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Melatonin Induces Apoptotic Cell Death via p53 in LNCaP Cells

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In this study, we examined whether melatonin promotes apoptotic cell death via p53 in prostate LNCaP cells. Melatonin treatment significantly curtailed the growth of LNCaP cells in a dose- and time-dependent manner. Melatonin treatment (0 to 3 mM) induced the fragmentation of poly(ADP-ribose) polymerase (PARP) and activation of caspase-3, caspase-8, and caspase-9. Moreover, melatonin markedly activated Bax expression and decreased Bcl-2 expression in dose increments. To investigate p53 and p21 expression, LNCaP cells were treated with 0 to 3 mM melatonin. Melatonin increased the expressions of p53, p21, and p27. Treatment with mitogen-activated protein kinase (MAPK) inhibitors, PD98059 (ERK inhibitor), SP600125 (JNK inhibitor) and SB202190 (p38 inhibitor), confirmed that the melatonin-induced apoptosis was p21-dependent, but ERK-independent. With the co-treatment of PD98059 and melatonin, the expression of p-p53, p21, and MDM2 did not decrease. These effects were opposite to the expression of p-p53, p21, and MDM2 observed with SP600125 and SB202190 treatments. Together, these results suggest that p53-dependent induction of JNK/p38 MAPK directly participates in apoptosis induced by melatonin.

Key Words: Melatonin, p53, p38, JNK, LNCaP cells

INTRODUCTION

Apoptotic cell death in androgen-sensitive LNCaP prostate cancer cells is mediated by p53. The combination of metformin and 2-deoxyglucose induces p53-dependent apoptosis in LNCaP cells [1]. Emodin, 1,2,8-trihydroxy-6-methylanthraquinone, is an active component contained in the root and rhizome of *Rheum palmatum L.* (Polygonaceae). Wogonin is one of the primary active compounds of *Scutellaria baicalensis*, and decreases prostate cancer cells in a p53-dependent mechanism [2,3]. The MDM2 antagonist Nutlin-3 specifically inhibits proliferation of LNCaP cells through cell cycle arrest and apoptosis by increasing levels of p53-responsive p21 and MDM2 expressions, demonstrating that MDM2 antagonists retain functional p53 and androgen receptor signaling in human prostate cancers [4].

Melatonin, a circadian indoleamine hormone secreted by the human pineal gland, is able to directly induced cell death of several types of human tumor cells [1-9]. Many recent reports have shown that melatonin inhibits the growth of androgen-sensitive human LNCaP prostate cancer cells [9-12]. In our previous report, we demonstrated that melatonin is able to induce apoptotic cell death in LNCaP cells via the p38 and c-JUN N-terminal kinase (JNK) pathways [13]. Here, we determined whether melatonin induced apoptosis via signaling mediated by p53 in LNCaP cells.

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METHODS

Cell culture

LNCaP cells, an androgen-sensitive human prostate adenocarcinoma cell line, were cultured in Dulbecco's modified Eagle's medium (DMEM, GibcoBRL, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, GibcoBRL) at 37°C with 5% CO_2 in a humidified incubator.

Treatment

Cells in exponential growth were plated at a density of 1×10^6 cells/100 mm dish (Corning, Corning, NY, USA) and cultured in 5% FBS DMEM medium. Melatonin (Sigma, St Louis, MO, USA) was dissolved in dimethyl sulphoxide (DMSO), and cells were treated with varying doses of melatonin (0 to 3 mM) for 0 to 48 hr. In experiments to determine the effects of MAPK inhibitors (PD98059, SP600125 and SB202190 all from Calbiochem, San Diego, CA, USA) on cell growth inhibition, cytotoxicity and apoptosis, cells were treated with the kinase inhibitors for 1 hr and then co-exposed to melatonin for varying time points.

ABBREVIATIONS: PARP, poly(ADP-ribose) polymerase; MARK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-JUN N-terminal kinase.

