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Short report

Direct antiproliferative effects of melatonin on two metastatic cell sublines of mouse melanoma (B16BL6 and PG19)

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The effects of melatonin on the growth of two highly tumorigenic rodent melanoma cells were studied *in vitro*. PG19, an amelanotic mouse melanoma cell line, and B16BL6, a melanotic melanoma cell line selected for its invasive potential *in vitro*, were cultured in the presence of different concentrations of melatonin (10 μM to 0.1 pM). Five days later, viable cells were determined in a haemocytometer by the trypan blue exclusion test. Melatonin at concentrations of 1 nM and 10 pM (within the range of concentrations that correspond to physiological night-time and daytime levels in human blood) significantly inhibited proliferation in both melanoma cell lines. Subphysiological (0.1 pM) or supraphysiological (10 μM to 100 nM) concentrations of melatonin lacked this effect. These results support the hypothesis that, at physiological concentrations, melatonin exerts a direct inhibitory effect on PG19 and B16BL6 cells proliferation. © 2001 Lippincott Williams & Wilkins

Key words: B16BL6, melanoma, melatonin, PG19, pineal

Introduction

The effects of melatonin on the proliferation of melanoma cells have been studied *in vitro* in different rodent and human melanoma cell lines. In one of the first *in vitro* studies,[1] millimolar concentrations of melatonin were shown to cause a 25% inhibition in the growth of a cloned hamster melanoma cell line (B7), whereas micromolar concentrations stimulated growth and either physiological (nM) or subphysiological (pM) ones were ineffective, suggesting that melatonin may have a dose-dependent biphasic effect on melanoma cell proliferation. This dose-dependent nature of the melanoma cell response to melatonin has been corroborated by other authors. Thus, Bartsch and Bartsch[2] found, in human melanoma cells, that micromolar concentrations of melatonin caused a 60% inhibition of cell proliferation, while a 10-fold higher dose produced only a moderate stimulation of cell growth. In S91 mouse melanoma and AbC1 hamster melanoma cell lines, pharmacological doses (100 μM) stimulated cell proliferation, whereas physiological (0.1–10 nM) concentrations of melatonin inhibited it.[3] The dose-dependent inhibitory effects of melatonin at concentrations in the physiological range have also been described in melanoma M2R cells[4] and in Cloudman S91 murine melanoma cells.[3] However, Ying *et al.*[5] described only antiproliferative effects for melatonin and the agonist 6-chloromelatonin (0.1 mM to 1 nM) in M-6 melanoma cells.

The aim of this study was to continue this research by examining the effects of different melatonin doses on other melanoma cell lines as yet not studied. We chose two highly tumorigenic rodent melanoma cell lines: PG19, an amelanotic mouse melanoma cell line, and B16BL6, a melanotic melanoma cell line selected for its invasive potential *in vitro*.

Materials and methods

Cells and culture conditions

PG19 and B16BL6 melanoma cells (donated by Dr I.J. Fidler from The University of Texas MD Anderson Cancer Center, Houston, Texas, USA) were maintained as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) (Sigma Chemical Co., St Louis, Missouri, USA) supplemented with 5% fetal

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bovine serum (FBS) (Gibco, France), penicillin (20 units/ml) and streptomycin (20 μg/ml), at 37°C in a humid atmosphere containing 5% CO_2.

In each experiment stock subconfluent monolayers (80%) of cells were incubated with 5 mM disodium ethylene diamine tetra-acetic acid (Na_2 EDTA) in phosphate buffered saline (PBS) (pH 7.4) at 37°C for 5 min, resuspended in DMEM supplemented with 5% FBS and passed repeatedly through a 25 gauge needle to produce a single cell suspension. Cell number and viability were determined by staining a small volume of the cell suspension with 0.4% trypan blue saline solution and examining the cells in a haemocytometer. Cells were plated at a density of 3.0×10^5 cells per dish (60×15 mm culture dishes) in DMEM supplemented with 5% FBS, penicillin (20 units/ml) and streptomycin (20 μg/ml). After the cells had firmly attached to the dishes (4 h), the plating medium was replaced with fresh medium containing the appropriate concentrations of melatonin (10 μM, 100 nM, 1 nM, 10 pM and 0.1 pM) (Sigma Chemical, UK) dissolved in ethanol (0.001%), and an equivalent volume of ethanol was added to the control plates. Five days later, the cells were harvested by treatment with 5 mM Na_2 EDTA in PBS (pH 7.4) at 37°C for 5 min, passed through a 25 gauge sterile needle to produce a single cell suspension, and counted with a haemocytometer. Viability was determined by the trypan blue exclusion test.

