Growth-Inhibitory Activity of Melatonin on Murine Foregastric Carcinoma Cells *In Vitro* and the Underlying Molecular Mechanism

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**ABSTRACT**

Melatonin (MLT) is an indolic hormone produced mainly by the pineal gland. Recent human and animal studies have shown that MLT exerts obvious oncostatic activity both *in vitro* and *in vivo*. The purpose of this study was to investigate the antiproliferative effect of MLT on the murine foregastric carcinoma (MFC) cell and to determine the underlying molecular mechanism. Cell viability was determined using the Cell Counting Kit-8 (CCK-8) and the results revealed that MLT exhibited a dose- and time-dependent inhibitory effect on MFC cell growth. Our studies also demonstrated upregulation of p21 and Bax and downregulation of Bcl-2 at both the mRNA and the protein levels in response to MLT treatment of MFC cells. These changes in the expression of these molecules were consistent with the results of the CCK-8. Furthermore, the mRNA and protein expression of membranous MLT receptors was also upregulated. Taken together, these results confirm the oncostatic effect of MLT in MFC cells and the expression of membranous MLT receptors is a potential approach to tumor cells in gastric cancer therapeutic treatment. Anat Rec, 296:914–920, 2013. © 2013 Wiley Periodicals, Inc.

**Key words:** melatonin; MT1 receptor; MT2 receptor; p21; Bax; Bcl-2; gastric cancer

Gastric cancer is one of the most common forms of cancer in Asia and worldwide. In China, gastric adenocarcinoma is the third leading cause of cancer mortality (Crew and Neugut, 2006; Hou et al., 2010). In general, gastric cancer patients respond poorly to conventional chemotherapies and hence more comprehensive therapy is required (Lissoni et al., 1993). Melatonin (N-acetyl-5-methoxytryptamine) (MLT), which is an indolamine that is primarily synthesized and secreted by the pineal gland, exerts a wide variety of biological activities (Cuzzocrea and Reiter, 2002; Carrillo-Vico et al., 2005; Claustrat et al., 2005; Lardone et al., 2006). MLT has been reported to exert oncostatic effects both *in vitro* and *in vivo* (Cos et al., 2006; Dauchy et al., 2009; Girgert et al., 2009).
However, the intracellular mechanisms by which MLT influences gastric cancer growth remain largely unknown. It has been demonstrated that MLT exerts its complex actions by binding and activating two distinct receptor types: membrane receptors MT1 and MT2 and the nuclear orphan RZR/RORα receptors (Winczyk et al., 2002; Kadekaro et al., 2004; Carbajo-Pescador et al., 2009). MLT membrane receptors mediate their functions through a G-protein-coupled second messenger pathway and nuclear receptor signaling appears to be mediated via the transcription factor RZR/ROR, which is an orphan member of the nuclear receptor superfamily.

In this study, the antiproliferative effects of MLT and its potential correlation with changes in MLT receptor expression were investigated in the murine foregastric carcinoma (MFC) cell line in vitro.

MATERIALS AND METHODS

Materials

MLT was purchased from Sigma (St. Louis, MO). Primary antibodies for MEL-1A-R, MEL-1B-R, p21, and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); Bax and Bcl-2 were from Cell Signalling (Beverly, MA). CCK-8 Kit was purchased from Dojindo Bio (Kumamoto, Japan). All cell-culture reagents were from Gibco (Invitrogen, Carlsbad, CA).

Cell Culture and Treatment

The murine MFC cell line, derived from the 615 mouse strain, was purchased from the Chinese Academy of Sciences Shanghai Institute for Biological Science. MFCs were cultured in RPMI-1640 medium that was supplemented with 10% fetal bovine serum. The cells were maintained at 37°C in a humidified incubator with 5% CO₂, passaged every 3 days and digested using trypsin. Cells were used in experiments after three to six passages. MLT was dissolved in 100% ethanol and diluted in RPMI 1640 to the desired concentrations, with a final ethanol concentration of 0.1% v/v for in vitro studies. The MFCs were treated with 2 mM MLT, 4 mM MLT (Liu et al., 2011) and medium containing 0.1% ethanol was used as a control.

