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Evidence for Biphasic Effects and Differential Expression of Melatonin (MLT) Receptors in Oral Squamous Cell Carcinomas.

Farnoush M[1], Swint D[1], Kingsley K[1*]

[1] Department of Biomedical Sciences,School of Dental Medicine, University of Nevada, USA

Corresponding author: Karl Kingsley, Associate Professor of Biomedical Sciences, University of Nevada, Las Vegas - School of Dental Medicine, 1001 Shadow Lane, B313, USA, Tel: (702) 774-2623; Fax: (702) 774-2721; E-mail: Karl.Kingsley@unlv.edu

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Abstract

Objectives: Previous work from this group has demonstrated that melatonin (MLT) is capable of significantly altering the growth and viability of oral cancer cell lines. The objective of this study was to explore the receptors that may modulate MLT effects in oral cancers.

Methods: Polymerase Chain Reaction (PCR) primers specific for the primary, cell-surface MLT receptors (MT1 & MT2) were obtained and used to analyze the mRNA expression in CAL27, SCC15 and SCC25 oral cancer cell lines. Extracted RNA following MLT administration was also analyzed.

Results: Expression of the primary MLT receptor MT1 was absent in all cell lines, although expression of the secondary receptor (MT2) was observed. MLT administration within the physiologic range was sufficient to induce MT1 expression in all cell lines, suggesting the MT2 receptor may initially facilitate this process. Saturation of both MT1 and MT2 receptors observed within the supraphysiologic ranges correlated with the induction of apoptosis-related caspase signaling and the activation of the intranuclear MLT receptor RZR.

Conclusions: This may be the first study to demonstrate the down-regulation of MT1 mRNA in oral cancers, as well as novel evidence of the specific role of melatonin and the cognate receptors in modulating oral cancer growth.

Keywords: Oral Cancer; Melatonin.

Introduction

Melatonin (MLT) is a hormone largely produced by the pineal gland in regular daily cycles in most mammals and humans, in particular [1]. Although melatonin may be intricately involved in the regulation of many diverse physiological processes, the most widely recognized and frequently studied is the role of MLT in the regulation of the sleep-wake cycle [2]. Although epidemiologic evidence has suggested that disruption of the sleep-wake cycle, frequently encountered with shift

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workers, may be linked with MLT dysregulation and increased risk for some cancers–more recent evidence has suggested that melatonin has more specific physiologic and pathologic functions specific to the oral cavity [3-5].

For example, recent studies have suggested melatonin and disruption of melatonin-specific pathways may be a critically important factor that increases risk of oral disorders, and more specifically for oral cancer [6]. In addition, new evidence has suggested that silencing and down-regulation of melatonin receptors may, in fact, be a common feature of many oral cancers [7-9]. Recent studies from this group have now demonstrated that administration of melatonin at physiologically-relevant concentrations can inhibit the growth of oral cancers in vitro, although these studies did not evaluate any effects on melatonin receptors, such as silencing or down-regulation [10].

On the basis of this information, the primary objective of the current study involved the examination of the expression of the cell-surface, membrane-bound melatonin receptors MT1 and MT2, as well as the intracellular receptor RZR in oral cancer cell lines. In addition, the experimental administration of melatonin at physiologic and supraphysiologic (supplementation equivalent) concentrations were evaluated with the specific aim of analyzing changes to MT1, MT2 and RZR expression to correlate with the observed changes to cellular behavior.

