Tracking the elusive antiestrogenic effect of melatonin: A new methodological approach

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Abstract

OBJECTIVES: Detection of the antiestrogenic effect of melatonin on various breast cancer cell lines and its dependence of the differential expression of estrogen receptors (ERα and ERβ) and melatonin receptors (mt1 and RZRα).

SETTING AND DESIGN: Dose-response curves of estradiol were determined in 6 different breast cancer cell lines using a colorimetric proliferation assay in the absence or presence of various melatonin concentrations.

METHODS: In order to detect the minor growth inhibitory effect of melatonin, a simple yet novel approach was employed: instead of incubating cells at single estradiol-concentrations at increasing melatonin levels, breast cancer cells were grown in microwell-plates for 4 days at increasing concentrations of estradiol (10^{-12} M – 10^{-10} M) in the absence or presence of melatonin (10^{-9} M – 10^{-8} M). Cell number was determined using Alamar blue and colorimetry.

RT-PCR was performed for the expression of ERα, ERβ, RZRα and mt1.

RESULTS: Melatonin at concentrations of 10^{-9} M and 5×10^{-9} M shifted the dose-response curves of estradiol to higher concentrations. Responsiveness to melatonin depended on expression of ERα but not on ERβ. mRNA of ERβ was not detectable in the breast cancer cell lines used. Only small amounts of mt1 transcripts were detectable in MCF-7 cells of one source. In MCF-7 cells transfected with the mt1 gene and in an ovarian cancer cell line mt1 was expressed at significant levels. RZRα was expressed in all tested cell lines at different amounts.

CONCLUSION: The growth of all ERα-positive breast cancer cell lines can be inhibited by melatonin. The effect in most cell lines is weak yet clearly reproducible. RZRα clearly contributes to the growth inhibitory effect of melatonin.

Introduction

Breast cancer is the most frequent malignant disease in women. The growth of most mammary carcinomas is stimulated by estradiol or other endogenous and exogenous estrogens. Detection of estrogen receptors (ER) in carcinoma tissue is widely used as a predictor for the efficacy of endocrine treatment and as a prognostic factor for the disease progress of breast cancer patients.

There is good evidence that the pineal gland influences the development and growth of breast cancer through its major hormone melatonin [1]. In pinealectomized rats the incidence of mammary carcinoma is increased and hormone replacement with physiological doses of melatonin reversed this phenomenon [2]. Further, by the administration of melatonin the onset of chemically induced breast cancer in rats treated with the carcinogen DMBA was delayed and the number of loci was reduced [3]. Two possible mechanisms may contribute to the anti-neoplastic effect of melatonin. A regulatory systemic action of melatonin is proposed since its enhanced secretion at night modulates the neuroendocrine release of prolactin and the regulation of 17β-estradiol secretion by the ovaries [4]. In addition, melatonin has immunostimulatory effects by inducing the secretion of IL-2 and IL-6 from mononuclear cells [5].

On cellular level some authors have shown that proliferation of breast cancer cells is inhibited by physiological concentrations of melatonin[6,7]. Others failed to reproduce this observation although these investigators nominally used the same cell line for their experiments [8,9,10,11]. Most of these experiments were performed with the widely used breast cancer cell line MCF-7. This cell line was established by Soule et al. [12] in 1973 and has since then been cultured in a large number of laboratories. It is evident that the various culture conditions used in different laboratories have led to a selection of various subclones of the original cell line MCF-7. In a comparative study, Ram et al. observed great differences in the responsiveness of these subclones to melatonin [13].

The effects of melatonin on the cellular level are triggered by two distinct classes of receptors for melatonin either membrane bound or located in the nucleus. Up to date three membrane bound melatonin receptors, Mt1, Mt2 and Mt3 have been cloned. These receptors are coupled to G-proteins that inhibit adenylyl cyclase activity after ligand binding [14]. The various melatonin receptors are expressed in different tissues. Mt1 is found in the mammalian brain and was detected in human embryonic kidney cells (HEK293) and prostate cancer cells [15]. Mt2 expression is limited to the retina and Mt3 was only cloned from birds. In addition, there are nuclear receptors that are homologous to retinoic acid receptors [16].