Morphological Observation of Cells

The cells were plated into six-well plates (3 × 10⁵ cells/well) and incubated for overnight at 37°C in a humidified incubator containing 5% CO₂. On the following day, the cells were treated with 2 and 4 mM of MLT. The cellular morphology was observed and imaged 24-hr post-treatment using an optical microscope (Olympus, Japan).

Cellular Proliferation and Viability Assay

MFC cells were plated in 96-well plates (5,000 cells/well). Cells were treated on the following day (control, 2 and 4 mM MLT) and cultured for 24, 48, and 72 hr. Cell viability and proliferation were assayed using a CCK-8 Kit according to the manufacturer’s protocol. Briefly, cells were incubated with CCK-8 solution (10 µL/well) for 1 hr before cell density was determined by measuring

Fig. 1. The effects of MLT on MFC cell morphology and viability. Cells were cultured in RPMI-1640 containing MLT (0 mM [control], 2 mM, and 4 mM). (A) Cellular morphology was observed by optical microscopy. (B) Cell viability was assayed using a CCK-8 assay kit. Values are expressed as means ± S.E.M. of three independent experiments.
the absorbance at 450 nm using a Varioskan Flash (Thermo Scientific, USA).

Western Blot Analysis

After treatment, cells were harvested, washed twice with phosphate-buffered solution (PBS), and lysed by adding ice-cold lysis buffer containing 1 mM phenylmethylsulphone fluoride, pH 7.4. Protein concentration was determined by the BCA method. For Western blot analysis, equal amounts of protein were separated by 12% SDS-PAGE gel electrophoresis (110 V, 1.5 hr) and the membranes were blotted by wet transfer (110 V, 1.5 hr, 4°C) on polyvinylidene fluoride (PVDF) membranes (Millipore, USA). Membranes were blocked in 5% nonfat milk solution in PBS. The membranes were then incubated with a primary antibody (dilution, 1:1,000) for overnight at 4°C. The membranes were washed with Tris-buffered saline-Tween (TBST) and then incubated for 1.5 hr at room temperature with a secondary antibody (dilution, 1:1,000). After washing with TBST, the membranes were exposed to X-ray film (1–15 min) for visualization of the immunoreactive bands. Densitometry analysis of specific bands was performed by Quantity One (Bio-Rad, USA). The quantity of target protein was calibrated with respect to β-actin, and control value and relative intensities were obtained.

Real-Time Reverse Transcriptase Polymerase Chain Reaction

Total RNA was isolated using Trizol reagent (Invitrogen, USA) according to the manufacturer’s instructions. First-strand cDNA was generated from 2 μg of each RNA preparation by reverse transcription using the first-strand cDNA synthesis kit (Promega, USA). Real-time quantitative polymerase chain reaction (PCR) for the analysis of MFC expression of MT1, MT2, p21, Bax, Bcl-2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes, cDNA was amplified using the dye SYBR Green (Stratagene, USA) on an StepOnePlus Real-Time PCR Systems (Applied Biosystems). The PCR cycling conditions (40 cycles) were as follows: 30 sec at 95°C; for 1 min at 60°C. The fold change in expression of each gene was calculated using the 2^(-ΔΔCt) method. Product quality of PCR was monitored using post-PCR melting curve analysis at the end of the amplification cycles. The primers were as follows: MT1, 5’-CCGCAAC AACGAGCTACCTGAGAATTCTC-3’ and 5’-CTCTGAGGAGGGCTTGGCATC-3’; MT2, 5’TACATCGGCTCTGACCTC-3’ and 5’TTCCTGAGGAGGGCTTGGCATC-3’; p21, 5’GCTGACTGACCTGACCTGTC-3’ and 5’TCCCTGACCTGACCTGTC-3’; Bcl2, 5’-GTGACTGACCTGACCTGTC-3’ and 5’TCCCTGACCTGACCTGTC-3’; Bax, 5’-GACCTGACCTGACCTGTC-3’ and 5’TCCCTGACCTGACCTGTC-3’; GAPDH, 5’-CCGGAATGGGAGAGGAACTG-3’ and 5’TCCCTGACCTGACCTGTC-3’.