Materials and Methods

Cell culture and cell lines

The human oral squamous cell carcinoma lines, SCC15 (CRL-1623), SCC25 (CRL-1628), and CAL27 (CRL-2095) were obtained from American Type Culture Collection (ATCC: Manassas, VA). CAL27 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 4.0 mM L-Glutamine adjusted to contain 3.7 g/L sodium bicarbonate, 4.5 g/L glucose, and 110 mg/L sodium pyruvate, obtained from HyClone (Logan, UT). SCC15 and SCC25 cells were maintained in a 1:1 mixture of DMEM and Ham's F12 medium obtained from HyClone (Logan UT) with 2.5 mM L-Glutamine, modified to contain 15 mM 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES), 0.5 mM sodium pyruvate, and 1.2 g/L sodium bicarbonate. All cell culture media was supplemented with 1% Penicillin (10,000 units/mL)-Streptomycin (10,000 ug/mL) solution and 10% fetal bovine serum (FBS) obtained from HyClone (Logan, UT). Cells were cultured in 75 cm2 BD Falcon tissue-culture treated flasks (Bedford, MA) at 37°C and 5% CO2 in humidified chambers.

Materials

Melatonin (N-acetyl-5-methoxy-tryptamine, MLT) was obtained from GNC Preventive Nutrition® (Pittsburgh, PA). Cellular assays were performed in the appropriate complete media, with and without the addition of MLT (10, 50 and 100 pg/mL). This concentration range approximates normal human physiologic serum concentrations (10.6 ± 1.7 – 94.8 ± 22.6 pg/mL) [11,12]. In addition, supraphysiologic concentrations of MLT were also utilized, to approximate serum and saliva concentrations of melatonin administered as an over-the-counter supplement (0.2 – 10 ug/mL) [11,12].

Proliferation

Proliferation assays were performed in the appropriate complete media, with and without the addition of MLT, prior to the start of each experimental assay. In brief, cells were plated in Corning Costar high-throughput, flat bottom, tissue culture-treated 96-well assay plates (Corning, NY) at a concentration of 1.2 x 105 cells per well, which roughly approximates 30-40% confluence per well at the onset of each assay. Proliferation was subsequently measured after three days. In brief, cultured cells were fixed after 72 hours, or day 3 (d3), using 50 uL of 10% buffered formalin for 24 hours at room temperature, and were subsequently processed as follows: liquid media (containing formalin) was aspirated in a chemical fume hood, cells were subsequently stained using 50 uL of crystal violet 1% aqueous solution (Fisher Scientific: Fair Lawn, NJ) for 20 minutes at room temperature. The stain was then aspirated and wells were then washed with 100 uL of 1X phosphate-buffered saline (PBS) solution for one (1) minute, followed by aspiration. The relative absorbance of each well was then measured at 630 nm using a Bio-Tek ELx808 microplate reader (Winooski, VT). The change in proliferation, measured as the change in absorbance between day 3 and day 1 (d3-d1) was calculated and compared with baseline controls (cells receiving media with no melatonin). The data were analyzed and graphed using Microsoft Excel (Redmond, WA) and SPSS (Chicago, IL). Three separate, independent replications of each experimental condition (physiologic, supraphysiologic) were performed on each cell line by the study authors in random order over the course of three consecutive weeks.

RT-PCR: Melatonin receptor expression

To determine whether the observed effects of melatonin on proliferation or viability were associated with changes to MT1, MT2 or RZR expression, RNA was isolated from 1.5 × 107 cells from all cell lines at 72 hours after growth inhibitory maximum (GIMAX) MLT administration from both the physiologic and supraphysiologic range (100 pg/mL and 10 mg/mL) using ABgene Total RNA Isolation Reagent (Epsom, Surrey,UK) and the procedure recommended by the manufacturer [10]. RT-PCR was performed with the ABgene Reverse-iT One-Step RT-PCR Kit (ReadyMix Version) and a Mastercycler gradient thermocycler (Eppendorf: Hamburg, Germany). The following primers for MT1, MT2 and RZR, were synthesized by SeqWright (Houston, TX) [13,14]:

MT1 forward primer, TGGCATATAGGTTGCAGTCTCGG;
MT1 reverse primer, TGTTCTGTAGGCTTGGGCAGTTG;
MT2 forward primer, ACATGGAAGCGAATCAATGGACTC;
MT2 reverse primer, AAGGACTCAAATTCTGTTGCCACC;
RZR forward primer, GATATTGGGGAACAACTGGAC;
RZR reverse primer, CATGTCATCATCCAGTTTGCA;

