Inhibition of ERK1/2 Signaling Pathway is Involved in Melatonin’s Antiproliferative Effect on Human MG-63 Osteosarcoma Cells

Lifeng Liu, Ying Xu, Russel J. Reiter, Yutao Pan, Di Chen, Yangzhou Liu, Xingyu Pu, Liguo Jiang, Zengchun Li

Abstract

**Background:** In a previous study, we found that melatonin inhibits MG-63 osteosarcoma cell proliferation; however, the underlying mechanisms remain elusive. Mitogen-activated protein kinase (MAPK) and Akt signaling pathways play key roles in the anticancer effects of melatonin. **Aims:** The present study investigated whether MAPK and Akt signaling pathways are involved in melatonin’s antiproliferative actions on the human MG-63 osteosarcoma cells. **Methods/Results:** Western blot analysis confirmed that melatonin significantly inhibited phosphorylation of ERK1/2 but not p38, JNK, or Akt. The expression of ERK1/2, p38, JNK, and Akt was not altered by melatonin. PD98059 and melatonin alone, and especially in combination, significantly inhibited cell proliferation. The changes included G1 and G2/M phase arrest of the cell cycle, and a downregulation of the expression at both the protein and mRNA levels of cyclin D1 and CDK4 (related to the G1 phase) and of cyclin B1 and CDK1 (related to the G2/M phase) as measured by flow cytometry after propidium iodide staining, and both western blot and real-time PCR, respectively. Furthermore, the combination of PD98059 and melatonin synergistically and markedly augmented the action of either agent alone. Co-immunoprecipitation further confirmed that there was an interaction between p-ERK1/2 and cyclin D1, CDK4, cyclin B1, or CDK1, which was blunted in the presence of melatonin or PD98059. **Conclusion:** These findings suggest that melatonin’s antiproliferative action is mediated by inhibition of the ERK1/2 signaling pathway rather than the p38, JNK, or Akt pathways.

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Zengchun Li
Department of Trauma Orthopaedics,
Shanghai East Hospital, Tongji University School of Medicine, Shanghai (China)
Tel./Fax: +86-21-5879-8999, E-Mail: drlzcz@163.com
Introduction

Since the introduction of chemotherapy, survival in osteosarcoma patients has improved considerably. Still, there is no worldwide consensus on the efficacy of treatment or a reduction in the severity or fewer side effects of the chemotherapy approach [1]. Importantly, a recent meta-analysis indicated that melatonin as an adjuvant therapy for cancer may lead to substantial improvements in tumor remission, 1-year survival, and alleviation of radiochemotherapy-related side effects [2]. Our previous study documents that melatonin inhibits cell proliferation of the human MG-63 osteosarcoma cell line [3]. This suppressive action involves the downregulation of cyclin D1 and CDK4 (related to the G1 phase) and of cyclin B1 and CDK1 (related to the G2/M phase). The current study is designed to help further clarify the mechanisms by which melatonin inhibits the activity of MG-63 osteosarcoma cells.

Mitogen-activated protein kinase (MAPK) and Akt signaling pathways are responsible for melatonin's antiproliferative actions in some cells, including human umbilical vein endothelial cells [4], human fibroblast-like synoviocyte [5], hepatocarcinoma HepG2 cells [6], human melanoma SK-MEL-1 cells [7], rat glioma cells [8, 9], human breast adenocarcinoma MCF-7 cells [10, 11], and human osteoblasts [12]. The MAPK family mainly consists of extracellular signal-regulated kinase (ERK1/2), p38, and c-Jun N-terminal kinase (JNK). The ERK1/2 cascade, activated by mitogenic stimuli, is critical for cell proliferation and survival [13, 14] and is required for normal progression into mitosis [15, 16]. The p38 and JNK pathways are activated in response to chemicals and environmental stress [17-19]. Akt (also known as Akt1), a mediator of growth factor-induced cell survival [20-22], may promote cell proliferation via phosphorylation [23].

Herein, we demonstrate that the inhibition of ERK1/2 signaling pathway involves melatonin-induced G1 and G2/M phase arrest in the human MG-63 osteosarcoma cell line by downregulating the expression of cyclin D1, CDK4, cyclin B1 and CDK1; this inhibitory action is potentially mediated via the ERK1/2 but not the p38, JNK, or Akt pathways. Information garnered from this study provides insight into the mechanisms of melatonin-mediated cell cycle arrest and has implications for exploring treatment of osteosarcoma with melatonin.