The membrane bound receptor Mt1 as well as the nuclear receptor RZRα were detected in MCF-7 cells by two different laboratories [17,18]. Reactivity of cells to melatonin depends on the presence of melatonin receptors but it is not yet clear what receptor is responsible for the antiproliferative activity of melatonin on breast cancer cells. Arguably, the activity of melatonin depends on the presence of estrogen receptors because ER-negative breast cancer cells are not growth inhibited by melatonin. But up to date there is no convincing clue how the signaling pathways of melatonin cross-talk with the transactivation of the estrogen receptor. Neither has it been elucidated whether ERα or ERβ or both are involved in the antiproliferative effects of melatonin.

In order to convincingly demonstrate a reproducible antiproliferative effect of melatonin on breast cancer cells we developed a new sensitive detection assay as a new research tool to measure the effects of melatonin on the proliferation of breast cancer cells.

Material and Methods

The breast cancer cell lines MCF-7, EFM-19 and MDA-MB-361 were obtained from ATCC., (Manassas, USA). A second MCF-7 clone (MCF-7 p181) was from the lab of Christian Bartsch. A MCF-7 cell line transfected with a vector containing the mt1 gene was generated as described [19].

Cells were maintained in Dulbecco's modified MEM supplemented with 5% fetal calf serum (FCS), 2 mM glutamine, 50 U/ml penicillin/streptomycin, 2,5 μg/ml amphotericin B and 1:100 non-essential amino acids (Biochrom, Berlin, Germany).

Mt1 transfected MCF-7 cells were periodically selected under growth pressure of 1000 μg/ml Zeocin (Invitrogen, Karlsruhe, Germany).

For proliferation tests FCS in the culture medium was replaced by 10% charcoal depleted fetal calf serum. Charcoal depleted serum (CD-FCS) was prepared according to the procedure described by Stanley et al.[20]. 750 cells/well were seeded in 100 μl CD-FCS medium into 96-well plates. After attachment of the cells either 50 μl CD-FCS medium (control) or 50 μl estradiol solution (final concentrations increasing from 10^{-12} to 10^{-10} M) were added to six replicate wells for each concentration. To test the effect of melatonin, 50 μl melatonin-solution (Sigma Chemicals, M 5250) were added to parallel series of estradiol concentrations to give a final concentration of either 10^{-9} M or 5×10^{-9}M or 10^{-8}M melatonin. In the control experiment another 50 μl CD-FCS medium were added to all estradiol concentrations instead of 50μl melatonin-solution. Cells were grown for 4 days at 37 °C, 5% CO_2 and saturated humidity. At the end of the incubation period the cell number was determined by a colorimetric method using Alamar Blue (Biosource, Solingen, Germany). Alamar blue is a water soluble blue dye that is reduced by mitochondrial reductases of living cells to yield a red color. The amount of the red color is proportional to the number of living cells and is assessed after 4 hours at 37 °C in a microplate reader at 570 nm vs. 630 nm.

Proliferation assays with each cell line were performed at least three times with six replicates for

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each concentration of estradiol. Means and standard deviations of the OD of the six replicates were calculated. The proliferative effect (PE) for each estradiol concentration was determined by dividing the average OD reached at the certain estradiol concentration by the average OD of the control for each independent experiment as previously described [21]. Dose-response curves for estradiol at each melatonin concentration were obtained by plotting the mean relative PE (RPE = PE-1 at the test concentration /PE-1 at maximal stimulation) versus the concentration of estradiol on a half-logarithmic scale. The EC_{50} values for half maximal growth stimulation were calculated from these dose-response curves by a VBA program for EXCEL 5 written by Josef Greve at the Fraunhofer Institute for Molecular Biology and Applied Ecology, Schmallenberg, Germany.

