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The impact of different extracellular matrices on melatonin effect in proliferation and stemness properties of ovarian cancer cells

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ABSTRACT

Aim: Endogenous melatonin has numerous physiological roles on modulating the function of different organs. Recent studies revealed oncostatic and protective effects of this molecule on tumor development. In this study, we examined the impact of melatonin and key underlying mechanisms on stemness, morphology, invasiveness and viability of SKOV3 ovarian cancer cells in different types of extracellular matrix.

Methods: Cell viability was evaluated by MTT Assay. Colony-forming assay was performed by seeding 4×10^3 cells on different matrices in six well-plate. The percentage of cancer stem like cells was determined by flow cytometric assay after applying antibodies against stemness markers, CD133 and CD44. Different types of extracellular matrix including fibronectin, gelatin, collagen and matrigel were applied to incubate the cells in the presence of melatonin. Downstream gene expressions including VEGF and E-cadherin were determined by Real-time PCR.

Results: Melatonin (0.1 mM) decreased the percentage of viable cells up to $61.79 \pm 8.2\%$ ($p < 0.05$). Colony formation assay revealed the significant impact of melatonin in inhibition of colony formation in these cells. The maximum effect was shown in the cells incubated with melatonin on gelatin ($p < 0.05$). Identification of stemness markers showed that applying matrigel caused significant increase in the percentage of cancer stem like cells compared to other types of extracellular matrix ($p < 0.05$). However melatonin slightly diminished the number of stem cell like cells in all incubated matrices. Our results from gene expression assays revealed that melatonin induced a marked increase in E-cadherin along with decrease in VEGF expression levels ($p < 0.05$).

Conclusion: Our results suggest that interaction between ovarian cancer cells and neighboring matrices determines the subsequent anti invasive activities of melatonin.

1. Introduction

Ovarian-related cancers are considered as highly lethal gynecological cancers distributed globally in which an approximately 70% of affected individuals are clinically distinguished in stage III–V [1]. Although, surgery excision and following platinum base chemotherapy are currently used as the most common therapy for ovarian cancer patients, tumor relapse can be observed in most of the cases [2]. Melatonin is an indolamine agent factor that preferentially synthesized in the pineal gland, however some studies discovered the presence of this hormone in other organs, including skin, retina and intestine with varying degree of synthesis [3]. Melatonin initiates unique intracellular signaling in target cells *via* G-protein coupled receptors, MT1A and B [4]. In addition to critical various regulatory physiologic roles, such as sleep, circadian rhythm, sexual maturation, reproduction, immune response, which is originated by melatonin, a line of

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evidence highlighted the potential oncostatic effects of this hormone on numerous cancers such as prostate, breast and ovary [5–10].

Melatonin has also known as a safe drug in the clinic thereby the anti-tumorigenic properties of this component have been greatly attracted lots of interest in cancer therapy [8,11]. In addition to various complex mechanisms reported before, melatonin can be considered as a suitable target in cancer therapy. Cell cycle arrest, an enhanced intracellular basal free radical generation, anti-migratory and −invasion abilities of melatonin orchestrated by MMP-2 and -9 enzyme down-regulation and induction of pro-apoptotic changes [12,13].

Multiple studies indicated that there is a small subset of cancer cells residing in various tumor environments, named cancer stem cells (CSCs) [14,15]. Most of the common cell surface-markers, including CD133, CD44, and CD117 along with ALDH1 have been agreed to apply for identifying, isolating and expanding CSCs in a cancerous milieu and in *in vitro* conditions [1,15,16]. CSCs are thought to be involved in tumor initiation, maintenance, chemo-resistance, radio-resistance, recurrence and metastasis phenomena through their clonogenicity, self-renewal and multi-differentiation capacities [16]. Therefore, targeting CSCs can be applied to be an emerging strategy in cancer therapy. A few number of studies have been exploited to discriminately detect the effective role of numerous hormones, especially melatonin on CSCs population either in *in vivo* or *in vitro* conditions [17,18]. A natural endogenous hormone which selectively breaks CSCs chemo-resistance can be considered as a promising target in controlling tumor progression and cancer treatment [19].

In this study we investigated the possible role of melatonin on viability, morphological change and clonogenicity of ovarian epithelial like cells incubated in various type of extracellular matrix (ECM) including fibronectin, gelatin, collagen and matrigel. The impact of melatonin in modulation of key signaling genes including E-cadherin and VEGF were also examined.