Materials and Methods

Cell culture and reagents

The human osteosarcoma cells (MG-63 cells) were maintained in a Dulbecco's Modified Eagle's Medium (Gibco, Life Technologies, Carlsbad, USA), supplemented with 10% fetal bovine serum (FBS) (HyClone, Thermo, Fremont, USA), in a humidified 5% CO2 atmosphere at 37 °C; the medium was changed every other day. The MG-63 cells were plated at 10^4 cells/cm^2 for 24 h before treatment. Melatonin solution was prepared as follows: a melatonin stock solution in 100% dimethyl sulfoxide (DMSO) was serially diluted with culture media in accordance with the tested doses; the culture media contained 0.2% DMSO and 10% serum in every concentration of melatonin or vehicle group.

Melatonin, PD98059, the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT), and propidium iodide (PI) were obtained from Sigma (St. Louis, MO, USA). Primary monoclonal antibodies for ERK1/2, phospho-ERK1/2, p38, phospho-p38, JNK, phospho-JNK, Akt, phospho-Akt, IgG, and β-actin were purchased from Cell Signaling Technology (Danvers, MA, USA) and cyclin B1, cyclin D1, CDK1, and CDK4 were from Lab Vision (Thermo, Fremont, CA, USA).

Western blotting

After treatment with 4 mM melatonin for 4 h, 24 h, or 48 h or 50 µM PD98059 for 24 h, the cells were extracted with lysis buffer (150 mM NaCl, 1% NP-40, 0.1% SDS, 2 µg/mL aprotinin, 1 mM PMSF) for 30 min at 4 °C. Extracts were centrifuged at 12,000 g for 15 min at 4 °C. Supernatants containing total protein were harvested. Aliquots, each containing 50 µg of protein, were separated by a 10% SDS–PAGE and
transferred to PVDF membranes at 60 V (Akt, phospho-Akt, and cyclin B1) or 40 V (the others) for 2 h at low temperature. The membranes were blocked in 5% bovine serum albumin (phospho-) or skimmed milk (the others) for 2 h. Subsequently, proteins were detected using monoclonal antibodies at 1:200 (cyclin B1, cyclin D1, CDK1, and CDK4), 1:500 (phospho-JNK), 1:1900 (phospho-ERK1/2), or 1:1000 (the others) dilution overnight at 4 °C; they were then visualized using anti-mouse or anti-rabbit IgG conjugated with horse radish peroxidase (HRP) at 1:6000 (cyclin B1, cyclin D1, CDK1, and CDK4) or 1:8000 (the others) dilution for 2 h at room temperature, respectively. An EC3 Imaging System (UVP LLC, Upland, CA, USA) was used to identify the specific bands, and the optical density of each band was measured using Gel-Pro Analyzer 4.0 software (Media Cybernetics, Inc., Bethesda, MD, USA). The difference between the proteins of interest and β-actin of the same sample was calculated as relative content and presented graphically.

**Cell proliferation assay**

Cell proliferative activities were determined using MTT method. Briefly, MG-63 cells were seeded onto 96-well plates (4,000 cells/well) for 24 h and the medium was then replaced with 10% serum medium containing 0.2% DMSO, 50 μM PD98059, 4 mM melatonin, or both 50 μM PD98059 and 4 mM melatonin for 24 h, 48 h or 72 h. After treatment, culture media were changed for serum-free culture media. MTT dissolved in phosphate buffer saline (PBS) was added to each well and incubated for 4 h at 37 °C. Then, the serum-free culture media containing MTT were discarded and DMSO was added to each to dissolve the precipitate. The optical densities were measured at 490 nm spectral wavelength using a microplate reader (Spectra Thermo, Männedorf, Switzerland). Viability results were expressed as percentages.

**Cell cycle analysis**

After treatment with 0.2% DMSO, 50 μM PD98059, 4 mM melatonin, or both 50 μM PD98059 and 4 mM melatonin for 24 h, cells were harvested and washed twice with ice-cold PBS, and fixed in 75% ethanol for overnight at 4 °C. The fixed cells were again rinsed with PBS and resuspended in 0.5 mL PI staining solution containing 50 μg/mL PI, 0.25% Triton X-100, and 0.2 mg/mL DNase-free RNase for 30 min at room temperature in the dark. 10,000 events per sample were acquired with a FACS SCAN flow cytometer (Becton-Dickinson, San Jose, CA, USA) and the percentage of cells in G0/G1, S and G2/M phases of the cell cycle was determined using Modfit LT 3.0 (Becton-Dickinson).

