Melatonin and Doxorubicin synergistically induce cell apoptosis in human hepatoma cell lines

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RESULTS: Treatment with Melatonin (10^{-8}-10^{-5} \text{ mol/L}) alone had a dose-related inhibitory effect on cell proliferation but no cytotoxic effect on hepatoma cell lines HepG2 and Bel-7402. Interestingly, when combined with Doxorubicin, Melatonin significantly increased the effects of cell growth inhibition and cell apoptosis. Furthermore, TUNEL staining and flow cytometry revealed that cooperative apoptosis induction was associated with decreased expression of Bcl-2 as well as increased expression of Bax and Caspase3.

CONCLUSION: The synergism of Melatonin and Doxorubicin inhibits hepatoma cell growth and induces cell apoptosis.

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Key words: Melatonin; Doxorubicin; Human hepatoma cell line; Apoptosis

INTRODUCTION

Melatonin, a chief secretary product of the pineal gland, has diverse physiological functions. It plays a crucial role in regulating circadian rhythm, and is involved in immunomodulation, hematopoiesis, and antioxidative...
Cytotoxicity was measured using MTT assay. HepG2 and Bel-7402 cells in exponentially growth phase were cultured at a density of 1 × 10⁴ cells/well in a 96-well plate. After treatment with various concentrations of drug for 48 h, MTT solution (5.0 mg/mL in phosphate-buffered saline) was added (20.0 μL/well), and the plates were incubated for another 4 h at 37°C. The purple formazan crystals were dissolved in 150.0 μL Dimethyl Sulfoxide DMSO per well. After 10 min, the plates were read on microplate reader (American Bio-Tek) at 490 nm. The cells without drugs were used as control. Assays were performed in three independent experiments. The percentage of cytotoxicity was calculated as follows: Cytotoxicity (%) = (1 - Ao of experimental well)/Ao of control well. The median inhibitory concentration (IC₅₀) (defined as the drug concentration at which cell growth was inhibited by 50%) was assessed from the dose-response curves.

Analysis of in vitro drug interaction
The coefficient of drug interaction (CDI) was used to analyze the synergistical inhibitory effect of drug combination. CDI is calculated as follows: CDI = AB/(A × B). According to the absorbance of each group, AB is the ratio of the combination groups to control group in Ao, A or B is the ratio of the single agent groups to control group in Ao. Thus CDI value less than, equal to or greater than 1 indicates that the drugs are synergistic, additive or antagonistic, respectively. CDI less than 0.7 indicates that the drugs are significantly synergistic.

TUNEL assay
The cells were cultured in 6-well plates containing cover slips overnight. After treatment with various concentrations of Melatonin or Doxorubicin or in combination for 48 h, the cover slips were washed twice with PBS and fixed in 4% paraformaldehyde solution for 25 min at room temperature. Apoptotic cells were detected by terminal deoxynucleotidyl-transferase-mediated dUTP nick end-labeling (TUNEL) assay using DeadEnd™ Colorimetric TUNEL System Kit from Promega (Madison, WI), following the manufacturer’s instructions. The TUNEL assay results were quantitatively analyzed by biological image analysis system which consists of Nikon ECLIPSE 80i biological microscope, Nikon Digital Camera DXM 1200F, ACT-1 version 2.63 software (Japan).

Immunocytochemistry
Cells were seeded onto glass coverslips overnight. After incubation with various concentrations of the drugs for 48 h, the cover slips were washed twice with PBS and added 4% paraformaldehyde solution for 30 min at room temperature. Immunohistochemical staining for Bcl-2, Bax and caspase-3 was measured using the standard S-P method. The detailed manipulation was conducted according to the manufacturer’s instructions. As negative control, PBS was used instead of primary antibody, and other steps were followed in the same way. The immunocytochemical results were quantitatively analyzed by biological image analysis system which consists of Nikon
ECLIPSE 80i biology microscope, Nikon Digital Camera DXM 1200F, ACT-1 version 2.63 software (Japan), and JEOA 801D morphological biological image analysis software version 6.0 (Jie Da Technologies, Inc., China). The average absorbance value was analyzed by 5 randomly selected optical fields under microscopy (× 400).

**Flow cytometry**

The cells were grown in 6-well plates and then treated with Melatonin and/or Doxorubicin at the desired concentrations. After exposure to drugs for 48 h, cells were trypsinized, washed twice with cold PBS and centrifuged. The cell pellet was resuspended in 1 mL cold PBS and fixed in 9 mL of 70% ethanol at -20°C for at least 12 h. Then cells were centrifuged and resuspended in 500 μL PBS, RNase A was added and the cells were incubated at 37°C for 30 min. Propidium iodide (PI) staining buffer was added in the dark at room temperature for 30 min (according to the procedure program of the Cell Apoptosis PI Detection Kit). A minimum of 1 × 10⁶/mL cells for each group was analyzed using an EPICS XL-MCL model counter (Beckman Coulter, Fullerton, CA, USA).