Expression of estrogen receptors and melatonin receptors was determined by RT-PCR. RNA of nontreated cells was purified by RNeasy-kit (Qiagen, Hilden, Germany). 200 ng RNA were transcribed by Superscript reverse transcriptase (Invitrogen, Karlsruhe, Germany) for 50 min at 42 °C. 5 μl of the resulting cDNA were amplified with 1 U Taq polymerase (Roche, Mannheim, Germany) at 200 μM dNTPs and 200 nM of the appropriate primers.

ERα, primer A:
ACTCGCTACTGTGCAGTGTGCAAT
Primer B:
CCTCTTCGGTCTTTTCGTATCCCA
35 cycles, annealing: 52 °C
ERβ, Primer A:
GCCTCCATGATGATGTCCCTG
Primer B: GATCATGGCCTTGACACAGAG
40 cycles, annealing: 57 °C
mt1, Primer A:
CTGGCCTGCGTCCTCATCTTCACCATC-GTG
Primer B:
CCATGCTGGCGGGGTCAGAGGC
44 cycles, annealing: 58 °C
RZRα, Primer A:
AACGGCGAGACTTCCCCAACTG
Primer B:
CAAAGGCACGGCACACTCTGATAA
40 cycles, annealing: 57 °C
β2-microglobulin, Primer A:
CATCCAGCGTACTCCAAAGA
Primer B: GACAAGTCTGAATGCTCCAC
40 cycles, annealing: 54 °C
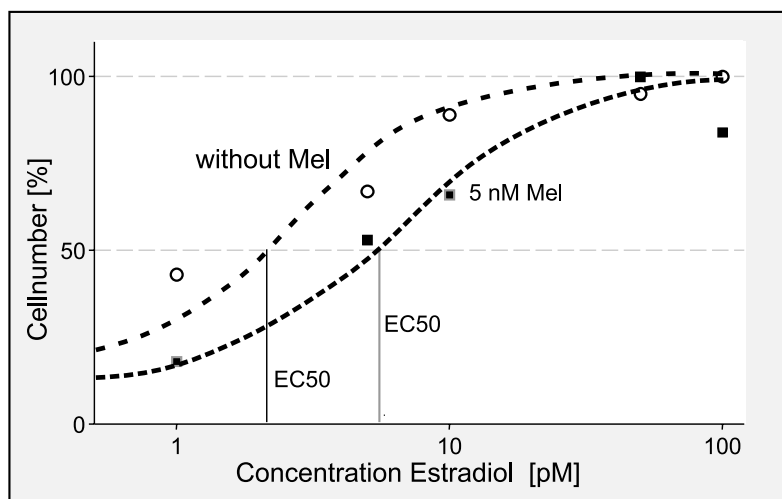
Results

Effect of melatonin on the growth stimulation by estradiol

The effect of melatonin on the growth of five different breast cancer cell lines was only minimal when it was measured at the optimal growth stimulating estradiol concentration (10^{-10} M). Cell number after treatment with 10^{-9} M or 10^{-8} M melatonin for four days varied by only $\pm15\%$ compared to the growth in the absence of melatonin (data not shown). To increase the sensitivity of the test system and to eliminate the influence of statistical variation a modified test system was established: instead of incubating cells at steady single estradiol-concentrations and increasing melatonin-concentrations, the opposite approach was employed. In the absence of or at three separate melatonin concentrations growth curves of breast cancer cells were determined as a dose response to increasing estradiol concentrations. Comparing estradiol-driven cell growth in the presence or absence of melatonin, 5 nM melatonin shifted the dose-response curve of estradiol to higher concentrations (Fig. 1). In MCF-7 cells (subclone p40) 5×10^{-9}M melatonin almost doubled the EC_{50} of estradiol from $1,9\times10^{-12}$M to $3,6\times10^{-12}$M.

