss of ~50% of RMS cells in comparison to vehicle-treated cells, indicating that melatonin has cytotoxic effects. This latter result was confirmed by immunoblotting analysis of Bax and Bcl-2 expression, two proteins that can be either pro- or anti-apoptotic, respectively (46-48).

As shown in Fig. 2, treatment of RD and RH30 cells with 1 mM melatonin promoted an increase in the pro-apoptotic Bax expression, while the expression levels of anti-apoptotic Bcl-2 were downregulated in comparison to those in untreated cells. These results indicated that melatonin, not only behaves as a cytostatic factor on RMS cell growth, but also impairs the survival of different RMS lines by triggering an apoptotic program. Thus, at the ultrastructural level, the pro-apoptotic effects of melatonin, RH30 cells treated or untreated were analyzed by electronic and confocal microscopy.

Control cells showed an obvious healthy morphology characterized by the presence of intact subcellular structures, as observed by means of SEM (Fig. 3A) and TEM (Fig. 3B and C), respectively. In addition, AO/PI double staining showed a uniform green labeling suggestive of cellular healthy structures (Fig. 3D). After 1 mM melatonin for 24 h a heterogeneous situation developed: some cells maintained good cell viability similar to the control condition, while other cells showed a round apoptotic-like morphology (Fig. 3E and F). In particular, TEM analyses revealed some cells with an intense chromatin condensation, a typical apoptotic pattern (Fig. 3G). At the confocal microscopy level some cells appeared rounded and early apoptotic features were evident (Fig. 3H). After 48 h, melatonin-treated cells were almost all detached showing a round apoptotic morphology while only a small number of adherent cells exhibited an atrophic behavior, due to cytoplasm shrinkage (Fig. 3I). In addition, rounded cells suggestive of necrotic features also appeared (Fig. 3J). These cells were characterized by typical apoptotic features, including the presence of chromatin condensation, cytoplasm vacuolization and secondary necrosis as confirmed by TEM analysis (Fig. 3K). Consistent with this, an increased number of apoptotic cells showed orange areas due to PI permeability suggestive of cells in late apoptosis (Fig. 3L). After 72 h, the melatonin-treated cells observed at the SEM level exhibited apoptotic and necrotic features, being completely detached and showing a rounded morphology, with disruption of cell membranes in those that were necrotic (Fig. 3M and N). As shown by TEM, necrotic cells were characterized by cytoplasmic vacuolization due to membrane disruption and loss of cell components during the necrotic process (Fig. 3Q). The few adherent apoptotic cells exhibited large orange areas (Fig. 3P, inset) as observed during late apoptosis, whereas suspended cells showed bright-green nuclei predictive of apoptotic bodies (Fig. 3P).

RH30 cells treated with 2 mM melatonin were characterized
by round, blebbed cells after 24 and 48 h (Fig. 3Q and R, respectively). TEM analysis at 48 h of treatment highlighted dark areas predictive of DNA condensation and cytoplasmic vacuolization (Fig. 3S). Using AO and PI double-staining, melatonin-treated cells showed orange staining at the nuclei due to late apoptosis and necrosis already after 24 h (Fig. 3T), as also observed after 48 h (data not shown).

Melatonin impairs the myogenic differentiation in embryonal RD cells. Forced differentiation of tumor cells induced by
The use of anticancer agents has been widely exploited to limit the growth of tumor masses. In this regard, melatonin has been hypothesized to promote a differentiated phenotype in some tumors, such as gastric (49) and prostate cancer (50). Thus, to understand whether melatonin influences the myogenic differentiation of RMS, we employed human RD cells, which commonly exhibit a consistent myogenic potential in comparison to alveolar RH30 cells. For RD cells, the DM in the absence or presence of melatonin was replaced daily using different concentrations of melatonin. The extent of myogenic differentiation reached by cells in the different conditions was measured by immunoblotting analysis of markers that are normally increased during the differentiation of myoblasts, including caveolin-3 (Cav-3) and myosin heavy chain (MHC). Melatonin treatment led to a dose-dependent impairment of myogenic differentiation, since Cav-3 and MHC levels were reduced in comparison to the controls. In particular, melatonin at a concentration of 1 mM completely abolished the myogenic differentiation in RMS cells, as both Cav-3 and MHC levels were undetectable (Fig. 4A). We also administered melatonin to RD cells after which they were differentiated for 4 days and then analyzed for caspase-3 proteolytic activation, which is commonly utilized as a readout of the cell apoptotic program. As observed by immunoblotting analysis, melatonin-treated differentiated RD cells were characterized by increased levels of caspase-3 cleaved fragments (~19 and 17 kDa) in comparison to vehicle-treated cells after treatment for 24 and 48 h (Fig. 4B). These experiments demonstrated that melatonin has no positive effects on RMS differentiation, but behaves as a cytotoxic drug by triggering a caspase-dependent apoptosis.

