Effects of MT_1 melatonin receptor overexpression on the aromatase-suppressive effect of melatonin in MCF-7 human breast cancer cells

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Abstract. A major mechanism through which melatonin reduces the development of breast cancer is based on its anti-estrogenic actions by interfering at different levels with the estrogen-signalling pathways. Melatonin inhibits both aromatase activity and expression *in vitro* (MCF-7 cells) as well as *in vivo*, thus behaving as a selective estrogen enzyme modulator. The objective of this study was to study the effect of MT_1 melatonin receptor overexpression in MCF-7 breast cancer cells on the aromatase-suppressive effects of melatonin. Transfection of the MT_1 melatonin receptor in MCF-7 cells significantly decreased aromatase activity of the cells and MT_1-transfected cells showed a level of aromatase activity that was 50% of vector-transfected MCF-7 cells. The proliferation of estrogen-sensitive MCF-7 cells in an estradiol-free media but in the presence of testosterone (an indirect measure of aromatase activity) was strongly inhibited by melatonin in those cells overexpressing the MT_1 receptor. This inhibitory effect of melatonin on cell growth was higher on MT_1 transfected cells than in vector transfected ones. In MT_1-transfected cells, aromatase activity (measured by the tritiated water release assay) was inhibited by melatonin (20% at 1 nM; 40% at 10 μM concentrations). The same concentrations of melatonin did not significantly influence the aromatase activity of vector-transfected cells. MT_1 melatonin receptor transfection also induced a significant 55% inhibition of aromatase steady-state mRNA expression in comparison to vector-transfected MCF-7 cells ($p<0.001$). In addition, in MT_1-transfected cells melatonin treatment inhibited aromatase mRNA expression and 1 nM melatonin induced a higher and significant down-regulation of aromatase mRNA expression ($p<0.05$) than in vector-transfected cells. The findings presented herein point to the importance of MT_1 melatonin receptor in mediating the oncostatic action of melatonin in MCF-7 human breast cancer cells and confirm MT_1 melatonin receptor as a major mediator in the melatonin signalling pathway in breast cancer.

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Introduction

Estrogens are involved in the growth and differentiation of the normal mammary gland and have an important role in the genesis and growth of breast cancer (1). Melatonin, the main secretory product of the pineal gland, has been demonstrated to have oncostatic properties on hormone-dependent mammary cancer (2-4). *In vivo*, experimental manipulations activating the pineal gland, or the administration of melatonin, reduce the incidence and growth rate of chemically-induced mammary tumors in rodents, while pinealectomy or situations which implicate a reduction of melatonin production usually stimulate mammary carcinogenesis (2). Melatonin reduces the incidence and development of breast cancer by down-regulating the synthesis of some of the hormones responsible for the normal and pathological growth of the mammary gland (2,3), and throughout direct actions at the tumor cell level (2,3,5,6). Evidence from *in vivo* studies on animal models and *in vitro* studies on human breast cancer cell lines supports the hypothesis that melatonin oncostatic effects on hormone-dependent mammary tumors are mainly dependent on its ability to interact with the estrogen-signalling pathway of tumor cells (5,6). At the mammary tumor cell level, melatonin interacts with the estrogen-response pathway and counteracts the effects of estrogens, thus behaving as a selective estrogen receptor modulator. Furthermore, melatonin has anti-aromatase activity, thus behaving also as a selective estrogen enzyme modulator (5,6).

In the present study, we aimed to expand our understanding of the antiaromatase properties of melatonin. Our group previously demonstrated, by using MCF-7 human breast cancer cells in culture, which express aromatase (7,8) and MT_1 melatonin receptor (9,10), that melatonin, at physiological concentrations (1 nM) reduces aromatase activity in these cells both under basal conditions and when aromatase activity was stimulated by cAMP or cortisol (11). By RT-PCR (Reverse transcriptase polymerase chain reaction analysis), we have also demonstrated that melatonin down-regulates aromatase mRNA steady state levels in MCF-7 cells (11). This modulator effect of melatonin on the enzyme which controls the conversion from androgenic precursors to estrogens has also been described *in vivo*, in rats bearing DMBA-induced mammary tumors (12).

In general, the actions of melatonin have been explained by a mechanism either dependent on its binding to receptors (in membrane or nuclei of target cells) or independent of receptors (binding to calmodulin or antioxidant effects) (9,13,14) (Burns DM, *et al*, 77th Annual Meeting of the Endocrine Society, abs. 151, p62, 1990). To explain the antiaromatase action of melatonin on MCF-7 cells we hypothesized the binding of melatonin to MT_1-receptors as a first step (6,11). The reasons for this hypothesis were twofold. First, MCF-7 cells express the MT_1 melatonin receptor (9). The activation of this Gi-protein coupled membrane receptors reduces cAMP (15). In mammary cancer cells aromatase genes contain promoters II and I.3 regulated by cAMP (16-18). Consequently, agents such as melatonin which are able to decrease cAMP levels could also decrease aromatase activity. Second, the overexpression of the MT_1 receptor enhances the growth-inhibitory and gene-modulatory effects of melatonin in ERα-positive (MCF-7) human breast cancer cells (19).

The objective of this study was to assess the role of the MT_1 melatonin receptor on the antiaromatase activity of this molecule. To this purpose, we analyzed the effects of the stable overexpression of the MT_1 receptor in MCF-7 human breast cancer cells, on the aromatase-suppressive effects of melatonin.

