Abstract. Melatonin is synthesized by the pineal gland and is released into the blood. In the last several years, some studies have shown that melatonin has anticancer properties; however, the mechanisms behind the antitumour traits are unclear, especially in pancreatic cancer. Therefore, in the present study, we investigated the antitumour effects of melatonin on the human pancreatic carcinoma cell line MIA PaCa-2 and explored its biological mechanisms. MIA PaCa-2 cells were treated with melatonin, and we used a CCK-8 assay to evaluate the cell viability. We also used flow cytometry to observe cell apoptosis and western blot analysis to assess the protein expression. Our study found that melatonin inhibited cell viability, suppressed colony formation and reduced cell migration and invasion and induced cell apoptosis in MIA PaCa-2 cells. Our results showed that melatonin treatment inhibited NF-κB p65 activation. Moreover, melatonin treatment activated the mitogen-activated protein kinase pathways (c-jun N-terminal kinase and extracellular-regulated kinase 1/2), which increased Bax protein expression and caspase-3 cleavage and decreased Bcl-2 protein expression. These new developments demonstrate that melatonin plays a potential role in anticancer treatment and may act as an effective therapeutic agent in the future.

Introduction

It is estimated that approximately 277,000 cases of pancreatic cancer are diagnosed worldwide every year, accounting for 2.2% of all cancers (1). Pancreatic cancer is a multifactorial disease and the seventh leading cause of cancer-related death in China (2). Moreover, pancreatic cancer has lower rates of diagnosis and the 5-year survival rate is <5% (3). Pancreatic cancer has limited treatment options. At present, surgery is considered as the only effective therapeutic measure. However, only 20% of the patients are suited for this approach (4). Therefore, more effective therapies are needed.

Melatonin is a pineal gland hormone and adjusts sleep and circadian rhythm (5). Melatonin also plays a part in biological processes including immunomodulation, antioxidative stress and anti-inflammation (6-9). Researchers have revealed that melatonin is an anticancer hormone (10). The anticancer mechanisms of melatonin involve activation of antioxidation stress, inhibition of migration and induction of cell apoptosis (11-13). Researchers showed that melatonin had an apoptotic effect on the hepatocarcinoma HepG2 cell line (14). Research has found that using melatonin has no significant side-effects (15). In conclusion, we postulated that melatonin may exert a protective effect against cancer.

The mitogen-activated protein kinase (MAPK) pathway plays an important role in cell survival and proliferation (16). The MAPK family has been categorized into three groups: c-jun N-terminal kinase (JNK), extracellular-regulated kinase 1/2 (ERK1/2) and p38MAPK. Some studies have suggested that the JNK substrate is involved in cell growth.
and apoptosis (17), which take part in tumor progression and are affected by melatonin (18).

Nuclear factor-κB (NF-κB) of the Rel family includes p50, p52, p65 (RelA), c-Rel and Rel B. NF-κB, as a transcription factor, stimulates the expression level of many genes closely related to oxidative stress and anti-apoptosis (19). Tamura et al found that melatonin suppressed the NF-κB pathway in rat endothelial cells (20). Gilad et al also revealed that melatonin suppressed the NF-κB pathway in murine macrophages (21).

At present, it is unclear whether melatonin has an effect on the apoptosis of the human pancreatic carcinoma cell line MIA PaCa-2 through the JNK and ERK pathways. Therefore, we examined the function of melatonin on the viability and apoptosis of MIA PaCa-2 cells via the MAPK signaling pathways and we investigated whether melatonin induces cell apoptosis through a decrease in NF-κB.

Materials and methods

Cell culture and reagents. Human pancreatic carcinoma cell line MIA PaCa-2 was purchased from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO, USA) supplemented with 1% penicillin/streptomycin and 10% heat-inactivated fetal bovine serum (FBS; Sigma) in 5% CO₂ in a humidified incubator. Cells were seeded at a density of 5x10⁵ cells/100-mm dish. Melatonin (Sigma) was dissolved in DMSO (Sigma) at 0.2% dimethyl sulfoxide (DMSO) and cells were treated with different doses of melatonin (0-4 mM) from 0 to 72 h.

Colony-forming assays. MIA PaCa-2 cells were seeded into 6-well plates (5x10⁵ cells/well) at 37°C with 5% CO₂ in a humidified environment. On the second day, the cells were treated with 1 and 2 mM melatonin for 8 days. Then each well was washed twice with PBS and stained with crystal violet. During the incubation period of 8 days, the culture medium was replaced every 3 days in all wells.

Cell viability assay. The Cell Count Kit-8 (CCK-8; Dojindo, Japan) was used to evaluate the effects of melatonin on cell viability. For the CCK-8 assays, MIA PaCa-2 cells were seeded into a 96-well culture plate (3x10⁴ cells/well) in 200 µl of complete medium. The plating medium was replaced with new culture medium after 24 h. Then melatonin was dissolved in the new medium at different doses (0.25-4 mM). Vehicle control cells were cultured in DMEM with 0.2% DMSO. Each group included three parallel wells. After exposure for 12, 24, 48 and 72 h, CCK-8 was added to the culture media. For the CCK-8 assays, MIA PaCa-2 cells were treated with 1 and 2 mM melatonin for 8 days. Then each well was washed twice with PBS and stained with crystal violet. During the incubation period of 8 days, the culture medium was replaced every 3 days in all wells.

