Involvement of melatonin in autophagy-mediated mouse hepatoma H22 cell survival

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The role of autophagy in cancer is controversial. Melatonin has been linked to several aspects of cancer progression and also to regulation of autophagy. Whether melatonin is involved in an autophagy-induced tumor suppressor mechanism or a cyto-protective mechanism is unknown. Therefore, we investigated the effects of melatonin on autophagy and its upstream regulator. We found that melatonin triggers an autophagic process by enhancing Beclin 1 expression and inducing a conversion of microtubule-associated protein 1 light chain 3 (LC3)-I to LC3-II, the protein associated with the autophagosome membrane, in hepatoma H22 tumor-bearing mice. Moreover, melatonin inhibits the phosphorylation of the mammalian target of the rapamycin (mTOR) and Akt. Knockdown of Beclin 1 by either RNA interference or co-treatment with the autophagy inhibitor, 3-methyladenine (3-MA), significantly enhanced the melatonin-induced apoptosis in mouse hepatoma H22 cells. Our data provides the first evidence that melatonin induces protective autophagy that prevents mouse hepatoma H22 cells from undergoing apoptosis. A combination of melatonin with an autophagy inhibitor might be a useful therapeutic strategy for hepatocellular carcinoma.

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1. Introduction

Autophagy is generally viewed as a cell survival response to starvation or a toxic environment. On the other hand, autophagy can be a non-apoptotic route of type II programmed cell death [1]. The role of autophagy in cancers is controversial. The induction of autophagy may be a cyto-protective mechanism in response to cytotoxic agents or the nutrient-poor tumor micro-environment. However, autophagy may also function as a tumor suppressor mechanism and induce cell death when apoptosis is blocked [2,3]. There are two different pathways linking autophagy and cancer. Dysregulation of Class I phosphatidylinositol 3-kinase (PI3K) phosphatidylinositol Pi(4)P and Pi(4,5)P2, which in turn inactivates Akt/PKB, has been implicated in tumorigenesis and resistance to therapy in breast, prostate, pancreatic, ovarian, and stomach cancers [4]. The binding of Beclin-1 (Atg6 or BECN1) to Class III PI3Ks regulates autophagosome formation and also plays a role in tumorigenesis [5]. Despite current controversies on the possible role of autophagy in the process of cancer progression, further investigations on pharmacologicals which target the autophagic pathway may give new insight into cancer therapy.

Melatonin (MLT) is a natural compound that is produced in the pineal gland and has been known to have physiological antitumor activity. Melatonin acts as an anticancer agent through several biological mechanisms: antiproliferative action, stimulation of anticancer immunity, modulation of oncogene expression, and anti-inflammatory, antioxidant and anti-angiogenic effects [6]. Furthermore, melatonin has been shown to protect normal cells from a variety of insults [7,8], including toxin exposure [9,10]. Melatonin has few relevant side effects even when high concentrations are given to animals or humans [11,12]. On the contrary, many studies have described melatonin’s ability to reduce the side effects of chemotherapeutic drugs [13]. Melatonin has also been shown to induce apoptosis in the SK-N-MC cancer cell line [14]. Furthermore, melatonin acts to prevent many of the H2O2-induced alterations in the MAPK and mTOR signaling pathways in H4IE hepatoma cells [15]. Melatonin down-regulates Akt leading to TGFβ-1-dependent growth inhibition of breast cancer cells [16,17]. Yet the exact roles of melatonin on autophagic pathway may give new insight into cancer progression.

Hepatocellular carcinoma is one of the most common malignant tumors in the world. Although advances have been made in its detection and treatment, prognoses have not improved yet because of the aggressive invasion of such cancers, resistance to existing chemotherapeutic agents, and lack of specific symptoms [18]. The elucidation of its mechanism should be helpful in developing more effective treatments for hepatoma.

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Here, we investigate whether melatonin could induce autophagy in mouse hepatoma in vivo. We report that melatonin induces autophagy in H22 tumor-bearing mice. Induction of autophagy was associated with inhibition of the PI3K/Akt/mTOR signaling pathway. Furthermore, the disruption of the autophagic process enhances the biological effects of melatonin in H22 cells in vitro.