**Statistical analysis**

Biostatistical analyses were done using the SPSS 11.5 software package. All experiments were repeated at least three times. The results of multiple experiments are given as the mean ± SE. Non-parametric Kruskal-Wallis test was used to detect differences among the different experimental groups. The Mann-Whitney U-test was subsequently used for statistical evaluation in two-group comparisons. Pearson correlation coefficient was used for continuous independent and dependent variables. A level of $P < 0.05$ was accepted as statistically significant.

**RESULTS**

**Inhibitory effect of Melatonin on hepatoma cell proliferation**

HepG2 and Bel-7402 cells incubated with various concentrations of Melatonin alone for 48 h showed a dose-dependent reduction of cell proliferation. The inhibitory rate of Melatonin (10⁻⁵, 10⁻⁶, 10⁻⁷ and 10⁻⁸ mol/L) on HepG2 cells was 5.27% ± 1.53%, 13.53% ± 2.73%, 24.91% ± 6.60%, and 30.78% ± 3.28%, ($r = 0.993$, $P < 0.01$) and that on Bel-7402 cells was 5.35% ± 2.05%, 17.06% ± 5.51%, 26.09% ± 4.23%, respectively ($r = 0.952$, $P < 0.01$). The IC₅₀ of Melatonin on HepG2 and Bel-7402 cells were 7.623 × 10⁻⁵ and 4.693 × 10⁻⁵, respectively.

**Synergistic cytotoxicity of combined Melatonin and Doxorubicin**

To investigate the synergistic inhibitory effects of Mela-
Melatonin and Doxorubicin, four different doses of Melatonin ($10^{-8}$, $10^{-7}$, $10^{-6}$ and $10^{-5}$ mol/L) and four different concentrations of Doxorubicin (0.31, 0.63, 1.25 and 2.5 mg/L) were chosen and assessed. As depicted in Figure 1, Melatonin increased the cytotoxicity of Doxorubicin on HepG2 (Figure 1A and B) and Bel-7402 cells (Figure 1C and D). CDI was used to evaluate the nature of the interaction. When $10^{-5}$ mol/L Melatonin was combined with 1.25 mg/L Doxorubicin, the synergistic inhibitory effects is the strongest on HepG2 (Figure 1B). Doxorubicin and Melatonin had the strongest synergism on Bel-7402 when $10^{-5}$ mol/L Melatonin was combined with 2.5 mg/L Doxorubicin (Figure 1D).

**Proapoptotic effect of combined Melatonin and Doxorubicin**

HepG2 and Bel-7402 cells were treated with different concentrations of Melatonin, Doxorubicin or a combination...
of both for 48 h, respectively, and apoptosis was assessed by TUNEL and FACS methods. We found that in the combination treatment group, there was a greater degree of induction of apoptosis than that caused by either agent alone. In HepG2 cells, the number of apoptosis cells in Melatonin (10⁻⁵ mol/L) or Doxorubicin (1.25 mg/L) alone treatment group was only slightly increased when compared with the cell control group. However, the apoptotic rate increased greatly when the cells were treated with combined Doxorubicin and Melatonin (Figure 2A and B). Similar results were found in Bel-7402 cells (Figure 3A and B). These results were confirmed by FCM assay. As shown in Figure 2C and Figure 3C, the sub-G1 peak, which appeared before the G1 phase that represents apoptotic cell population, was slightly increased in these two cell lines treated with Doxorubicin or Melatonin alone. The
apoptotic peak was dramatically increased when the cells were exposed to combined Melatonin and Doxorubicin.

The standard positive Bcl-2 and Bax expressions were stained brown or yellow mainly in cytoplasm or membrane. After treatment for 48 h, the expression of Bcl-2 decreased (Figure 4A and Figure 5A) and in contrast, there was a significant increase of Bax expression, especially in the combination groups (Figure 4B and Figure 5B). The results were quantitatively analyzed by biological image analysis system and the ratio of Bcl-2/Bax decreased correspondingly (Figure 4C and Figure 5C).

To determine whether caspase-3 plays a role in Melatonin and/or Doxorubicin mediated apoptosis of HepG2 and Bel-7402 cells, we assessed the activated caspase-3 protein level of the two cell lines before and after treatment with Melatonin and/or Doxorubicin using S-P method. The standard positive caspase-3 expressions were stained brown or yellow mainly in cytoplasm or nucleus. As shown in Figure 6, the expression of activated caspase-3 increased especially in the combination group after the two cell lines were exposed to drugs for 48 h.