**Melatonin sensitizes RD and RH30 cells to cell death induced by chemotherapeutic agents doxorubicin and cisplatin.** Previous findings have suggested that combination therapies including melatonin and conventional cancer drugs enhance success by increasing drug efficacy while reducing their side effects. In most clinical trials where melatonin was used in conjunction with chemotherapeutic drugs, improved overall
survival and patient conditions were observed (51,52). This suggests that melatonin enhances the efficacy of chemotherapy and reduces side effects (53,54). A combination of melatonin and doxorubicin was reported to enhance the growth inhibitory effect and induction of apoptosis in human hepatoma cells in comparison to melatonin or doxorubicin used alone (55). To verify the combined effects of melatonin and chemotherapy drugs on RMS chemoresistance, RD and RH30 cells were concurrently treated with 1 mM melatonin or 0.15 ng/ml doxorubicin or a combination of the two. Histograms show the absorbance values reflecting the amount of viable cells that have incorporated the crystal violet. (A) Cells were either treated with 1 mM melatonin or 0.15 ng/ml doxorubicin or a combination of the two. (B) Cells were treated with 1 mM melatonin or 2 µg/ml cisplatin or a combination of the two. The histograms show absorbance values reflecting the amount of viable cells that incorporated the crystal violet. Results are representative of four independent experiments. *P<0.05; **P<0.001; ***P<0.0001 vs. untreated cells; #P<0.05; ##P<0.001 vs. cells treated with melatonin or doxorubicin or cisplatin alone. RMS, rhabdomyosarcoma.

Discussion

Results obtained in the present study indicate that melatonin, when used at concentrations varying from 0.01 to 2 mM, profoundly affect the cell survival of rhabdomyosarcoma (RMS), the most frequent myogenic sarcoma affecting children and adolescents (4). In human cell lines representative of the most frequent RMS categories, i.e., the eRMS and aRMS subtypes, we observed that melatonin limited cell proliferation and triggered morphological and subcellular changes typically recognizable in apoptotic cells, such as DNA fragmentation, disruption of cell membranes and proteolytic cleavage of caspases. We also observed similar effects in primary mouse tumor RMS cultures which, having been derived from mice with specific genetic backgrounds, faithfully recapitulate the onset of RMS genesis (41). In the cell cultures, the ability of melatonin to increase apoptosis was not exclusively correlated with cell cycle-dependent effects, since we observed melatonin to be effective in triggering cell death even in RMS cells that had withdrawn from the cell cycle to attempt differentiation. These observations suggest a potential efficacy of melatonin towards undifferentiated and more differentiated tumor histotypes. Studies on cancer cells have shown that antiproliferative and pro-apoptotic effects of melatonin were achieved with high doses, as compared with those detected in the blood at night. However, it is known that the intracellular levels of melatonin may be much higher than in blood (56). To explain the reason for melatonin often requiring to be added at pharmacological concentrations to produce inhibitory effects, a regulatory
mechanisms by which its accumulation in cell membranes acts as a reservoir, limiting the net amount of the biological active indoleamine has been suggested (57).

Previous findings have indicated that melatonin produces no consistent adverse effects over various concentrations (58), suggesting that it may be useful to improve the efficacy of conventional cytotoxic agents. In this regard, we showed that melatonin synergized with chemotherapeutic drugs in human RMS cell lines. To the best of our knowledge, this is the first study showing indolamine to be effective in the enhancement of cell death on myogenic tumor cells using a complementary approach with doxorubicin or cisplatin drugs, as already observed in clinical trials on different types of cancer (51-53,59).

The mechanisms underlying the effect of melatonin on apoptosis have not been clarified and appear to be, to some extent, context-specific (60-62). Some melatonin actions are mediated by specific membrane receptors, known as MT1 and MT2, that are known to be expressed in RMS tumor samples. Melatonin acts through specific membrane receptors, known as MT1 and MT2, that are known to be expressed in RMS tumor samples and to mediate effects on cell proliferation. Thus, RMS cells likely have a non-receptor-mediated action, since melatonin has been shown to have a non-receptor-mediated action, since melatonin has been shown to have a non-receptor-mediated action, since melatonin has been shown to permeate into cells by means of receptor-independent processes. Notably, differentiated RD cells seemingly exhibited a marked responsiveness to melatonin, since already at 0.01 and 0.1 mM concentrations we observed a negative effect on myogenic differentiation. In this context, whether the expression levels of melatonin receptors may differ between proliferating and differentiated cells, thus accounting for the different observed sensitivities should be investigated. Whereas in normal cells melatonin and its metabolites act as efficient radical scavengers (63), it has been suggested that changes in the oxidative status account for the ability of melatonin to induce apoptosis in cancer cells (62,64,65). In this regard, a correlation between the increase in ROS production and the induction of melatonin-driven apoptosis has been reported in several cell lines (66). Consistent with this evidence, we have preliminarily observed a reduction in the melatonin cytotoxic effect by pretreating RMS cells with vitamin E (data not shown), a lipid-soluble antioxidant molecule. Although these observations are under investigation, it remains to be established whether the potential changes in the redox status is the cause rather than the consequence of the increased cell death.

In conclusion, the molecular mechanisms underlying the cytotoxicity on RMS cells, as observed for other types of cancer, deserve attention for establishing whether a rationale occurs for the introduction of melatonin as an adjuvant in the multimodality approach currently used against RMS.

Acknowledgements

We are grateful to Charles Keller (Oregon Health and Science University, USA) for providing the primary mouse tumor cultures of RMS.

References