**Fig. 1.** Tumor suppression in mice. In each H-22 tumor-bearing mice model, mice were divided into 3 groups (n = 10 for each group) and intraperitoneally given with MLT per day for 14 days. Data are mean ± SD. **P < 0.01 compared with normal saline control group.

**Fig. 2.** MLT induces autophagy in tumor cells come from H22-bearing mice. Autophagic vacuoles were observed using a transmission electron microscope. Tumor cells come from mice treated with (a) normal saline, (b) MLT (10 mg/kg), (c) MLT (20 mg/kg); arrows indicate multiple autolysosome-like vesicles in the cytoplasm. The formation of autophagic vacuoles was indicated by several autophagic profiles. Stage 1: An early stage of autophagic vacuoles formation, identified as membranes from the rough endoplasmic reticulum containing cytosolic components. This process will lead to the formation of the autophagosomes, which characterized by double membrane vesicles. Stage 2: Autophagophosomes [7], adjacent to lysosomes [8], contain some vacuoles bounded by a small inner vesicle enclosing residues of the lytic organelles. Scale bar: 0.2 μm for first panels. (d) Tumor cells from melatonin and saline-treated mice were imaged at random and the total number of autophagic vacuoles per cell profile was determined for each condition. Results shown in d are the mean ± SD of six profiles for each condition.
2. Materials and methods

2.1. Reagents

Melatonin from Sigma-Aldrich (St. Louis, MO, USA) was dissolved in the sterile normal saline to yield a dose of 10 or 20 mg/kg b.w. during the treatment or was dissolved in serum-free RPMI 1640 (Life Technologies, Bedford, MA) for cell culture and treatment.

2.2. Cell culture and tumor model establishment

Mouse hepatoma cell line H22 were kindly donated by School of Medicine, Tsinghua University. These cells were grown in RPMI 1640 (Life Technologies, Bedford, MA) containing 10% heat-inactivated FCS, 100 units/mL penicillin, and 100 units/mL streptomycin in a humid chamber at 37 °C under 5% CO₂.

The H22 tumor models were established in 8-week-old female BALB/c mice as described previously[19]. Briefly, mice were subcutaneously injected 0.1 mL of cell suspensions containing 1.0×10⁷ cells/mL in the dorsal area. All these mice were purchased from Department of Animal, Health Science Center of Peking University. All experimental protocols described in this study were approved by the Ethics Review Committee for Animal Experimentation of Xiyuan Hospital, China Academy of Chinese Medical Sciences.

2.3. 3-methyl-adenine (3-MA) inhibition

H22 cells were seeded at 1×10⁵ cells/well in 6-well plates, incubated overnight and then exposed to the indicated concentrations of MLT or 3-MA (Sigma-Aldrich Chemical) for 24 h, and then analyzed for Cell viability assay or analysis of apoptosis.

2.4. Lentiviral Vectors and shRNA transduction

The shRNA was designed with the published guidelines [20]. Lentiviral vectors pGCL-LTR-RRE-U6 shRNA-CMV Luciferase-WRE-LTR (LV-shBeclin1) were generated as previously described [21]. One unrelated sequence TTCTCGAAGCTGTACGT that shows no significant homology to any mice known gene (analyzed using BLAST search) was used as a control (LV-control). Cells were transduced with LV-shBeclin1 or LV-control at a multiplicity of 10⁴ viral particles/cell. Two days after transfection, a fraction of the cells was subjected to Western blotting for Beclin-1. The remaining cells were plated in 96-well flat bottom plates at a density of 1×10⁴ cells/mL. After 24 h of incubation, the
cells were incubated in RPMI supplemented with 0.1% FBS for 12 h and were treated with growth media containing or not containing MLT for 24 h, and then analyzed for Cell viability assay or apoptosis assay.

2.5. Cell viability assay

H22 cells following which 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma-Aldrich Chemical) solution (0.5 mg/mL) were added for 3 h at 37 °C in a humidified incubator with 5% CO2. The supernatant was removed, 150 μl DMSO was added and shaken for 5 min at room temperature. The absorbance at 570 nm was measured with Universal Microplate Spectrophotometer (BioTek mQuantTM), using wells without cells as blanks. The following formula was used: cell viability = (OD of the experimental sample/OD of the control group) × 100%.