2. Material & methods

2.1. Cell line culture

Human ovarian epithelial-like cell line, SKOV3, was obtained from the National Cell Bank of Iran, Pasteur Institute (NCBI, C209). The cells were then cultured in RPMI 1640 medium (Sigma-Aldrich, USA) containing with 10% fetal bovine serum (FBS, Gibco, USA) and 1% Penicillin-Streptomycin solution (Gibco, USA) and maintained in a humidified atmosphere of 95% air and 5% CO_2. All the cells used in the current experiment were at passage 3–6.

2.2. Cell seeding on different extracellular matrix surfaces

To elucidate the possible effect of different extracellular matrices (ECM) on $CD133^+/CD44^+$ cancer stem-like cells proportion, the cells were incubated up to six days on each of fibronectin (Promocell, Germany), collagen, gelatin (Sigma-Aldrich, USA) and Matrigel (Corning, USA). The results were compared to non-ECM-coated surfaces.

2.3. Collagen extraction from rat tail

Type I collagen of a rat tail was extracted as explained before [20]. Briefly, adolescent male Wistar rats were euthanized with a combined overdose of Ketamine and Xylazine. The rat tail soaked in 70% ethanol for 15 min, deskinned, tail tendons were then collected and dissolved in 0.1% acetic acid solution at 4 °C for 48 h. To obtain clear collagen solution, the primary viscous solution was subsequently centrifuged at 15,000 rpm for 90 min at 4 °C. Ultimately, the pH was adjusted into neutral range, and the final protein concentration was assessed.

2.4. Melatonin preparation

Melatonin (Sigma Aldrich, China) was dissolved in Dimethyl Sulfoxide (DMSO, Merck, Germany) up to 2 M as our stock solution. Thereafter, stock solution was diluted with DMEM/LG (Gibco, USA) to achieve a final concentration of 0.1 mM as a working concentration. The maximum concentration of DMSO for each experimental condition was less than 0.01%.

2.5. Coating of culture plate with different ECM

Cell culture plates, 24-well, (SPL, Korea) were coated with 250 μl of 0.1% gelatin, collagen, 1 μg/ml fibronectin or matrigel (1:10 dilution). To achieve efficient coating, the plates were incubated for next 1 h at 37 °C. Subsequently, the extra coating solutions were discarded and air dried. Non-coated wells were used as a control group. SKOV3 cells were plated at initial density of 10^5 cells/well in DMEM/LG which supplemented with 0.1 mM melatonin, 0.4% bovine serum albumin (BSA, Sigma-Aldrich, USA), 10 ng/ml epidermal growth factor (EGF, Sigma Aldrich, USA) and 10 ng/ml basic fibroblast growth factor (bFGF, Sigma Aldrich, USA) and 0.5% B27(Gibco, USA). The medium was replenished every 3 days. The experiment was performed in triplicate and repeated at least three times. The control group has no melatonin in cultured condition.

2.6. Morphological changes

After six days incubation, morphological properties of melatonin treated and non-treated cells on different types of ECM were evaluated by an inverted microscope (Labomed, USA). To further quantification the morphological changes of the cells on each matrix were measured using an image-analyzing software (AxioVision Version Rel 4.8 Software; Carl Zeiss Micro imaging, Inc.).

2.7. Cell viability assay

Cell survival rate were determined by the convenient colorimetric assay using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (Sigma-Aldrich, USA)) [13]. Briefly, a total of 10^4 cells per well were seeded in 96-well cell culture plate (SPL, Korea) which was pre-coated with each matrix. The cells were further exposed to DMEM/LG medium containing 0.4% BSA, 10 ng/ml EGF, 10 ng/ml bFGF and 0.5% B27 at the presence or absence of 0.1 mM melatonin. The medium was replenished every 3 days. For MTT assay, the supernatant was further discarded and 20 μl of MTT solution (5 mg/ml) added to each well and incubated for 4 h. Then, the solution was replaced with 200 μl DMSO solution. Finally, the absorbance value was measured between 570 – 630 wavelengths using a Micro-plate reader system (BioTek, USA). The relative levels of non-viable cells were determined by comparison of mean optical density values (OD) with control group. Three independent experiments were performed and repeated in triplicate.

2.8. Flow cytometric immunophenotyping

Flow cytometric Immunophenotyping was performed based on cancer stem-like cell feature with a panel of the surface markers including, FITC conjugated mouse-anti human CD133/2 (Miltenyi Biotec, Germany) and PE conjugated mouse-anti human CD44