Apoptosis assay using Annexin V FITC/PI. To observe early apoptosis and necrosis, the cells were stained with FITC-conjugated Annexin V and propidium iodide (PI) (MultiSciences Biotech Co., Ltd., Hangzhou, China). The cells (4x10⁶) were plated in 6-well plates and treated with 0 and 2 mM melatonin for 0 to 72 h. Cells were harvested by trypsinization, cleared with PBS, centrifuged at 1,000 rpm for 5 min and the supernatant was discarded. The pellet was resuspended in 1X binding buffer. A total of 500 µl of the sample solution was added to 5 µl of FITC-conjugated Annexin V and 10 µl of PI and the solution was incubated for 5 min in the dark at room temperature. The cells were visualized and sorted using FACS (Becton Dickinson, San Jose, CA, USA) and quantified using FlowJo software. Positioning of quadrants on the Annexin V FITC/PI dot plots was used to distinguish living cells (Annexin V FITC+/PI-), early apoptotic cells (Annexin V FITC+/PI-) and late apoptotic/secondary necrotic cells (Annexin V FITC+/PI+) (18).

Western blot analysis. After 2 mM melatonin treatment, the cells were washed three times with cold PBS and lysed by adding ice-cold RIPA buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, sodium orthovanadate, sodium fluoride, EDTA, leupeptin and PMSF and PhosSTOP (Roche, Basel, Switzerland) for 15 min on the table concentrator minus four degrees. Then cells were scraped off the plate. The extracts were transferred to a microfuge tube and centrifuged at 12,000 x g for 15 min. The protein concentration was determined using the BCA assay (Beyotime). Equal amounts of total protein (40 µg) were separated by SDS-PAGE and transferred to a PVDF membrane (Bio-Rad, Hercules, CA, USA). The membranes were blocked at room temperature for 1 h in blocking buffer and the proteins were incubated with primary antibodies targeted against: phospho-ERK (1:1,000), ERK (1:1,000), phospho-JNK (1:1,000), JNK (1:1,000), phospho-p65 (1:1,000), p65 (1:1,000) (Cell Signaling Technology, Beverly, MA, USA) and GAPDH (1:1,000) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 12-16 h. After washing with TBST three times, the membranes were incubated for 1 h at room temperature with the secondary HRP-conjugated antibody (1:5,000; Bioworld Technology, Inc., USA) and visualized using WesternBright ECL detection kit (Advansa, Menlo Park, CA, USA). The density of the specific bands was quantified using Image Lab software with an imaging densitometer (Bio-Rad ChemiDoc MP) (both from Bio-Rad).

Statistical analysis. The results were analyzed using SPSS software (version 13) (SPSS, Inc., Chicago, IL, USA) and are presented as the mean values ± SEM. Data comparisons were performed using analysis of variance (ANOVA). When the...
Results

Melatonin affects MIA PaCa-2 cell viability, colony formation, invasion and apoptosis. We used the human pancreatic carcinoma cell line MIA PaCa-2 to evaluate the antitumour effects of melatonin on pancreatic cancer. The effect of melatonin on MIA PaCa-2 cell viability was observed by the CCK-8 assay and the results showed that cell viability was significantly reduced by 2 and 4 mM melatonin after 12–72 h of treatment (Fig. 1A). When analysing the number of colonies formed, melatonin treatment (1 and 2 mM) caused a significant decline in colony formation (Fig. 1B).

Flow cytometry and Annexin V FITC/PI staining, which can identify early apoptotic cells, were used to assess apoptosis in the human pancreatic carcinoma cell line MIA PaCa-2 after exposure to 2 mM melatonin for 0, 24, 48 and 72 h. As shown in Fig. 2A, after treatment for 48 h the percentage of early apoptotic cells was 1.79-fold of that noted in the control cells. The early apoptotic rate (Annexin V FITC/PI staining) was significantly increased after 72 h (23.9%, Fig. 2B).

MIA PaCa-2 cells were treated with various concentrations of melatonin at various times to assess the effects of melatonin on cell migration. As shown in Fig. 3, 2 mM of melatonin markedly reduced MIA PaCa-2 cell migration (20.1% of the control at 48 h). To examine the inhibition of cell motility by melatonin by invasion assay, we found that melatonin also suppressed cell invasion compared with the control group (Fig. 4).

Effects of melatonin on the phosphorylation of MAPK pathway components. Melatonin was found to inhibit cell viability and migration and induce cell apoptosis in the MIA PaCa-2 cells. Considering the possible mechanisms, we evaluated the function on the elementary activation status of MAPKs. JNK and ERK phosphorylation was significantly induced at 24 and 48 h (Fig. 5A); the levels of JNK and ERK were used as internal controls and the phosphorylated proteins were quantified using the control. The results revealed that the levels of p-JNK and p-ERK were increased at 24 and 48 h (Fig. 5B).