Statistical Analysis

Results are presented as mean values ± standard error of mean (S.E.M.). Significance between experimental values was determined by Student paired t tests, and one-way ANOVA was used to test differences in repeated measures across experiments. Differences were considered to be statistically significant at \( P < 0.05 \). Values were analyzed using the statistical package SPSS16.0 (Statsoft, Tulsa, OK).

RESULTS

Effects of MLT on Cell Morphology and Cellular Viability

Evaluation of cellular morphology showed that, compared to the normal control treatment, the cells were significantly sparser after MLT treatment. Furthermore, analysis of cell viability revealed a time- and dose-dependent inhibition of MFC cell growth after MLT treatment (Fig. 1A,B).

Effects of MLT on the mRNA and the Protein Expression of p21

To investigate the mechanism of this G0/G1 block, the protein and gene expression of p21 was analyzed by Western blot and real-time PCR. The results demonstrated a upregulation of p21 in MLT-treated MFC cells in a dose- and time-dependent manner (Fig. 2A,B).

Effects of MLT on the mRNA and the Protein Expression of Bax and Bcl-2

To investigate the effects of MLT on apoptosis and inhibition of cell proliferation, the expression of the
antiapoptotic gene, Bcl-2, and the proapoptotic protein, Bax was analyzed. MLT was shown to decrease the transcription of Bcl-2, but also increased the transcription of Bax (Fig. 3A–D).

Effects of MLT on the mRNA and the Protein Expression of MT1, MT2

Treatment of MFC cells with MLT (2 and 4 mM) for 24, 48, and 72 hr resulted in elevated expression of both MT1 and MT2 at the mRNA and protein levels compared with that of the control group. Furthermore, treatment of MFC cells with 2 mM MLT (72 hr) and 4 mM MLT (48 and 72 hr) resulted in significantly higher expression of MT1 compared with that in the control group. Treatment of MFC cells with 2 mM MLT (72 hr) and 4 mM MLT (24, 48, and 72 hr) resulted in significantly higher expression of MT2 compared with that in the control group (Fig. 3A–D).

DISCUSSION

MLT is an indolic hormone produced mainly by the pineal gland. Both physiological and pharmacological levels of MLT have been shown to exert substantial anticancer activity (Cos et al., 2006; Girgert et al., 2009). MLT has also been shown to exert marked antiproliferative activity in numerous experimentally induced tumors in vivo as well as in both animal and human cell lines in vitro. In this study, we investigated the effect of MLT on cellular proliferation and viability of an MFC cell line. Our confirmed that MLT exerted a dose- and time-dependent antiproliferative effect on MFC cells. These data agree with those reported for MLT in other cancer
The oncostatic effects of MLT have been reported in human breast cancer MCF-7 cells (Girgert et al., 2009), 22Rv1 human prostate cancer cells (Tam et al., 2007), or human choriocarcinoma JAr cells (Shiu et al., 1999) at physiological concentrations. Other related studies, using human hepatocarcinoma HepG2 cells (Martín-Renedo et al., 2008) or CT-26 mouse colon cancer cells (Farriol et al., 2000) have found its anticancer effects at a range of 1–6 mM. Moreover, studies have shown that the local concentrations of MLT, such as the immune system (Carrillo-Vico et al., 2005) or the gastrointestinal tract (Motilva et al., 2001), are higher than the physiological lever of MLT in serum, which suggests a beneficial role of MLT in gastric disease and in the immune system. In our study, we detected the antiproliferative effect of MLT at both the concentrations of 2 and 4 mM.

