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Melatonin sensitizes human malignant glioma cells against TRAIL-induced cell death

Vanesa Martín [a,b,*], Guillermo García-Santos [a,b], Jezabel Rodriguez-Blanco [a], Sara Casado-Zapico [a,b], Ana Sanchez-Sanchez [a], Isaac Antolín [a], Maria Medina [b,c,d], Carmen Rodriguez [a,b]

[a] Departamento de Morfología y Biología Celular, Spain
[b] Instituto Universitario de Oncología del Principado de Asturias (IUOPA), Spain
[c] Departamento de Cirugia, Area de Fisioterapia, Facultad de Medicina de la Universidad de Oviedo, Spain
[d] Servicio de Rehabilitacion del Hospital de Jove, Gijon Principado de Asturias, Spain

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ABSTRACT

Despite the common expression of death receptors, many types of cancer including gliomas are resistant to the death receptor ligand (TRAIL). Melatonin antitumoral actions have been extensively described, including oncostatic properties on several tumor types and improvement of chemotherapeutic regimens. Here, we found that melatonin effectively increase cell sensitivity to TRAIL-induced cell apoptosis in A172 and U87 human glioma cells. The effect seems to be related to a modulation of PKC activity which in turns decreases Akt activation leading to an increase in death receptor 5 (DR5) levels and a decrease in the antiapoptotic proteins survivin and bcl-2 levels.

1. Introduction

Glioblastoma, the most common primary brain tumor, is a highly aggressive malignancy considered to be one of the most lethal cancers [1]. Despite aggressive approaches, treatment of glioblastoma patients represents an ongoing challenge, as complete surgical resection of the tumor is difficult and glioblastoma are refractory to most current chemotherapy regimens. Thus, after the use of conventional treatments, including surgery and radiotherapy, which may be followed by chemotherapy with temozolomide, the average life expectancy of glioma patients remains less than 1 year pointing out the necessity of novel therapeutic strategies for malignant gliomas treatment.

Apoptosis-based therapies gained interest as promising experimental treatment strategies. Thus, the death ligand TRAIL/Apo2L might be a useful tool to trigger apoptosis in cancer since TRAIL kills tumor cells of diverse cellular origin without severe toxic side effects [2]. TRAIL kills cells by binding one of the two cancer cell surface receptors, DR4 or DR5 [3]. Once activated, these transmembrane receptors trimerize and assemble a death-inducing signaling complex (DISC) constituted by their death domains, the Fas-associated death domain (FADD) adaptor protein, and the inactive proenzymatic form of the apoptosis-initiating proteases caspase-8 or -10. These caspases self-activate by proteolysis and in turn activate the effector caspase-3, either by direct processing (type I cells), or through cleavage of the proapoptotic bcl-2 family member Bid, and engagement of mitochondrial apoptotic pathway, which

* Corresponding author. Address: Departamento de Morfología y Biología Celular, Facultad de Medicina, c/ Julian Claveria, 33006 Oviedo, Spain.
E-mail address: martinvanesa@uniovi.es (V. Martín).

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involves release of cytochrome c, formation of the apoptosome and activation of caspase-9 (type II cells). However, several studies have showed that, despite the common expression of death receptors, many types of cancer including gliomas are resistant to TRAIL due to the blockage of apoptotic signaling cascades. Thus, the use of sensitizers capable of overcome TRAIL resistance is needed in order to obtain more effective TRAIL-based cancer therapies.

Melatonin is a neurohormone mainly synthesized in pineal gland, although enzymes for its synthesis are widespread. It has been described to regulate circadian rhythms and seasonal reproduction at nanomolar concentration due to its binding to melatonin membrane receptors [4,5]. Reports on new properties of melatonin have been made through the last two decades, being its antioxidant abilities one of the most interesting [6]. *In vivo* long-term experiments in rodents have proved the lack of any noteworthy side effect and also showed some protection against several degenerative events occurring with aging, including the incidence of malignant tumors [7,8]. Melatonin inhibition of cell growth and invasive and metastasic properties of tumoral cell cultures, mainly endocrine tumors, have been described at nanomolar concentrations related to its membrane receptors activation [9,10]. During the last 3 years, also a reduction of tumor cell proliferation and differentiation has been showed using millimolar melatonin concentrations related to its antioxidant properties [11,12]. In this way, our group has described that melatonin induces an important reduction in rat glioma cells proliferation. The effect seems to be related to the antioxidant properties of the neurohormone, decreasing intracellular peroxides and inhibiting some intracellular effectors like Akt and the transcription factor NFkB. The effect also seems to be related to an inhibition in PKC activity [12].