2.6. Analysis of apoptosis

Cells were collected and double stained with FITC-conjugated Annexin-V (25 μg/mL) and propidium iodide (PI) (50 μg/mL) (Beckman Coulter) for 20 min in the dark. After that, cells were collected on a Epic XL flow cytometer equipped with a 488-nm argon laser and analyzed using the Expo32 (Beckman Coulter).

2.7. In vivo antitumor activity

After inoculation with H22 cells on day 0, mice were randomly assigned into three groups (n = 10 for each group). Tumor-bearing mice were given an intraperitoneal injection with MLT (10, 20 mg/kg per day for 14 days) or sterile normal saline (0.2 mL per day for 14 days). Tumor size was determined by caliper measurement of the largest and perpendicular diameters every 3 days. Tumor volumes were calculated according to the formula: V = a × b × 0.52, where a is the largest superficial diameter and b is the smallest superficial diameter. On day 15, mice were sacrificed; tumors were dissected and weighed. To detect the Beclin 1 and LC3 expression, tumors tissues excised were fixed in 10% formalin or frozen in −80 °C.

2.8. Transmission electron microscopy

Transmission Electron Microscopy (TEM) was used to evaluate the ultrastructural change. Tumor fragments were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 2 h at 4 °C and post-fixed with 1% OsO4 in 0.1 M cacodylate buffer for 1 h at 4 °C. After dehydration in an ethanol series, they were embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate and examined under a transmission electron microscope (JEM-2100, Japan Electron Optics Laboratory Co., Ltd., Tokyo, Japan).

Fig. 4. Decreased phosphorylation of Akt and mTOR proteins in MLT treated H22-bearing mice. An antibody for β-actin was used to show equal protein loading. (a) Western blot analysis of the phosphorylated forms of Akt (p-Akt-ser473); total Akt; phosphorylated mTOR(p-mTOR-ser2448) or total mTOR. (b) Bar graphs show quantitative evaluation. Data are mean ± SD. Nonparametric test was used for statistical analysis. *P < 0.05 compared with normal saline group. Lane 1: normal saline; lane 2: MLT (10 mg/kg); lane 3: MLT (20 mg/kg).

Fig. 5. (a) Prior to 100 μM of MLT treatment for 24 h, H22 cells were transfected with LV-Beclin 1 or LV-control for 48 h and the expression of Beclin 1 was verified by western blotting. After that cell viability was measured by using MTT assay. Values are means ± SD (**P < 0.01 vs. cells transfected with LV-control). (b) H22 cells were exposed to either 100 μM of MLT or 10 mmol/L 3-MA, or a combined treatment of MLT and 3-MA for 24 h, and cell viability was measured by MTT assay. Values are means ± SD (*P < 0.05, **P < 0.01 vs. untreated cells). (c–d) Apoptosis was analyzed by flow cytometry following PI/Annexin V FITC double staining. The percentages of apoptotic cells (early apoptotic cells: Annexin V FITC+/PI−; late apoptotic/necrotic cells: Annexin V FITC+/PI+) are indicated (values were averaged from three independent experiments. *P < 0.05 vs. cells transfected with LV-control; **P < 0.05 vs. cells treated with MLT + LV-control in c; *P < 0.05 vs. untreated cells, **P < 0.05 vs. cells treated with MLT in d).
Fig. 5 (continued).
2.9. Immunochemical staining

Paraffinized sections were stained for immunofluorescence using the antibodies including rabbit polyclonal anti-autophagy APG8a (MAP1LC3A) antibody (Abgent, San Diego, CA, USA) and anti-rabbit IgG-fluorescein isothiocyanate (FITC) (1:100; Sigma-Aldrich Chemical), and apoptotic morphological changes in the nuclear chromatin were detected by DNA-binding fluorochrome Hoechst 33258 staining. Confocal images were taken under Zeiss LSM-510 microscope.