Cell viability assay

Cell survival was determined using the Cell Counting Kit-8 (CCK-8) (Dojindo, Tokyo, Japan). Briefly, LNCaP cells were cultured in a 96-well plate (Corning Inc.) at a density of 5×10^3 cells per well, with or without dissolved melatonin. After 48 hr, the cells were washed and treated with CCK-8 solution, and the plate was incubated in the dark for an additional 4 hr. The absorbance at 450 nm was read using a microtiter plate reader (Molecular Device, Sunnyvale, CA). Percent viability was calculated as follows: (absorbance of melatonin-treated cells/absorbance of control cells) ×100.

Western blot analysis

Cells were harvested, washed two times with ice-cold PBS, and then suspended in 20 mM Tris-HCl buffer (pH 7.4) containing protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml pepstatin A, and 1 μg/ml chymostatin) and phosphatase inhibitors (5 mM Na_3VO_4, 5 mM NaF). Whole cell lysate was prepared using 20 strokes of a Dounce homogenizer, followed by centrifugation at 13,000× *g* for 20 min at 4°C. Protein concentration was determined using the BCA assay (Sigma). Proteins (50 μg) were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was incubated with antibodies directed against the following proteins, as indicated: PARP (1 : 1,000 dilution) (Cell Signaling Technology, Beverly, MA, USA); caspase-3, -8, and -9 (1 : 1,000) (Cell Signaling Technology); Bax (1 : 500) and Bcl-2 (1 : 500) (Santa Cruz Biotechnology, Santa Cruz, CA, USA); p53 (1 : 1,000), p21 (1 : 1,000), and p27 (1 : 1,000) (Cell Signaling Technology); phospho-p53 (serine 15) (1 : 500) and MDM2 (1 : 500) (Santa Cruz Biotechnology); and GAPDH (1 : 1,000) (Assay Designs, MI, USA). Immunoreactive proteins were visualized by exposure to X-ray film. The protein bands were shown by image-scanning.

Statistical analysis

Significant differences were determined by ANOVA, followed by Tukey's test for multiple comparisons. Analysis was performed with Prism Graph Pad v4.0 (Graph Pad Software Inc., San Diego, CA, USA). Values are expressed as means±SD. A p value of <0.05 was considered statistically significant.

RESULTS

Melatonin significantly decreases cell survival dependent on dose and time in LNCaP prostate cancer cells

To evaluate any anti-proliferative effects of melatonin on human LNCaP prostate cancer cells, the number of viable cells in culture was examined after a 48 hr melatonin treatment (0 to 3 mM) (Fig. 1A). The time dependence on cell viability was examined for 48 hr using 3 mM melatonin (Fig. 1B). Cell viability (%) was significantly decreased in both dose- and time-dependent manners (Fig. 1). Doses of melatonin greater than 1 mM decreased cell growth by more than 50% after 48 hr in culture; the lowest cell viability was observed at 48 hr (≅16.2%). The marked decrease of cell viability by 3 mM melatonin beyond 48 hr of treatment most likely reflected the induction of cell death by melatonin.

Melatonin induces apoptotic cell death in LNCaP cells

Clearer evidence for apoptosis was identified by western blot analysis using antibodies against PARP, caspase-3, -8, -9, Bax, and Bcl-2. In the presence of melatonin (0 to 3 mM), the fragmentation of poly(ADP-ribose) polymerase (PARP) and activations of caspase-3, caspase-8, and caspase-9 proteins in LNCaP cells occurred in a dose-dependent manner (Fig. 2A). Moreover, melatonin markedly activated Bax expression and decreased Bcl-2 expression according to dose increment (Fig. 2B, C). These results indicate apoptotic cell death is induced by melatonin.