To investigate the effects of MLT on apoptosis and inhibition of cell proliferation, we analyzed the expression of Bcl-2 and Bax. Bcl-2 mediates antiapoptotic effects by binding to the proapoptotic protein, Bax (Jacobson et al., 1993; Adams and Cory, 2007), which is essential for the formation of pores in the mitochondrial membrane. This induces the release of cytochrome C as the final triggering of apoptosis (Saito et al., 2000; Harada and Grant, 2003). In this study, we found that MLT not only decreased transcription of Bcl-2, but also increased the transcription of Bax. These results suggest that MFC cell death is related, at least in part, to the apoptosis, which is induced by the changes in Bax and Bcl-2. In this research, the results showed that protein levels were decreased but mRNA was not for the 2-mM treatments at 48 and 72 hr, and the same for 4 mM at 72 hr. This may be owing to different mechanisms of protein translation; for example, the activity of promoter being inhibited by methylation or acetylation.

The Cip/Kip family member, p21 is a negative regulator of the G1/S transition of the cell cycle (Gong et al., 2002). In our study, p21 was also analyzed to investigate the antiproliferative effects of MLT. A significant increase was observed in p21 expression.
Many pathways are involved in the oncostatic effects of MLT, some of which can be attributed to the fact that its receptors are expressed in a wide variety of tissues. MT1 and MT2 receptors are expressed together and in isolation in various tissues. The MT1 MLT receptor (MT1R) is expressed in the suprachiasmatic nucleus of the hypothalamus (SCN), cardiac vessels, kidney, immune system, skin, ovary, or cancer tissue. MLT MT2 receptors are found mainly in the brain, retina, and cancer tissues (Witt-Enderby et al., 2003; Pandi-Perumal et al., 2008).

Numerous studies have shown that MLT inhibits the proliferative activity of tumor cells via MT1R-mediated inhibition of the cAMP signal transduction cascade, resulting in decreased Protein Kinase A activity and decreased CAMP-response element binding phosphorylation (Sauer et al., 2001). The MT1 and MT2 receptors also mediate the inhibition of tumor linoleic acid metabolism to the growth of signaling molecule 13-hydroxyoctadecadienoic acid (Blask et al., 2004), stimulate c-Jun oligomerization to the growth of signaling molecule 13-hydroxyoctadecadienoic acid (Blask et al., 2004), stimulate c-Jun N-terminal kinase activity (Martin-Renedo et al., 2008), decrease MAP kinase activity (Dauchy et al., 2007), and inhibit telomerase activity in breast cancer cells (Leon-Blanco et al., 2004).

Similarly, MLT also exerts antiproliferative activity in tumor cells via MT2R mechanism shown to involve inhibition of cAMP formation and stimulation of PI hydrolysis in various transfected models (Kanishi et al., 2000). In gastric ulcer rats, enhanced expression of MT2 involved in biosynthesis of MLT has been shown to contribute to the acceleration of ulcer healing (Konturek et al., 2008).

In our study, both the MT1 and MT2 subtypes of G protein-coupled MLT receptor were identified in MFC cells. Furthermore, MLT was shown to upregulate gene transcription and protein expression of both MT1 and MT2 in MFC cells.

In conclusion, our results confirm the antitumor effects of MLT in MFC cells, and indicate that the oncstatic effect of MLT is mediated by upregulation of gene and protein expression of Bcl-2 and p21 protein as well as downregulation of gene and protein expression of Bcl-2 in MFC cells. It can be speculated that the altered expression of Bcl-2, Bax, and p21 is mediated via different cellular receptors and that the oncstatic properties of MLT are related, at least in part, to changes in the expression of the MLT membrane receptors subtypes. This study indicates that MLT is a suitable adjuvant for use in gastric cancer therapy. However, further studies on MFC cells, using specific inhibitors and siRNA, are necessary to elucidate the contribution of each MLT receptor to the oncstatic effects of MLT.

**LITERATURE CITED**


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