2.10. Western blotting detection in tumor tissue

Protein from tumor tissues was collected and diluted into 0.5 mg protein/mL for the measurement of Beclin 1 and the phosphorylation of Akt and mTOR. Protein content was determined with BSA as a standard according to Bradford assay. Proteins samples (20 ng/μl) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and were transferred to PVDF membranes (Millipore, Billerica, MA) through electroblotting. Blots were stained with phosphorylated-Akt (Ser473), total Akt, phosphorylated mammalian target of rapamycin (mTOR, Ser2448), total mTOR (Cell Signaling Technology, Beverly, MA) and rabbit polyclonal anti-Beclin 1 antibody (Cell Signaling, Boston, MA, USA). The blots were then developed by enhanced chemiluminescence using SuperSignal west femto maximum sensitivity substrate (Pierce, Rockford, IL). Bio-Rad Image Lab™ Version 3.0 software was used to calculate the numerical value of every blot, and mean densitometric × areal values were depicted as bar graphs.

2.11. Statistical analysis

Data were expressed as mean ± SD. The analysis of variance (ANOVA), regression–linear test and nonparametric test were used in the program SigmaStat (SPSS Software Products, Chicago, IL, USA) to determine significant differences between groups. P values less than 0.05 were considered significant. Western blots were repeated at least three times for each sample and subjected to semiquantitative analysis to ensure maximal accuracy of the conclusion drawn from these data.

3. Results

3.1. Antitumor effect of MLT in vivo

The antitumor efficacy of MLT was directly observed in tumors excised from H22-bearing mice. Tumor volume was more suppressed in MLT-treated groups than saline control. It was clear that MLT-treated mice showed significant inhibition to the tumor growth (as shown in Fig. 1).

3.2. MLT induced autophagy in H22-bearing mice

The formation of autophagic vacuoles was further assessed and confirmed by transmission electron microscopic test. In contrast to saline-treated H22-bearing mice, MLT-treated animals showed increased autophagous morphology, such as autophagic vacuoles in the cytoplasm (Fig. 2). These results indicate that MLT induced autophagy formation.

3.3. Effects of MLT on autophagy-associated proteins

Beclin-1 is a member of the lipase signaling complex which is essential in the induction of autophagy [22]. An elevated level of Beclin-1 can be detected in tumor tissue from H22-bearing mice treated with MLT. In contrast, only a low level of Beclin-1 can be detected in vehicle-treated H22-bearing mice. Atg5 and LC3 are constituents of the conjugation system and play a role in the extension of the autophagosomal membrane [23]. The levels of LC3-II were significantly enhanced in H22-bearing mice treated with MLT (Fig. 3a). LC3 immuno-fluorescence could result in two staining patterns: diffuse cytoplasmic staining (basal) and punctate staining (AV-related) [24] (Fig. 3d). The redistribution of LC3 from diffuse cytoplasmic staining to punctate staining is a reliable marker of autophagosome formation. As shown in Fig. 3d, only a few LC3-positive puncta were observed in tumor tissue from saline-treated H22-bearing mice. However, in the tumor tissue from MLT-treated H22-bearing mice, LC3 punctuated staining was increased.

3.4. Reduced Akt/mTOR signaling in H22-bearing mice treated with MLT

It has been shown that the inhibition of the PI3K/Akt/mTor pathway induces autophagy in human malignant glioma cells [26]. Therefore, we investigated the effects of MLT treatment on this signaling pathway in H22-bearing mice by analyzing the activation status of Akt and mTOR signaling proteins. In the present study, decreased phosphorylation of Akt and mTOR could be detected in H22-bearing mice treated with MLT (Fig. 4). These results suggest that modulation of PI3K/Akt/mTOR signaling may be at least one of the pathways by which MLT exerts its effects on H22-bearing mice.

3.5. Disruption of MLT-induced autophagy enhances the biological effects of MLT

To identify whether the role of MLT-induced autophagy in hepatoma cells is a protective or death promoting mechanism, we evaluated the consequences of the disruption of autophagy by treatment with Beclin-1 RNAi or 3-MA (an inhibitor of autophagy) on the antitumor effects of MLT. To inhibit autophagy, cells were pretreated with Beclin-1 RNAi (LV-control, or LV-shbeclin-1) prior to administration of MLT (100 μM) or co-treated with MLT (100 μM) and 3-MA (10 mM/L). Fig. 5a shows that the level of Beclin 1 was significantly decreased by Beclin 1 RNAi. Compared with LV-control, Beclin 1 RNAi decreased MLT-inhibited cell viability and enhanced MLT-induced apoptosis (Fig. 5a and c). Further studies showed that co-treatment with MLT (100 μM) and 3-MA (10 mM/L) significantly decreased cell viability, compared with treatment with MLT alone (Fig. 5b). Co-treatment with MLT and 3-MA also significantly increased the apoptotic population, especially late apoptotic cells, compared with the cells treated with MLT alone (Fig. 5b-d).