Melatonin induces apoptotic cell death via p53 activation in LNCaP cells

To identify mechanisms that might lead to melatonin-mediated apoptosis, the expression of p53, p21, and p27 proteins was examined by western blot analysis (Fig. 3). p53, p21, and p27 proteins were significantly activated by melatonin according to the dose increase (Fig. 3B, C, D). To further investigate the downstream signals of p53 such as p-p53, p21, and MDM2 by melatonin treatment, LNCaP cells were treated with the mitogen-activated protein kinase (MAPK) inhibitors PD98059 (ERK inhibitor), SP600125

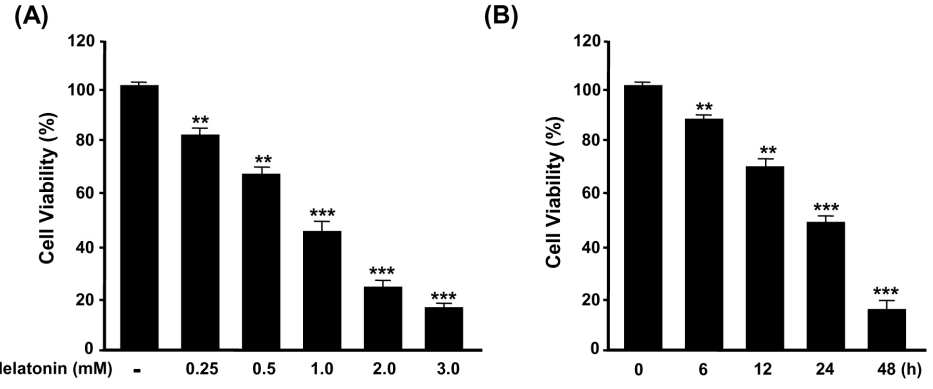

Fig. 1. Viability of melatonin-treated LNCaP cells. LNCaP cell viability was determined using the Cell Counting Kit-8 assay (A) 48 hr after exposure to melatonin at varying doses and (B) at varying times after exposure to 3 mM melatonin. In (A) and (B), results for cells not treated with melatonin are shown for comparison. Results are the means of 3 independent experiments (bars represent SD). **$p<0.01$, ***$p<0.001$ vs. control.

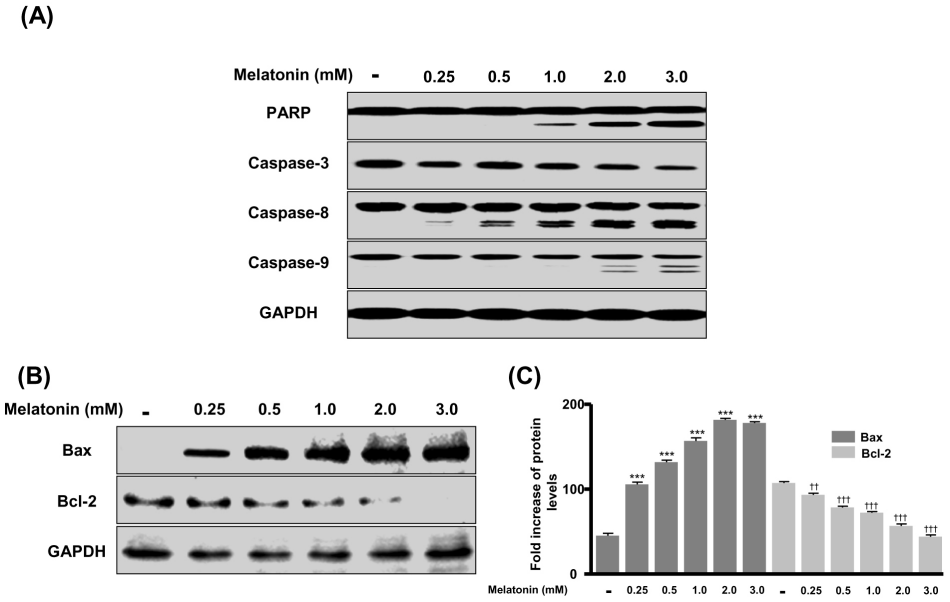

Fig. 2. Induction of LNCaP cell apoptotic cell death by melatonin. LNCaP prostate cancer cells were cultured in DMEM containing 10% FBS and then treated with melatonin at varying doses for 48 h. (A) Cell lysates prepared at the indicated culture times were separated by 10% SDS-PAGE and immunoblotted with antibodies to PARP, caspase-3, -8, -9, and GAPDH. (B) Cell lysates prepared at the indicated culture times were separated by 12% SDS-PAGE and immunoblotted with antibodies to Bax, Bcl-2, and GAPDH. (C) The relative amounts of Bax and Bcl-2 were quantified as described in Materials and Methods. ***$p<0.001$ vs. control. $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$ vs. control.