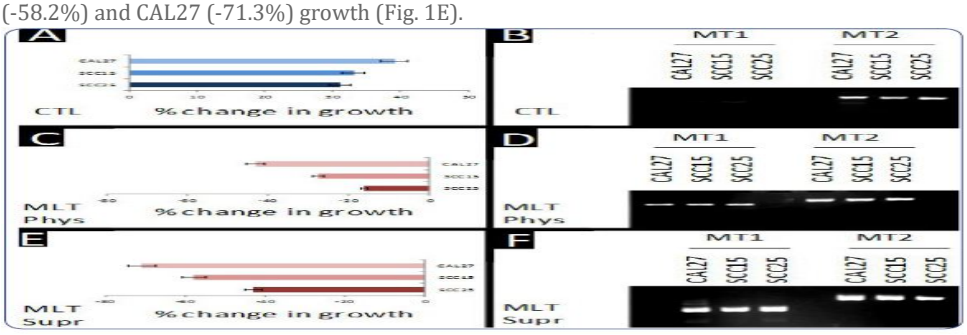
In brief, one µg of template (total) RNA was used for each reaction. The reverse transcription step ran for 30 minutes at 47°C, followed by denaturation for 2 minutes at 94°C. Thirty five amplification cycles were run, consisting of 20 second denaturation at 94°C, 30 seconds of annealing at 58°C, and 6.5 minutes of extension at 72°C. Final extension was run for 5 minutes at 72°C. Reaction products were separated by gel electrophoresis using Reliant 4% NuSieve R3:1 Plus Agarose gels (Lonza: Rockland, ME). Bands were visualized by UV illumination of ethidium-bromide-stained gels and captured using a Kodak Gel Logic 100 Imaging System and 1D Image Analysis Software (Eastman Kodak: Rochester, NY). Quantification of RT-PCR band densitometry was performed using Adobe (San Jose, CA) Photoshop imaging software, Image Analysis tools.

Results and Discussion

Growth of SCC25, SCC15 and CAL27 oral cancer cell lines, with and without the addition of MLT, was assessed for comparison with the expression of MT1, MT2 and RZR (Figure 1). More specifically, the control cells (no MLT) increased in number by approximately one third (31.5%, 33.2% and 39.1%, respectively) (Fig. 1A). An assessment by RT-PCR from extracted RNA revealed the absence of MT1 mRNA in all three cell lines, although mRNA specific for MT2 was observed (Fig. 1B).

The administration of MLT at the previously determined growth inhibitory maximum (GIMAX) of 100 pg/mL within the normal physiologic range (Phys) was sufficient to inhibit SCC25 growth by -16.1%, SCC15 y -36.5%, and CAL27 by 43.1% (Fig. 1C). Furthermore, the addition of MLT at this physiologic concentration was associated with a slight up-regulation of MT2 mRNA expression, as well as the introduction of MT1 mRNA expression (Fig. 1D).

Administration of MLT at concentrations that approximate the GIMAX for the supraphysiologic dose (Supr) observed among those taking oral supplements also inhibited SCC25 (-42.9%), SCC15 (-58.2%) and CAL27 (-71.3%) growth (Fig. 1E).



Farnoush et al. Figure 1. Growth of SCC25, SCC15 and CAL27 oral cancer cell lines, with and without the addition of MLT in both physiologic (Phys) and supraphysiologic (Supr) concentrations using cell surface, membrane-bound receptors MT1 and MT2.

A marked up-regulation in both MT1 and MT2 mRNA expression was also observed (Fig. 1F). This increase ranged between 15-fold (CAL27: MT1; SCC25: MT2) and 30-fold (SCC25: MT1; CAL27: MT2).

As MLT is an amphipathic hormone that possesses the ability to bind both cell surface as well as intracellular receptors, the expression of the intracellular receptor RZR was also assessed (Figure 2).