Table I displays the EC_{50} of estradiol observed at various melatonin concentrations in 5 different cell lines. In all three different MCF-7 subclones the effect

Table I. Shift of the EC50 of estradiol by melatonin in breast cancer cell lines

Cell line	without melatonin	10^{-9} M melatonin	5×10^{-9} M melatonin	10^{-8} M melatonin
MCF-7 p40	$1,9\times10^{-12}$	$1,4\times10^{-12}$	$3,6\times10^{-12}$	$1,0\times10^{-12}$
MCF-7 p 180	$3,0\times10^{-12}$	$2,3\times10^{-12}$	$3,9\times10^{-12}$	n.d.
MCF-7 Mel1a	$2,2\times10^{-12}$	$2,2\times10^{-12}$	$3,1\times10^{-12}$	$2,5\times10^{-12}$
EFM-19	$4,1\times10^{-12}$	$5,1\times10^{-12}$	$4,0\times10^{-12}$	n.d.
MDA-MB-361	$1,3\times10^{-13}$	$6,0\times10^{-12}$	$3,7\times10^{-12}$	n.d.

Fig.1. Shift of the EC_{50} of estradiol by melatonin in MCF-7 cells.
Dose-response curves of estradiol in MCF-7 (p40) cells in the absence (dotted line) and in the presence (solid line) of 5×10^{-9} M melatonin.
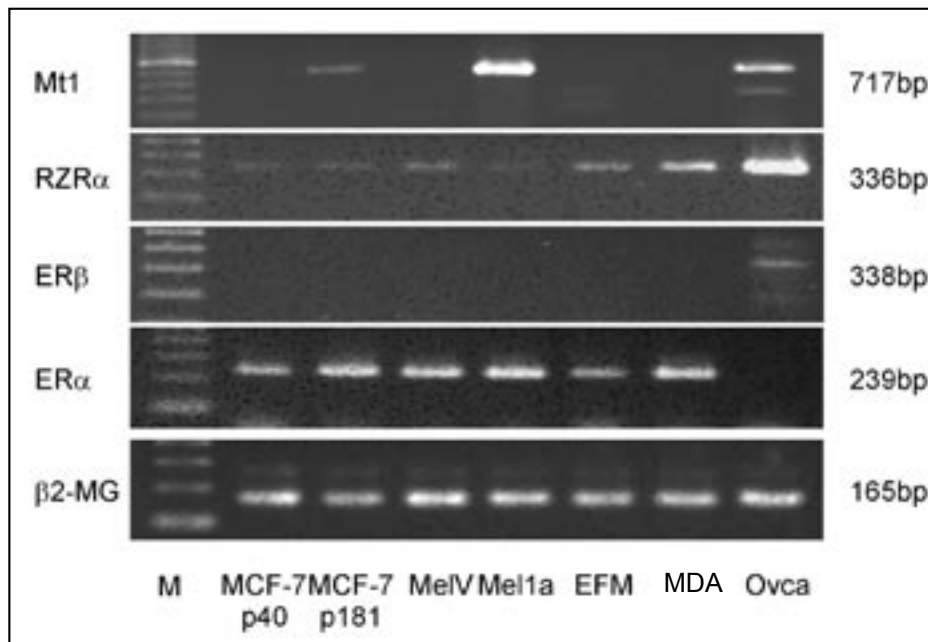
Fig. 2. Expression of melatonin and estrogen receptors in breast cancer cell lines.
RNA (0.2 µg) of each cell line was amplified for ERα by 35 cycles, for ERβ, RZRα and
β2-microglobulin by 40 cycles and for mt1 by 44 cycles of PCR.
Cell lines: MCF-7 (p40): low passage cell line from ATCC, MCF-7 (p181): high passage cell line from
Dr. Christian Bartsch, Tübingen, MelV: MCF-7 cells transfected with an empty vector, Mel1a: MCF-7
cells transfected with the mt1 gene from Dr. Steven Hill, New Orleans, EFM: cell line EFM 19,
MDA: cell line MDA-MB-361, OVCA: ovarian carcinoma cells.
Representative agarose gels of three independent experiments.

of melatonin was maximal at 5×10^{-9} M melatonin. At 10^{-8} M melatonin the effect of melatonin was reduced to a lower level or even neutralized.

The growth of the cell lines EFM-19 and MDA-MB-361 was only weakly stimulated by estradiol. Relative proliferation factor (RPF) for MDA-MB-361 at 10^{-10} M estradiol was only $1,5 \pm 0,2$ compared to the RPF $= 2,3 \pm 0,3$ in MCF-7 cells. Due to the low proliferation induction by estradiol in these cell lines the effect of melatonin was almost indiscernable. The shift of EC_{50} in the dose-response curves of these cell lines was maximal at 10^{-9} M melatonin.