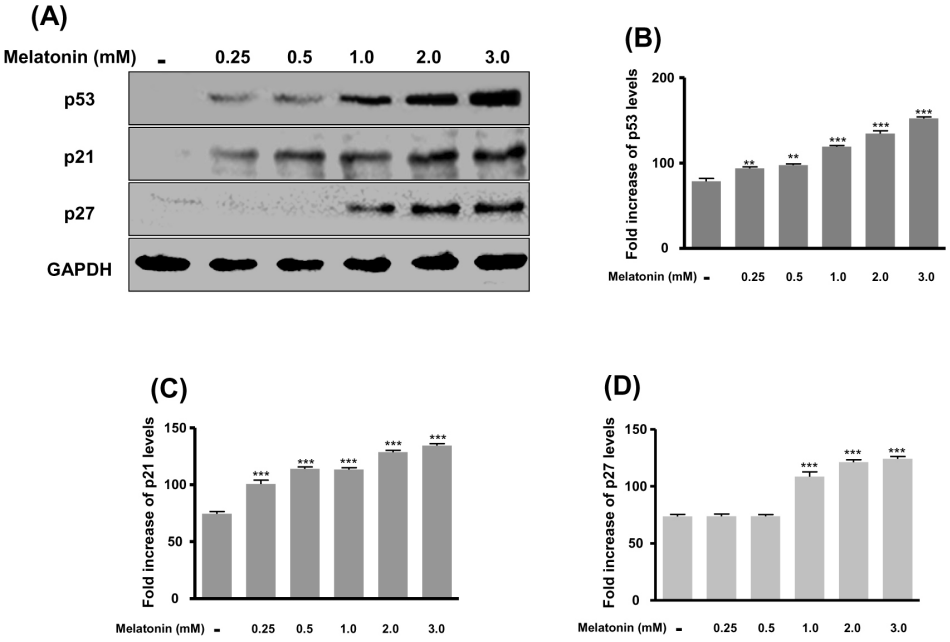

Fig. 3. Activation of p53, p21, and p27 in melatonin-treated LNCaP cells. Cells were cultured in DMEM medium containing 10% FBS and then treated with melatonin at varying doses for 48 h. (A) Cell lysates prepared at the indicated culture times were separated by 12% SDS-PAGE and immunoblotted with antibodies to p53, p21, p27, and GAPDH. The relative amounts of p53 (B), p21 (C), and p27 (D) were quantified as described in Materials and Methods. **$p<0.01$, ***$p<0.001$ vs. control.

(JNK inhibitor), and SB202190 (p38 inhibitor) in the presence of melatonin (Fig. 4). Melatonin treatment increased the expression of p-p53, p21, and MDM2 in a dose-dependent manner (Fig. 4A). Co-treatment of melatonin and PD98059 did not inhibit the expressions of p-p53, p21, or MDM2; suggesting PD98059 does not influence their expression (Fig. 4B). However, treatment of melatonin plus SP600125 or SB202190 suppressed the expression of p-p53, p21, and MDM2; indicating treatment of SP600125 or SB202190 inhibits their expression in a dose-dependent manner (Fig. 4C and Fig. 4D). These results indicate that melatonin effectively induces apoptosis via the p53 pathway and is directly involved with the JNK and p38 pathways, as was seen in the treatment profiles of MAPK inhibitors.