**Real-time PCR**

Total RNA was isolated from cells using TRIzol reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions. Total RNA was reverse transcribed using a reverse-transcription kit (TaKaRa, Dalian, China) according to the manufacturer’s protocols. Real-time PCR was performed on ABI Prism 7900HT Fast System (Applied Biosystems, Life technologies, Foster, CA, USA) using SYBR Premix Ex Taq II (TaKaRa). Amplifications were carried out in a total volume of 20 μL and cycled 40 times after the initial denaturation (95 °C for 30 s) with the following parameters: 95 °C for 5 s and 60 °C for 30 s. Primer sequences were reported previously [23] and β-actin was used as an internal control. The reliability of results was supported by analyzing the dissociation curve. Real-time PCR data were calculated using 2-ΔΔCT Method by the SDS 2.4 software package (Applied Biosystems).

**Co-immunoprecipitation**

After treatment with melatonin or PD98059 for 24 h, cells were extracted with lysis buffer (10 mM KCl, 1.5 mM MgCl2, 10 mM HEPES, 1 mM PMSF, 1 mM DTT) and homogenized for 30 min at 4 °C. The extracts were centrifuged at 12,000 g for 15 min at 4 °C and then the supernatants containing total protein were harvested. Equal amounts of protein were, respectively, exposed to equal amounts of monoclonal antibodies against p-ERK1/2, IgG, cyclin D1, CDK4, cyclin B1, or CDK1, which were immobilized on protein A/G beads (Beyotime, Haimen, China). Following 3-h incubation at 4 °C with gentle rotation, beads were washed extensively five times with lysis buffer, boiled, and microcentrifuged. Proteins were detected with monoclonal antibodies against p-ERK1/2, cyclin D1, CDK4, cyclin B1, or CDK1 by western blot. To ensure the data validity and repeatability, the MG-63 cells used in the experiments were from the same treated batch.
Statistical analysis

Data were analyzed using SPSS 16.0 software. An independent-samples t test or a one-factor analysis of variance was used to evaluate the differences between groups with various treatments, and least significant difference (LSD) test was used for post hoc subgroup analysis. All data were presented as mean ± S.E.M. of at least three independent experiments. Results were considered statistically significant when the p-value was less than 0.05. N-fold values in gene expression below 0.5 and up to 2 were taken as significant in accordance with values obtained from control genes [24].

Results

Melatonin significantly inhibited phosphorylation of ERK1/2 but not p38, JNK, or Akt

Previously, we confirmed that 4 mM melatonin has an antiproliferative effect by inducing G1 and G2/M phase arrest in human MG-63 osteosarcoma cells [3]. MAPK and Akt signaling pathways play a critical role in melatonin-induced antiproliferative events in other cells [4-12]. To determine whether MAPK and Akt signaling pathways are involved in the antiproliferative action of melatonin in MG-63 cells, the expression of MAPK components and Akt was evaluated using the western blot method. We found that melatonin significantly inhibited phosphorylation of ERK1/2 (p-ERK1/2) but not p38 (p-p38), JNK (p-JNK), or Akt (p-Akt) in a time-dependent manner, compared with control cells at various time points examined. The expression of ERK1/2, p38, JNK, and Akt was not altered (Fig. 1).

Inhibition of ERK1/2 signaling pathway is involved in melatonin-induced antiproliferative effect

To further test whether the inhibition of ERK1/2 signaling pathway is involved in melatonin-induced the antiproliferative action in this osteosarcoma cell line, the cells were treated with PD98059, a selective inhibitor of MEK that disrupts downstream activation of ERK1/2. Because of previously published data [25, 26] and the results of our preliminary experiment, PD98059 at 50 µM concentrations was used (inhibiting only the ERK pathway, data not shown). As shown in Fig. 2, treatment with PD98059 (50 µM) or melatonin for 24 h alone, and especially in combination, effectively blocked the ERK1/2 activation in MG-63 cells, compared with control cells. Also, the proliferation assay using MTT method showed...
that PD98059 or melatonin alone, and especially in combination, significantly inhibited osteosarcoma cell proliferation at various time points examined, compared with control cells (Fig. 3). The results are consistent with the fact that the inhibition of ERK1/2 signaling pathway is associated with melatonin-induced antiproliferative effect on osteosarcoma cells.

**Melatonin-induced G\textsubscript{1} and G\textsubscript{2}/M phase arrest is related to the inhibition of ERK1/2 signaling pathway**

To determine whether the inhibition of ERK1/2 signaling pathway alters cell cycle distribution, flow cytometry was performed after PI staining. We found that PD98059 and melatonin alone, and especially in combination, significantly increased the fraction of cells in G\textsubscript{0}/G\textsubscript{1} phase of the cell cycle while simultaneously reducing the proportion in the G\textsubscript{2}/M phase rather than the S phase, compared with control cells (Fig. 4). The combination synergistically and markedly reinforced the alterations in the cell cycle distribution (CompuSyn software analysis: CI values < 1). This suggests that melatonin-induced G\textsubscript{1} and G\textsubscript{2}/M phase arrest is related to the inhibition of ERK1/2 signaling pathway.