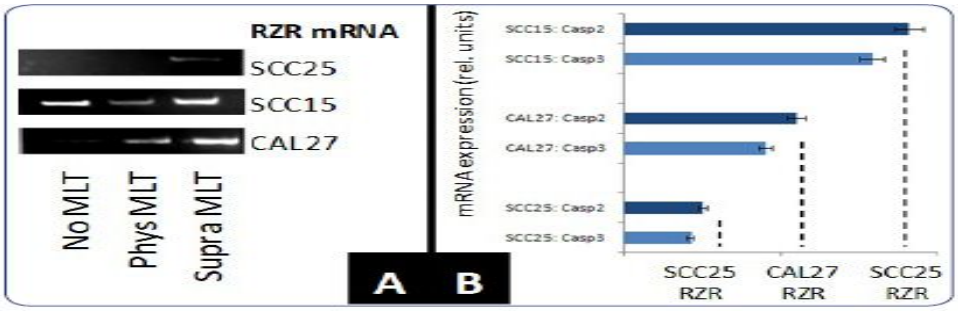


Figure 2. Expression of the intracellular receptor RZR using SCC25, SCC15 and CAL27 oral cancer cell lines, with and without the addition of MLT in both physiologic (Phys) and supraphysiologic (Supr) GIMAX concentrations.

These analyses revealed that RZR is not actively transcribed in either SCC25 or CAL27 cells under MLT-free conditions, although expression in SCC15 cells was noted (Fig. 2A). Administration of MLT at physiologically relevant concentrations induced expression of RZR in CAL27 cells but not SCC25 cells, while SCC15 expression was slightly down-regulated. However, supraphysiologic concentrations of MLT were the only concentration tested that induced RZR expression in SCC25 cells. Moreover, RZR expression in both SCC15 and CAL27 cells was substantially increased.

As previous studies from this group demonstrated that only supraphysiologic concentrations of MLT induced the expression of key apoptosis regulatory genes, Caspase-2 and Caspase-3, the relative expression of the intracellular RZR receptor, graphed on the X-axis, was plotted against the expression of these apoptosis-specific products on the Y-axis for comparison (Fig. 2B). These analyses revealed that the relative abundance of RZR mRNA transcription was strongly associated with activation of Caspase-2 and Caspase-3 expression in all three cell lines (R=0.731).

When combined, these data strongly suggest an interconnected and complex relationship between MLT administration and MLT receptor activation (Figure 3). For example, MT1 expression appears to be suppressed in these oral cancer cell lines, although MT2 expression was still observed. Administration of MLT within the normal physiologic concentration was sufficient to induce an up-regulation of MT2 expression, while MT1 expression appeared to be rescued. In addition, the apparent saturation of both MT2 and MT1 receptors with the supraphysiologic concentration was sufficient to allow consequent membrane crossing and direct intracellular transport of MLT to bind with and up-regulate expression of the RZR receptor. Finally, this activation of RZR appears to be sufficient to induce expression of apoptosis-specific regulators Caspase-2 and Caspase-3, which also appeared to exhibit expression strongly correlated with RZR expression. concentrations associated with supraphysiolgic dosages. This activation of RZR appeared to be strongly correlated with activation of the apoptosis-specific Caspase initiation pathway (Caspase-2, Caspase-3). This may suggest that only concentrations of MLT commensurate with supplementation are sufficient to saturate the