DISCUSSION

Our previous study demonstrated that melatonin results in apoptosis and induces oncostasis via modulation of signaling mediated by JNK and p38 MAPK in LNCaP prostate cancer cells and that melatonin may play an important role in androgen-dependent prostate cancer as an apoptotic and oncostatic anti-cancer agent [13]. The present report shows that melatonin also induces apoptotic cell death in LNCaP prostate cancer cells via signaling mediated by p53. Both pathways include a common pathway of p21 and MDM2

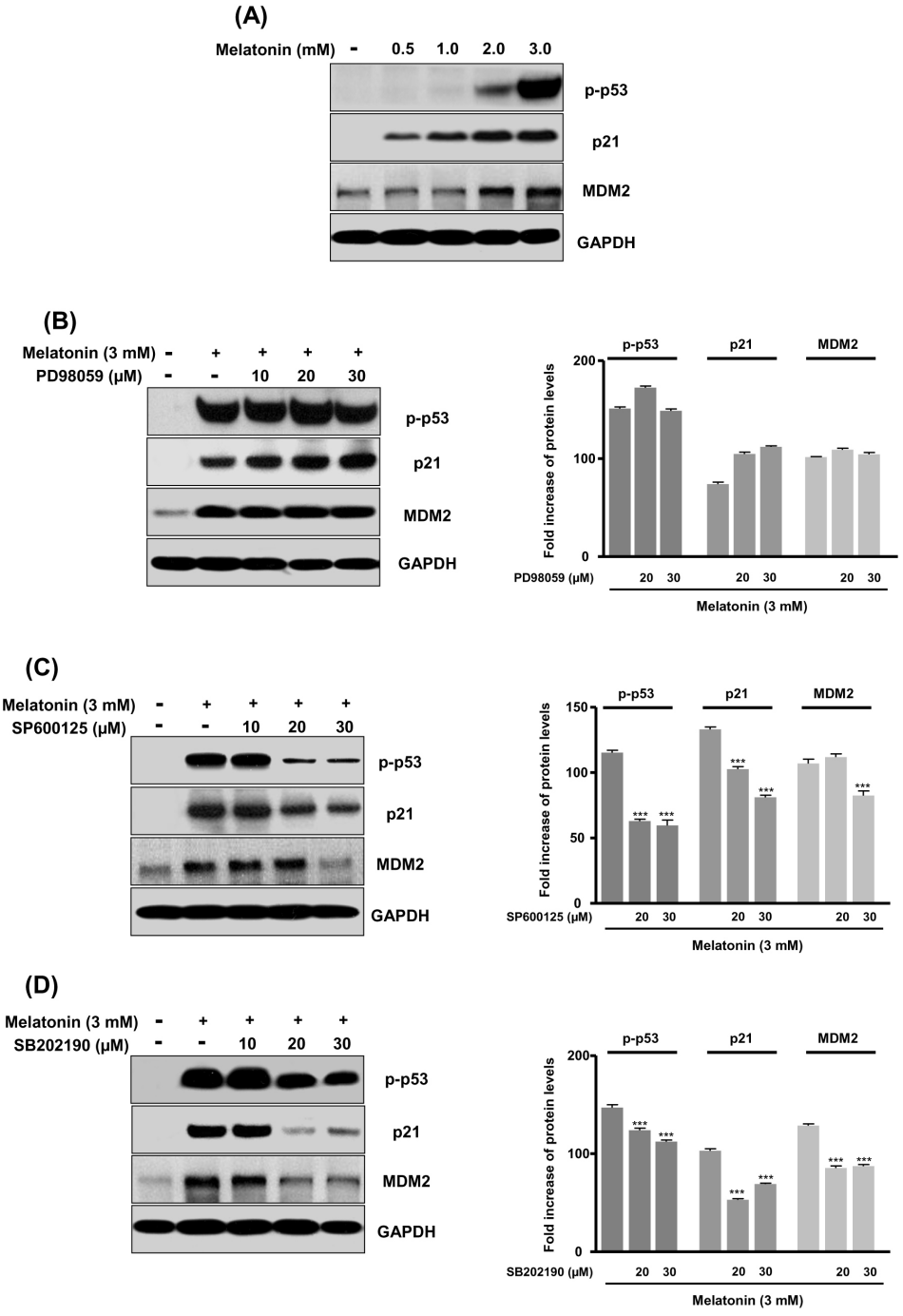

Fig. 4. Activation of p-p53, p21, and MDM2 by blockade with PD98059, SP600125, and SB202190 inhibitors on melatonin-induced apoptotic cell death. Inhibitor concentrations of 10 to 30 μM for PD98059, SP600125 and SB202190 were added to cells 1 hr before melatonin (3 mM) treatment for 48 hr. (A) LNCaP cells were cultured with melatonin at varying doses. (B), (C), and (D) Cells were treated with 3 mM melatonin for 48 hr in the presence of various concentrations of PD98059 (B), SP600125 (C), or SB202190 (D). Cell lysates prepared at the indicated culture times were separated by 12% SDS-PAGE and immunoblotted with antibodies to p-p53, p21, MDM2, and GAPDH. ***$p < 0.001$ vs. control.

and both result in the activation of caspase-3 and apoptotic cell death.

It is important that the concentration of melatonin used in treatment determines the induction of one of the two types of cell death pathways. For example, the signal pathway via the JNK and p38 MAPK in LNCaP cells is activated by melatonin [13]. In our previous research, we found that melatonin markedly activated JNK and p38 kinase. Treatment with PD98059 (ERK inhibitor), SP600125 (JNK inhibitor) and SB202190 (p38 inhibitor) confirmed that melatonin-induced apoptosis was JNK- and p38-dependent, but ERK-independent [13]. Here, we showed that melatonin increases the expressions of p53 and p21. Treatment with PD98059, SP600125 and SB202190, confirmed that the melatonin-induced apoptosis was p21-dependent, but ERK-independent. Together, these results strongly suggest that the melatonin induces the apoptotic cell death signaling pathway by p53-dependent induction of JNK/p38 MAPK in LNCaP cells.

Target p53 may be important for many cancer therapies. p53 has been shown to trans-activate a broad range of proapoptotic proteins from the Bcl-2 family (Bax, the BH3-only