Melatonin inhibits NF-κB activation. Following treatment with 2 mM melatonin, p65 phosphorylation was significantly induced in a time-dependent manner (Fig. 6A); the levels of p65 were used as internal controls and the phosphorylated proteins were quantified using the control. The results presented a clear decrease in p-p65 activity at 12, 24 and 48 h (Fig. 6B).
Discussion

Pancreatic cancer originates from pancreas tissue and has a high rate of metastasis (22). Pancreatic cancer is not only difficult to diagnose at an early stage, but also lacks effective therapeutic strategies (23). Recently, studies have revealed that melatonin has anticancer properties. However, the mechanisms of melatonin in regards to its antitumour properties are poorly
Figure 5. Effects of melatonin on the phosphorylation of MAPK pathway components. (A) MIA PaCa-2 cells were treated with 2 mM melatonin for 0 to 48 h and the proteins were incubated with primary antibodies targeted against the following: anti-phospho-JNK, anti-JNK, anti-phospho-ERK and anti-ERK and assessed by western blot analysis. Protein signals were visualized with an ECL detection system. (B) The levels of JNK and ERK were used as internal controls. By densitometric analysis the phosphorylated proteins were quantified using the control. Experimental data are presented as the means ± SEM of three independent experiments. *P<0.05; **P<0.01 vs. control group. p-ERK, phospho-ERK; p-JNK, phospho-JNK.

Figure 6. Melatonin inhibits NF-κB activation. (A) MIA PaCa-2 cells were treated with 2 mM melatonin for 0 to 48 h. The proteins were incubated with primary antibodies targeted against: anti-phospho-p65, anti-p65 and GAPDH and assessed by western blotting analysis. Protein signals were visualized with an ECL detection system. (B) The levels of p65 were used as internal controls. By densitometric analysis the phosphorylated proteins were quantified using the control. Experimental data are presented as the means ± SEM of three independent experiments. *P<0.05; **P<0.01 vs. control group. p-p65, phospho-p65.
understood. Induction of apoptosis is a potential antimour mechanism and it is a basic step in the regulation of different cell types. Therefore, we studied melatonin which induced cell apoptosis in the human pancreatic carcinoma cell line MIA PaCa-2.

We found that melatonin inhibited MIA PaCa-2 cell viability, migration and invasion (Figs. 1, 3 and 4). Moreover, we revealed that melatonin induced cell apoptosis at various concentrations. Joo et al demonstrated that apoptosis activates-caspases which are related to cell viability (18). Pro-apoptosis is viewed as the most suitable strategy for treating cancer. Some reports suggest that ERK pathway activation influences a survival signal that weakens pro-apoptotic effects via activating JNK (24,25). Cisplatin through ERK pathway activation, slows down cell growth and causes apoptotic cell death (26).

In summary, we considered whether melatonin through MAPK (JNK, ERK) pathways induces apoptosis in MIA PaCa-2 cells. We hypothesized that melatonin acts as a potential apoptotic inducer. Our western blot analysis of MAPK pathway components showed that phosphorylation of JNK and ERK was enhanced at 24 h and significantly increased at 48 h by melatonin (Fig. 5). Recent research suggests an important role for JNK and ERK in pathways related to apoptosis and growth-inhibitory pathways (27,28). In addition, JNK induces caspase-3 activation and JNK is necessary for the phosphorylation of proteins related to apoptosis in cancer cells, including Bcl-2 and Bax (29). In conclusion, we consider that melatonin caused MIA PaCa-2 cell apoptosis by activating JNK and ERK which promote caspase-3 overexpression and caspase-3-induced cell apoptosis.

The transcription factor NF-κB family is comprised of related protein dimers (30). Upon activation, NF-κB p65 or p50 is liberated from the IκB compound and NF-κB p65 translocates to the nucleus, where it causes the expression of a series of genes encoding different proteins which take part in suppressing apoptosis and causing inflammation, cellular invasion and proliferation (31). These target genes play key roles in malignant development and include apoptosis-suppressor proteins, such as Bcl-2 and Bcl-XL, and cell-cycle regulatory proteins, such as cyclin D1 (32). In malignant cells, stimulation of cell proliferation and protection against apoptosis are connected with abnormal NF-κB activation (33). Curcumin (34) and flavopiridol (35) have been suggested to inhibit NF-κB. In this study, we found that melatonin may suppress the phosphorylation of NF-κB p65 (Fig. 6).

In summary, melatonin may possess anticancer effects in several types of cancer, including gastric cancer. Research suggests that melatonin exerts an antitumour effect (36). The potential to target mechanisms that inhibit NF-κB p65 and promote ERK and JNK may offer effective strategies for chemotherapy. By analyzing the present and previous data, we established that melatonin induces apoptosis and suppresses migration and invasion via modulation of signaling mediated by the JNK and ERK MAPKs and NF-κB p65 pathways. The present study suggests the requirement for additional or adjunct therapies in combination with melatonin treatment to fully inhibit cancer progression. Our results suggest that melatonin may act as a potential anticancer agent against human pancreatic cancer.

Acknowledgements

This study was supported by the Science and Technology Bureau of Wenzhou, Zhejiang Province, China (no. 2014S0192).

References