In the present work, we show for the first time that melatonin effectively sensitize glioma cells to TRAIL-induced apoptotic cell death by modulating multiple components in the death receptor pathway, including DR5, bcl-2 and survivin.

2. Materials and methods

2.1. Cell culture and reagents

Human glioma cell lines, A172 and U87 MG (from the American Type Culture Collection, Manassas, VA) were maintained in DMEM/F-12 (1:1, vol/vol) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic–antimycotic mixture containing 10,000 U penicillin, 10 mg streptomycin and 25 μg amphotericin. Cells were maintained at 37 °C in a 5% CO_2 atmosphere. Cell culture reagents were from Sigma (Sigma Chemical Co., St. Louis, MO, USA), except for FBS, which was purchased from GIBCO (Invitrogen Life Technologies, Spain). Culture flasks and dishes were acquired from Falcon (Becton Dickinson Bio-Science, Le Pont de Claix, France). Melatonin was from Aldrich (Sigma Aldrich, Milwaukee, WI, USA) and TRAIL was purchased from R&D Systems. All other reagents were purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA), unless otherwise indicated.

2.2. Cell viability assays

2.2.1. MTT assay

3-(4,5-dimethyl-2-thiazolyl) 2,5-diphenyl-2H-tetrazolium bromide (MTT) is converted to formazan in living cells. To carry out the MTT assay, the method described by Denizot was followed [13]. Basically, cells were plated on 96-well dish at a density of 10^4 cells/well in a final volume of 100 μl of complete medium. Once the treatments were completed, 10 μl of a MTT solution in PBS (5 mg/mL) were added. After 4 h of incubation at 37 °C, one volume of the lysis solution [sodium dodecyl sulphate (SDS) 20% and dimetylformamide pH 4.7, 50%] was added. The mixture was incubated at 37 °C overnight and the samples were measured in an automatic microplate reader (μQuant, Bio-Tek Instruments, Inc., Winooski, VT, USA) at the wavelength of 540 nm.

2.2.2. Annexin-V binding

Cells were seeded in 6 well plates at a density of 2×10^5 cells/well. Once the treatments were completed, apoptosis was evaluated by the Annexin V-FICT Apoptosis Detection Kit (Sigma Chemical Co., St. Louis, MO, USA) according to the manufacturer's protocol. Briefly, cells were harvested by tripsinization and incubated in an annexin V solution (5 μg/ml)) for 15 min at room temperature. Ten microliters of propidium iodide solution (50 μg/ml) were added in order to evaluate cell viability. Apoptosis levels (annexin V positive cells) was determined in 10,000 cells per group using a Beckman Coulter FC500 flow cytometer (Beckton Dickinson).

2.3. Reverse transcriptase PCR

Total cellular RNA was extracted using RNeasy Mini Kit (Qiagen; Valencia, CA). One microgram was subjected to reverse transcription using SuperScript™ First-strand Synthesis System for RT-PCR (Invitrogen Corporation; Carlsbad, CA) according to the manufacturer's protocol. PCR was done for 40 cycles (94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min). The sequences of the sense and antisense primers for human DR4, DR5 and GADPH (as loading control) are the following: DR4, 5′-CGATGTGGTCAGAGCTGGTACAGC-3′ and 5′-GGACACGGCAGAGCCTGTGCCATC-3′; DR5, 5′-GGGAGCCGCTCATGAGGAAGTTGG-3′ and 5′-GG CAAGTCTCTCTCCCAGCGTCTC-3′ and GADPH, 5′-ATGGGG AAGGTGAAGGTCGG-3′ and 5′-GACGGTGCCATGGAATTT GC-3′.

2.4. Real time quantitative PCR (Q-RT-PCR)

Quantitative analysis of DR5 mRNA levels was performed by the SYBR Green real time PCR method using Green PCR Core Reagents (ABI systems; Foster City, CA) in an AB7700 Real-time system (ABI systems; Foster City, CA). An increase in the fluorescence of the reporter dye, SYBR green, during Q-PCR is due to the SYBR green binding to double-stranded DNA. The sequences of the sense and antisense primers used were same as described before. Each amplification cycle consisted of denaturation for 15 s at 95 °C and annealing/extension for 1 min at 60 °C.