**DISCUSSION**

Our results in the present study indicate that Melatonin inhibits growth of HepG2 and Bel-7402 cells in a dose-dependent manner. Meanwhile, combinations of Melatonin and Doxorubicin results in enhanced growth inhibitory effect and induction of cell apoptosis when compared with Melatonin or Doxorubicin used alone. These results suggest that chemotherapy combined with Melatonin may increase the therapeutic effect of anti-
cancer drugs. Our results demonstrated that Melatonin had a synergistic effect with Doxorubicin in inhibiting the proliferation of non-small cell lung cancer (A-549)\(^\text{[15]}\).

Moreover, combining with the study of Martin-Renedo et al\(^\text{[16]}\), our observation extend the possibility that Melatonin may provide a potential approach for the development of agents for the treatment and prevention of HCC.

Although the exact mechanism of the cytotoxicity of Melatonin against tumor cells is not very clear, many potential mechanisms have been proposed for the growth inhibition by Melatonin on cultured cells and animal models. These mechanisms include the induction of apoptosis\(^\text{[17]}\), the direct augmentation of natural killer (NK) cell activity\(^\text{[17]}\), which increases immunosurveillance, as well as the stimulation of cytokine production, e.g. interleukin IL-2, IL-6, IL-12 and interferon IFN\(^\gamma\)^\text{[18]}\). Apoptosis or programmed cell death is an essential physiological process that plays a critical role in development and tissue homeostasis. However, apoptosis is also involved in a wide range of pathological conditions\(^\text{[19]}\). Apoptotic defects are a common event in oncogenesis and contribute to drug resistance\(^\text{[20,21]}\). It is therefore interesting to search for ways to facilitate this apoptotic process after the use of chemotherapeutic drugs. Many studies have demonstrated that Melatonin could induce apoptosis in vitro and in vivo. To investigate the apoptosis-inducing effect of Melatonin as a single agent and combined Melatonin and Doxorubicin in hepatoma cells, the morphological changes and apoptotic rate were detected. The cells treated with the drugs showed the typical characteristics of apoptosis, which were more prominent in the combination group. Similarly, an apoptotic peak appeared before G1 phase when treated

Figure 5 Effect of Melatonin and/or Doxorubicin on the expression of Bcl-2 (A) and Bax (B) in Bel-7402 cells, the quantitative analysis (C) of Bcl-2 and Bax.

Quantitative analysis of Bcl-2 and Bax, expression by Biological Image Analysis System. Data are presented as mean ± SD (error bar). a: Untreated cells; b: 10\(^{-5}\) mol/L Mel; c: 2.5 mg/L Dox; d: 10\(^{-5}\) mol/L Mel plus 2.5 mg/L Dox. S-P (× 400). \(P < 0.01\) vs control of Bcl-2, \(P < 0.01\) vs control of Bax.
with Melatonin or Doxorubicin alone, and a significant synergistic effect on the induction of apoptosis was observed in the combination group.

Several factors contribute to apoptosis, but the key elements are categorized into two main families of proteins including caspase enzymes and Bcl-2 family. Bcl-2 family is a set of cytoplasmic protein members that regulate apoptosis. The two main groups of this family, Bcl-2 and Bax proteins, are functionally opposed: Bcl-2 acts to inhibit apoptosis, whereas Bax counteracts this effect. Caspases are crucial mediators of programmed cell death (apoptosis). Among them, caspase-3 is a frequently activated death protease, catalyzing the specific cleavage of many key cellular proteins. Thus, we studied bcl-2 and bax and caspase-3 protein in hepatoma cell lines. The current findings show that Melatonin and/or Doxorubicin increased the expression of Bax and caspase-3, decreased the expression of Bcl-2. Furthermore, a significant decrease in the ratio of Bcl-2/Bax was observed when Melatonin was treated in combination with Doxorubicin, which was correlated with the incidence of apoptosis.

In conclusion, these results indicate that Melatonin in combination with Doxorubicin has significantly synergistic growth-inhibitory and apoptosis-including effects on the human hepatoma cell lines HepG2 and Bel-7402, which may be related to down-regulation of Bcl-2, up-regulation of Bax, and activation of caspase-3. Melatonin is expected to be an adjuvant drug in HCC treatment in the future.

Figure 6 Effect of Melatonin and/or Doxorubicin on the expression of Caspase-3 in HepG2 (A) and Bel-7402 cells (B), and the quantitative analysis (C) of Caspase-3 expression. A: (a) Untreated cells; (b) 10^{-5} mol/L Mel; (c) 2.5 mg/L Dox; (d) 10^{-5} mol/L Mel plus 2.5 mg/L Dox. B: (a) Untreated cells; (b) 10^{-5} mol/L Mel; (c) 2.5 mg/L Dox; (d) 10^{-5} mol/L Mel plus 2.5 mg/L Dox. S-P (× 400); C: Quantitative analysis of Caspase-3 expression by Biological Image Analysis System. Data are presented as mean ± SD (error bar). ^P < 0.01 vs control of Caspase-3, ^P < 0.01 vs Dox alone.
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