Statistical analysis

Data were analysed by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls multiple comparisons test for differences between means. Differences between group means were considered significant at $P < 0.05$.

Results

Figures 1 and 2 show the effect of melatonin on PG19 and B16BL6 melanoma cell proliferation, respectively. Physiological concentrations (1 nM and 10 pM) of the indolamine significantly inhibited cell growth in both cell lines, whereas supraphysiological (10 μM and 100 nM) or subphysiological concentrations (0.1 pM) lacked these effects. In both cases, the highest antiproliferative effects were obtained with 1 nM melatonin, which reduced cell growth to 45% (B16BL6) and 62% (PG19) of control levels. The differences in cell number between the melatonin-treated plates and the controls after 5 days of incubation were significant at concentrations of 1 nM ($P < 0.01$) and 10 pM ($P < 0.05$) in both the cell lines tested.

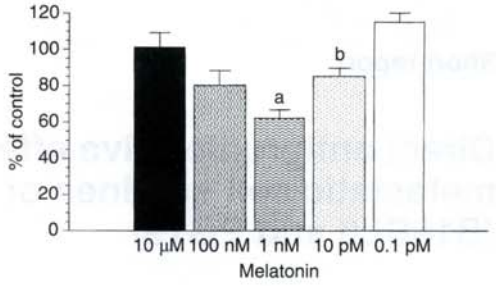

Figure 1. Effects of melatonin on PG19 mouse melanoma cell number. Data are expressed as the percentage of the controls (100%) on day 5 of growth. $^{a}P < 0.01$ and $^{b}P < 0.05$ versus control.

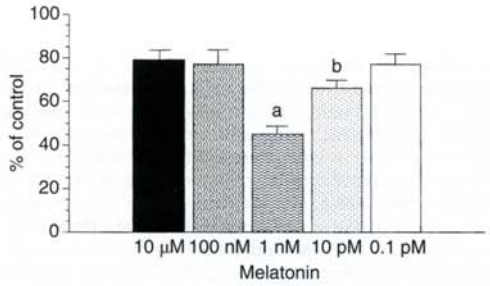

Figure 2. Effects of melatonin on B16BL6 mouse melanoma cell number. Data are expressed as the percentage of the controls (100%) on day 5 of growth. $^{a}P < 0.01$ and $^{b}P < 0.05$ versus control.

Discussion

Although most data supporting a role for melatonin as an oncostatic hormone are in the realm of breast cancer, a variety of other neoplasms, among them the melanomas, have also been the subject of studies for their possible sensitivity to the antiproliferative effects of this indolamine.[6-8] After the pioneering studies of Das Gupta's group, which showed that pinealectomy favoured the development of primary tumours and tumour metastases of pigmented malignant melanoma transplanted to Syrian hamsters,[9] and that these effects were reversed by melatonin,[10] numerous experiments have been performed in clonogenic melanoma cell lines, from both animal

and human tumours, to clarify the possible direct actions of melatonin on these tumour cells. In the present study we examined the effect of melatonin on two highly tumorigenic rodent melanoma cell lines: PG19, an amelanotic mouse melanoma cell line, and B16BL6, a melanotic melanoma cell line selected for its *in vitro* invasion through the urinary bladder wall and its high production of spontaneous metastases.[11] Cell proliferation of both cell lines was inhibited only by low (1 nM and 10 pM) concentrations of melatonin. Furthermore, melatonin's growth-inhibiting activity on these cells was restricted to concentrations that correspond to physiological night-time and daytime levels in human blood. This is in agreement with other reports demonstrating that melatonin at physiological, but not pharmacological or subphysiological, concentrations can inhibit proliferation of different cancer cells cultured *in vitro*.[3,12-14]