**Inhibition of ERK1/2 signaling pathway is implicated in melatonin-induced downregulation of expression levels of cyclin D1, CDK4, cyclin B1 and CDK1**

To examine whether the inhibition of ERK1/2 signaling pathway contributes to downregulation of the expression levels of cyclin D1 and CDK4 (related to the G\textsubscript{1} phase) and of cyclin B1 and CDK1 (related to the G\textsubscript{2}/M phase), western blot and real-time
PCR assays were performed. We found that PD98059 and melatonin alone, and especially in combination, significantly reduced their expression at both protein and mRNA levels, compared with control cells (Fig. 5). This shows that the inhibition of ERK1/2 signaling pathway is implicated in melatonin-induced downregulation of expression levels of cyclin D1, CDK4, cyclin B1, and CDK1.

**There is an interaction between p-ERK1/2 and cyclin D1, CDK4, cyclin B1, or CDK1**

Finally, to identify whether there is an interaction between p-ERK1/2 and cyclin D1, CDK4, cyclin B1, or CDK1, co-immunoprecipitation was performed. The MG-63 cells in the absence or presence of melatonin for 24 h were subjected to immunoprecipitation with antibodies against p-ERK1/2 or IgG, followed by western blotting for cyclin D1, CDK4, cyclin
B1, or CDK1. An evident interaction between p-ERK1/2 and cyclin D1, CDK4, cyclin B1, or CDK1 was observed. In contrast, this interaction was weakened when the cells were treated with melatonin for 24 h (Fig. 6A). Reciprocal immunoprecipitation with antibodies against cyclin D1, CDK4, cyclin B1, CDK1, or IgG was assessed by western blotting for p-ERK1/2 (B). The cells in the absence or presence of PD98059 (50 µM) for 24 h were subjected to immunoprecipitation with antibodies against p-ERK1/2 or IgG antibodies followed by western blotting for cyclin D1, CDK4, cyclin B1, and CDK1 (C). The graph labeled “A”, “B” or “C” respectively corresponds to the blot displayed as "A", "B" or "C". The results were normalized in accordance with the data of treated groups as a percentage of each corresponding control group. Each bar represents the mean ± S.E.M. of three independent experiments. * P < 0.05 or ** P < 0.01, compared with control cells.
Discussion

Melatonin plays key roles in the biologic regulation of circadian rhythms [27, 28], sleep [29, 30], antioxidant protection [31-35], cell aging [36], tumor growth [12, 37], reproduction [31, 38], and bone physiology [39, 40]. A growing amount of data indicates that the age-related decline in the level of melatonin is closely associated with bone diseases: osteosarcoma, adolescent idiopathic scoliosis and osteoporosis [1, 39]. Available information regarding the influence of melatonin, especially at mM concentrations, on abnormal osteoblast, such as osteosarcoma cells, is limited and poorly defined. Previously, we confirmed that melatonin at 4 mM concentrations has an inhibitory effect on osteosarcoma cell proliferation by inducing G_1 and G_2/M phase arrest [3]. In the current study, we further document that melatonin induces G_1 and G_2/M phase arrest by downregulating the expression of cyclin D1, CDK4, cyclin B1 and CDK1; this is potentially mediated via the ERK1/2, but no p38, JNK, or Akt, signaling pathway.

Several studies using other cell types have indicated that MAPK and Akt signaling pathways are associated with melatonin-mediated antiproliferative actions [4-12]. We examined whether 4 mM melatonin regulates the expression of MAPK components and Akt in a human osteosarcoma cell line MG-63. Our results suggest that melatonin significantly inhibited the phosphorylation of ERK1/2, which is consistent with previous reports [4, 5]. However, melatonin had no significant influence on the phosphorylation of p38, JNK, or Akt, which contrasts with the findings of some studies in other cell types [4, 6-11]; the results indicate that these pathways do not play a central role in melatonin-induced cell cycle arrest in osteosarcoma cells. Moreover, the differences in cell types, melatonin concentrations used and time points at which the endpoints were examined could contribute to the lack of an apparent change in the phosphorylation of p38, JNK, or Akt. These findings indirectly suggest that ERK1/2 signaling pathway plays a key role in melatonin-regulated abnormal or normal osteoblast proliferation, such as the human osteosarcoma cell line MG-63 or osteoblastic cell line hFOB 1.19.