Expression of the various receptors for estradiol and melatonin in the breast cancer cell lines

The expression level of the melatonin receptors mt1 and RZRα and the estrogen receptors ERα and ERβ was determined by RT-PCR in two subclones of MCF-7, in a MCF-7 cell line transfected with mt1 (Mel1a) or the empty vector (MelV), two further breast cancer cell lines (EFM-19 and MDA-MB-361) and an ovarian carcinoma cell line (Fig.2).

Only low amounts of mt1-RNA were detectable in the subclone of MCF-7 cells with higher passage number (p181). A strong signal of mt1 was observed in the MCF-7 cells transfected with the mt1 gene (Mel1a) and in the ovarian carcinoma cell line whereas the MCF-7 cells transfected with the empty vector (MelV) contained no mt1 RNA.

The mRNA of the low affinity receptor RZRα was expressed in all breast cancer cell lines at trace amounts. The different MCF-7 cell lines expressed less RZRα than the cell lines EFM-19 or MDA-MB-361. The strongest expression was observed in the ovarian carcinoma cells. All breast cancer cell lines expressed ERα. Expression was strongest in the MCF-7 cells with higher passage number (p181), the mt1 transfected MCF-7 cells expressed similar amounts of ERα. Lowest amounts of ERα mRNA were found in EFM-19 cells. In the ovarian carcinoma cell line no ERα was expressed.

ERβ mRNA was not detectable in any of the six breast cancer cell lines although four more cycles of PCR were performed. As positive control for the amplification reaction the mRNA from an ovarian carcinoma cell line shows the expected signal at 338 bp.

Discussion

The antiestrogenic effect of melatonin on breast cancer cells has been discussed controversially. Using a novel methodological approach, we were able to demonstrate the susceptibility of a number of cell lines to melatonin. The effect of melatonin on the proliferation of the breast cancer cell lines was only of minor scale and only detectable by comparing dose-response curves of estradiol in the absence and presence of melatonin. These dose-response curves were shifted by melatonin to higher concentrations. The estradiol concentration required to yield an equivalent proliferative reaction in the presence of melatonin was roughly doubled. The effect of melatonin was maximal at the concentration of 5×10^{-9} M for the various MCF-7 subclones tested. Higher and lower concentrations of melatonin yielded

a lesser antiproliferative effect. In the cell lines EFM-19 and MDA-MB-361 that both didn't express mt1 but considerable amounts of RZRα, melatonin was most effective at a lower concentration of 10^{-9} M. Such a bell shaped curve of the melatonin effect was observed by other authors, too [6,7,13]. After all, an antiproliferative effect of 70% and more as described by these authors was never observed in any of the cell lines we examined. Ram et al. [13] reported that a MCF-7 clone expressing a variant transcript of the ERα carrying a deletion of exon 5 was less responsive to melatonin than MCF-7 cells expressing wild-type ER.

It is not yet clear what receptor for melatonin is responsible for the antiproliferative effect of melatonin. Both, the membrane bound receptor Mt1 and the nuclear receptor RZRα were independently detected in MCF-7 cells [17,18]. Most investigations focused on the role of Mt1 in the antiproliferative action of melatonin [13,15,17]. Very recently, Winczyk et al. emphazised a possible involvement of the nuclear RZRα receptor in the antitumor action of melatonin on colon cancer [22]. Interestingly, the two breast cancer cell lines (EFM-19 and MDA-MB-361) that expressed substantial amounts of RZRα (Fig.2) were already maximally growth inhibited at 10^{-9}M melatonin (Table 1), whereas all MCF-7 clones we examined expressed only trace amount of RZRα and needed 5×10^{-9}M melatonin for antiproliferative reaction. This observation points to a more important contribution of RZRα in the antiproliferative activity of melatonin in breast cancer. It remains an unsolved question how RZRα influences the transactivation of ERα.

Acknowledgements

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