Although as far as we know the effects of melatonin on PG19 cells had not been studied previously, different effects of melatonin and methoxytryptamine on the proliferation of B16 mouse melanoma cells had been observed previously.[12,15,16] Thus, in BALB/c athymic mice inoculated with B16 melanoma cells, melatonin (5 μg/g body weight per day) given with the drinking water significantly reduced the size of the tumours formed 49 days after cell inoculation.[15] In other experiments, while continuous exposure to melatonin did not affect B16 cell proliferation, intermittent exposure (pulses of 8 h daily) at physiological concentrations (0.1 or 1 nM) modestly stimulated cell proliferation.[12] B16 melanoma sublines show a degree of phenotypic diversity, with different invasive potentials and various degrees of differentiation.[17,18] These phenotypic differences may explain the differential sensitivity to the melatonin-induced growth inhibition of B16BL6 and parental B16 cells. B16 melanoma cells also show a considerable heterogeneity in their response to other antiproliferative substances such as retinoic acid.[19]

The mechanisms involved in the antiproliferative effects of melatonin on melanoma cells are unknown. However, three possible hypotheses can be considered. The first could be an interaction with specific receptors in the cells. However, the existence of melatonin receptors in melanoma cells has been not completely clarified. Studies of [^{125}I]melatonin-specific binding to membrane suspensions from Syrian hamster RPMI 1846 melanoma cells revealed the expression of a melatonin receptor,[20] linked to phosphoinositide hydrolysis as a second messenger.[21] With the same probe (iodomelatonin), binding sites have also been observed in murine B16 melanoma as well as in SK Mel 28 and SK Mel 30 human melanoma cells.[12] However, saturation studies of iodomelatonin binding to membranes from normal human melanocytes as well as B16F10 mouse melanoma cells and amelanotic S91 revealed no high affinity binding sites,[22] and binding studies in M-6 human malignant melanoma membranes showed the coexistence of picomolar and nanomolar affinity binding sites.[5] Recent studies have demonstrated that melatonin (0.1 to 10 nM) inhibits, in a dose-dependent manner, the growth of human uveal melanoma cells *in vitro*.[23,24] Since these uveal melanoma cells express RNA encoding the Mel1b,[25] the hypothesis of the direct action of melatonin on specific receptors as the basis for its antiproliferative action seems acceptable, at least for these kind of melanoma cells.

The second hypothesis could be the interaction of melatonin with the oestrogenic response pathway, as described for some mammary cancer cell lines.[8,13,26] Controversy regarding the presence of oestrogenic receptors in melanoma cells persists despite numerous investigations of this subject. Nuclear oestrogen binding has been characterized in rodent and human melanoma cells,[27-30] whereas no evidence for oestrogen receptors has been reported in studies of ocular and other human melanomas.[31-34] The antiproliferative activity of tamoxifen, an anti-oestrogen, on B16 and HM-1 melanoma cells *in vitro* could support the role of oestrogen receptors;[12,27] however, not all authors agree with the idea that the effects of tamoxifen can be explained by its anti-oestrogenic actions.[30,35]

As an alternative hypothesis, melatonin antioxidant properties[36-38] could be involved in its antiproliferative effects on melanoma cells. In this regard, Roberts *et al.*[39] have recently described that melatonin and its receptor agonist 6-chloromelatonin block the proliferation of dermal and uveal melanoma cells by mechanisms involving redox reactions.

Our results demonstrate that melatonin can act directly at a cellular level to inhibit the proliferation of PG19 and B16BL6 mouse melanoma cells in culture, although the mechanisms involved in these antiproliferative actions are still unknown.

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