ERK1/2 activation has been well documented as necessary for cell proliferation, which is partly involved in G_1 and G_2/M phase progression [41-43]. As melatonin was capable of decreasing the expression of p-ERK1/2, we tested whether PD98059, a selective inhibitor of MEK that disrupts downstream activation of ERK1/2, would synergistically augment the antiproliferative effect of melatonin in MG-63 osteosarcoma cells. Our results demonstrated that PD98059 and melatonin alone, and especially in combination, significantly induced an antiproliferative effect, the G_1 and G_2/M phase arrest, and the downregulation of the expression at both protein and mRNA levels of cyclin D1 and CDK4 (related to the G_1 phase) and of cyclin B1 and CDK1 (related to the G_2/M phase) as measured by the MTT method, flow cytometry after PI staining, and both western blot and real-time PCR, respectively. Moreover, the combination synergistically and markedly enhanced the actions of PD98059 or melatonin alone. These findings strongly suggest that the prevention of ERK1/2 activation plays a central role in the observed melatonin-mediated actions.

Since a decline in both protein and mRNA levels of the cyclins and cyclin-dependent kinases was closely accompanied by a reduction in the expression of p-ERK1/2 after treatment with melatonin, we determined whether there is an interaction between p-ERK1/2 and cyclin D1, CDK4, cyclin B1, or CDK1. Co-immunoprecipitation and reciprocal immunoprecipitation results strongly suggested an interaction between p-ERK1/2 and cyclin D1, CDK4, cyclin B1, or CDK1. This interaction was weakened by inhibiting the ERK1/2 signaling pathway with melatonin or PD98059. Melatonin effectively reduced phosphorylation of ERK1/2 rather than downregulating total ERK1/2 levels. The MG-63 cells used in the experiments were from the same treated batch and the results of figure 6 were trustworthy given that the data of figure 1 and 2 account for the equalization of total protein and total ERK1/2 levels among groups. Hence, the loss of protein in the immunoprecipitation of figure 6 is due to the loss of affinity but not total protein. These results demonstrate that the effects of melatonin on cell proliferation, cell cycle distribution, and the expression at both protein and mRNA levels of
cyclin D1, CDK4, cyclin B1, and CDK1 can occur via the ERK1/2 pathway in human MG-63 osteosarcoma cells. In addition, a meta-analysis indicated that melatonin causes substantial improvements in tumor remission and alleviation of radiochemotherapy-related side effects [2]. Accordingly, our findings may well be helpful to exploring what it means in the context of inflammatory bone pain or bone metastases from all types of cancers: breast, ovarian, prostate, etc. Indeed, additional research should be encouraged.

Both the human MG-63 osteosarcoma cells and the normal mature osteoblast hFOB 1.19 cells are osteoblast-like cells, but they exhibit specific differential behaviors when they are exposed to identical concentrations of melatonin. When they were exposed to melatonin at 10 nM – 100 µM concentrations, the hFOB cells exhibited increased cell proliferation while the MG-63 cells exhibit no change. When both cell types were exposed to melatonin at 1 mM concentrations, by contrast, the proliferation of hFOB cells was markedly inhibited while the MG-63 cells exhibited no response; this only occurred in the latter cells when the concentration of melatonin reached 4 mM. Interestingly, the current data showed that melatonin-induced pro- or anti-proliferative actions in the hFOB [12] or MG-63 cells were mainly related to the ERK1/2 signaling pathway. Possible explanations for the apparent differences in this response may be a negative feedback loop or other regulated mechanisms having a role in the delayed response of osteoblast-like cells to melatonin. These findings suggest that there are obvious differences between the normal and cancer cells in the responsiveness to melatonin although they all are osteoblast-like cells. Additional research is necessary to mechanistically clarify these differential responses. These findings suggest that there are obvious differences between the normal and cancer cells in the responsiveness to melatonin although they all are osteoblast-like cells. Additional research is necessary to mechanistically clarify these differential responses. This study suggests that the inhibition of ERK1/2, but not p38, JNK, or Akt, signaling pathway is involved in melatonin-induced G1 and G2/M phase arrest of the human MG-63 osteosarcoma cell line. This information may help clarify the regulatory mechanisms of melatonin supplementation in preventing the progression of some cancers and may provide useful information regarding the treatment of osteosarcoma with melatonin.

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**Disclosure Statement**

The authors declare that they have no conflict of interest

**References**


Liu et al.: ERK1/2 Involves Melatonin-inhibited Osteosarcoma Cell Proliferation