4. Discussion

Here, we demonstrated that melatonin triggers an autophagic process in H22 tumor-bearing mice by reducing mTOR and Akt. Furthermore, we showed that disruption of the autophagic process by Beclin 1 RNAi or 3-MA enhanced the melatonin-inducing apoptosis in H22 cells in vitro.

Autophagy has been shown to be an important player in many critical biological processes such as cellular response to starvation, cell survival and death, and cancer [27]. It has been demonstrated that autophagy is activated by some anti-tumor drugs while they induce apoptosis [28,29]. Melatonin was first discovered as a regulator of circadian rhythms, a characteristic that may contribute to its antitumoral effects [30–33]. There are also several publications reporting
the proapoptotic effect of melatonin in cancer cell lines [34]. It was reported that melatonin administration prevented rigidity in the mitochondrial membrane and seemed to decrease age-related autophagy-lysosomal alterations [35]. Melatonin has also been reported to induce apoptosis [14] and down-regulate mTOR/Akt pathways in cancer cell lines [15–17]. Meanwhile, in our study a robust autophagy was observed in tumor tissue from H22 tumor-bearing mice treated with melatonin. Western blotting also showed a conversion of LC3-I to LC3-II. This is the first demonstration that melatonin could promote autophagy, increase expression of autophagy-associated proteins, and induce autophagy-associated morphologic changes in a tumor-bearing mouse model.

There are several molecular pathways of autophagy that play important roles in cancer, including the Class I and 3 PI3K pathways. In animal studies, inhibition of Class I PI3Ks/Akt or its downstream molecules mTOR is effective in decreasing tumor growth and angiogenesis [29,36]. PI3K/Akt signaling is also demonstrated to be involved in tumor growth and angiogenesis through reactive oxygen species [37,38]. In contrast to Class I PI3Ks, Class III PI3Ks stimulate autophagy [39]. An integral protein in the Class III PI3K pathway is Beclin-1, whose knockdown inhibits autophagy and sensitizes to starvation-induced cell death [40,41]. Studies have shown Beclin-1 depletion in 40–75% of breast, ovarian, and prostate cancers. In addition, forced expression of Beclin-1 in MCF-7 breast cancer cells induced autophagy, decreased proliferation, and inhibited tumorigenesis in nude mice [42]. Our findings show that melatonin could inhibit the phosphorylation of Akt and mTOR and also enhance Beclin 1 expression. These results suggest that melatonin may promote autophagy by modulation of PI3K/Akt/mTOR signaling pathway in H22 tumor-bearing mouse model.

Although autophagy is generally viewed as a cell survival mechanism, in some cases it can also be a backup cell death mechanism in the process of cancer progression, when other cell death mechanisms fail [43]. The role of autophagy in cancers is controversial. In this regard, the induction of autophagy may be a cytoprotective mechanism in response to the nutrient-poor tumor micro-environment. On the other hand, autophagy may act as a tumor suppressor mechanism and induce cell death when apoptosis is blocked [2,25,44]. We then investigated whether the autophagy induced by melatonin was a protective response or a process leading to death. We showed that 3-MA, which blocks autophagic response by the inhibition of Class III PI3K [39], reduced the melatonin-inhibited cell viability in a cultured H22 cell line, and also promoted melatonin-inhibited H22 cell apoptosis. Inhibition of autophagy by genetic knockdown of Beclin 1 also decreased melatonin-inhibited cell viability and enhanced melatonin-induced apoptosis. These data suggest that melatonin may activate a protective autophagic reaction by Class III PI3K/Beclin-1 pathway to adapt to the stressful conditions and protect H22 cells from death. Inhibition of protective autophagy may enhance the anti-tumor effect of melatonin. In addition, therapeutic drugs combined with autophagy inhibitors may have synergistic effects to inhibit tumor development. This elucidates the link between apoptosis and melatonin-induced autophagy, which could lead to the development of therapeutics strategies of cancer.

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