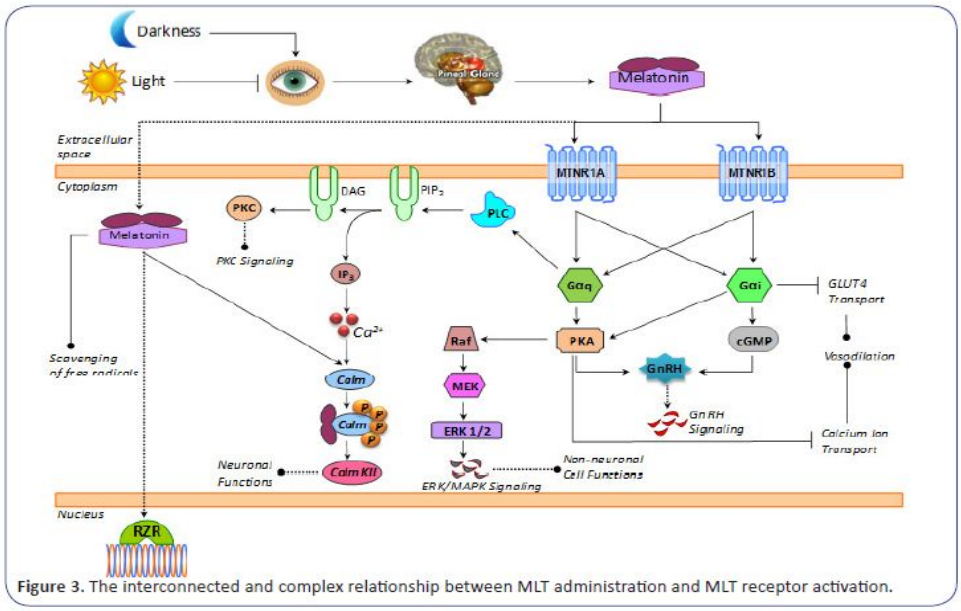


Figure 3. The interconnected and complex relationship between MLT administration and MLT receptor activation.

Conclusions

The primary purpose of this study was to explore the expression of the membrane-associated MLT receptors MT1 and MT2, as well as the intracellular receptor RZR in the context of previous results that suggested MLT administration at physiologic and supraphysiologic concentrations was sufficient to inhibit growth of oral cancer cell lines [10]. The main findings of this project suggest that expression of at least one MLT receptor, MT1, was suppressed in all three cell lines – although MT2 expression remained intact. Moreover, the effects of MLT administration appeared to function through the MT2 receptor, as the physiologic dose of MLT was sufficient to up-regulate expression of MT2, as well as to induce expression of MT1. This may suggest that transcriptional suppression of MT1 expression may not only be reversible but may also be dependent upon the activation of MT2 through its activation by MLT.

Interestingly, expression of the intracellular receptor RZR did not appear to be activated by physiologically relevant concentrations of MLT, but were significantly up-regulated by the super saturation cell surface receptors in order to facilitate the cross-membrane passage of MLT to directly activate the intracellular RZR receptor.

These phenomena are consist with other similar biphasic effects observed in other studies of MLT demonstrating that only the saturation of cell surface MLT receptors allowed excess MLT to move along the concentration gradient directly into the cell, subsequently binding the alternative intracellular ligands [15-17]. However, these results also appear to confirm previous observations that although apoptosis may be induced only at the supraphysiologic supplementation concentrations, even the lowest concentrations of physiologically relevant concentrations were sufficient to activate MT2 expression and to recover MT1 expression in all cell lines evaluated.

Although a number of clinical, biomedical and epidemiologic investigations have provided evidence that MLT may have significant effects on oral cancer risk, progression and even mortality, this study provides novel information about the receptors and associated mechanisms that may be involved in the cellular response of oral cancers to MLT administration. This study may the first to demonstrate that MT1 expression, lost in many oral cancers, may be recovered with MLT administration, an effect that could prove useful for complementary and alternative therapies for oral cancer. Finally, this study provided initial data to suggest that the induction of apoptosis following supraphysiologic MLT administration may be correlated with RZR expression, suggesting that this pathway may be an important and critical interlocutor that functions to activate these pathways in at least these oral cancer lines. Future studies that investigate these receptors, pathways and the mechanisms involved in their control may prove indispensible to oral health researchers and oncologists who search for new methods to control or inhibit the growth and spread of oral cancers.

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19C Trolley Square

Wilmington, DE 19806

Mail: info@bioaccent.org