proteins Bid, Puma and Noxa), and to downregulate anti-apoptotic proteins from the Bcl-2 family (Bcl-2, Bcl-$_{xL}$), as well as to induce the upregulation of proteins that localize to the mitochondria [14]. Knowledge of the p53-dependent pathway in prostate cancer could allow the development of selective and effective anti-cancer strategies involved with the apoptotic response [15].

We confirmed that melatonin caused apoptosis by p53 phosphorylation at serine 15, increasing the expression of downstream signals such as MDM2 and p21 in LNCaP cells; this is similar to our previous data, which indicated that melatonin induced caspase-3 activation and cytochrome c and Bax release into the cytosol [13]. However, MAPK inhibitors, SP600125 and SB202190, caused the down-regulation of p-p53, p21, and MDM2 after a co-treatment with melatonin, while PD98059 did not affect the expressions of p-p53, p21, or MDM2 during cell death induced by melatonin, indicating that the JNK and p38 MAPK pathways are closely associated with p53 phosphorylation. The phosphorylation of p53 can usually be induced at serine 15 or/and 18. Especially, the phosphorylation of p53 at serine 15 was reported to be a key phosphorylation target during the p53 activation process to apoptotic cell death [16,17]. Of the MAPK families, p53 can be phosphorylated either directly or indirectly by p38 kinase [18-21] and JNK [22,23]. Compared to several studies on the role of p38 kinase or JNK in p53 regulation, there is less evidence to indicate phosphorylation and/or accumulation of p53 by ERK. In the present study, JNK and p38 were able to mediate p53 phosphorylation leading to apoptosis by melatonin. In our previous study, SP600125 and SB202190 significantly disturbed the caspase-3 activation induced by melatonin, suggesting roles for JNK and p38 in the control of caspase-3 activation [13]. Finally, our previous and present studies confirm that melatonin induces apoptotic cell death through a common signal pathway including caspase-3-dependent pathway.

In conclusion, melatonin can induce apoptotic cell death through p53 activation in LNCaP cells. The MAPK families, JNK and p38, can play important roles in mediating apoptotic signal including p53 expression and caspase-3 activation during melatonin-induced apoptosis. Overall, these results suggest that melatonin can induce apoptotic cell death by p38/JNK pathway-dependent p53 activation in LNCaP cells.

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