Materials and methods

Cells and culture conditions. Vector-transfected and MT_1-transfected MCF-7 were a kind gift from Dr Steven M. Hill from the Department of Structural and Cellular Biology, Tulane University Medical School, New Orleans, LA, USA (19). Cells were cultured as monolayers in 75 cm^2 flasks and routinely maintained at 37˚C in a humidified atmosphere of 5% CO_2 in RPMI-1640 medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% FBS (Gibco, France), 2 mM glutamine, 50 mM MEM non-essential amino acids, 100 mM sodium pyruvate, penicillin (20 units/ml), streptomycin (20 μg/ml) (Sigma Chemical Co.), 10 mM BME and Zeocin (25 μg/ml) (Invitrogen S.A., Barcelona, Spain).

All cells were subcultured every 3-4 days by suspension in 5 mM Na_2EDTA in PBS (pH 7.4) at 37˚C for 5 min. Before each experiment, stock subconfluent monolayers (80%) of cells were incubated with 5 mM Na_2EDTA in PBS (pH 7.4) at 37˚C for 5 min, resuspended in medium supplemented with 10% 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 cell suspension with 0.4% trypan blue saline solution and examining the cells in a hemocytometer.

Measurement of MT_1 mRNA expression. Analysis of the MT_1 mRNA expression was carried out by reverse transcription PCR (RT-PCR) in vector-transfected and MT_1-transfected MCF-7 cells. The total cellular RNA was purified with the Aurum™ Total RNA mini kit (Bio Rad Laboratories Inc., Hercules, CA, USA) following the manufacturer's instructions. Integrity of RNA was assessed by electrophoresis in ethidium bromide-stained 1.2% agarose-Tris-borate EDTA gels. The absorbance ratio A_{260nm}/A_{280nm} was >1.8. For cDNA synthesis, 0.5 μg of total RNA were denaturated at 70˚C for 10 min and reverse transcribed 50 min at 37˚C with M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) in a final volume of 20 μl in the presence of 500 ng of oligo (dT)12-18 primer.

PCRs were performed using a set of MT_1 melatonin receptor specific primers [5'-TCCTGGTCATCCTGTCGG TGTATC-3' (forward) and 5'-CTGCTGTACAGTTTGTC GTACTTG-3' (reverse)] (Sigma Genosys Ltd., Cambridge, UK) (20,21). The coding sequence between the two PCR primer sites is interrupted by one intron in the gene to exclude the possibility of amplification of any contaminating genomic DNA present in the RNA preparation. As a control quantification, actin mRNA was also carried out by RT-PCR using a set of specific primers [5'AAATCTGGCACCACACCTC (forward) and 5'-TAGCACAGCCTGGATAGCAA (reverse)].

PCRs were performed for 40 cycles for semiquantitative analysis using the following temperature profile: 55˚C, 45 sec (annealing); 72˚C, 60 sec (extension); and 95˚C, 45 sec (denaturation). Each product was electrophoresed on ethidium bromide-stained 1.5% agarose-Tris-borate gels.

Measurement of cellular proliferation. Vector-transfected or MT_1-transfected MCF-7 were seeded in 96-multiwell plates at a density of 8.000 cells per well and incubated at 37˚C in RPMI-1640 medium supplemented with 10% of FBS, 2 mM glutamine, 50 mM MEM non-essential amino acids, 100 mM sodium pyruvate, penicillin (20 units/ml), streptomycin (20 μg/ml), 10 mM BME and Zeocin (25 μg/ml). Cells were cultured for 5 days. Medium was renewed 72 h after the beginning of treatment. Cell proliferation was measured by the MTT [3(4,5dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] method reading absorbance at 570 nm in a microplate reader (Molecular Probes Inc., Eugene, OR, USA).

Measurement of cellular aromatase activity. Indirect evidence of aromatase activity of estrogen-dependent cells, such as MCF-7, was obtained by evaluating cell proliferation in estrogen-free media in the presence of testosterone. Under these conditions, cell growth depends on the biotransformation of androgens to estrogens via the aromatase activity of the cells (22,23). To test this, vector and MT_1-transfected cells were seeded into 96-well culture plates at a density of 8000 cells/well, in RPMI-1640 medium supplemented with 10% of FBS, 2 mM glutamine, 50 mM MEM non-essential amino acids,

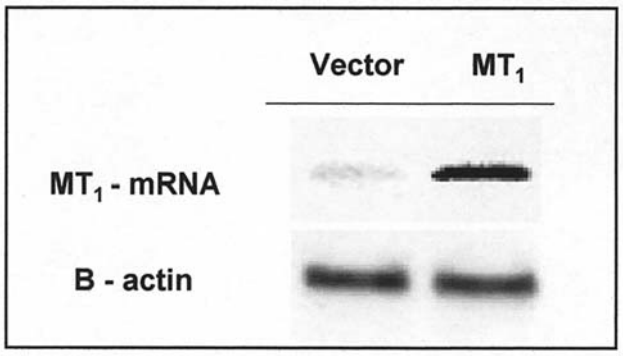

Figure 1. MT_1 melatonin receptor steady state mRNA expression in vector and MT_1-transfected MCF-7 cell lines. Total RNA was isolated to perform semiquantitative RT-PCR. cDNA was subjected to PCR using specific primers for MT_1 melatonin receptors or actin. The results of a representative experiment (chromatogram analysis) are shown.

100 mM sodium pyruvate, penicillin (20 units/ml), streptomycin (20 μg/ml), 10 mM BME and Zeocin (25 μg/ml), at 37˚C in a humid atmosphere containing 5% CO_2. After 48 h of incubation to allow a correct attachment of the cells, media were changed to the ones supplemented with 5% charcoal-stripped FBS (sFBS) containing either testosterone (1 μM), melatonin (1 nM or 10 μM), the aromatase inhibitor aminoglutethimide (100 μM) (all from Sigma-Aldrich Química S.A., Madrid, Spain), or the diluent of these drugs (ethanol, at a final concentration <0.0001% per plate). Cell proliferation was assessed at 3 and 5 days of culture, by using the MTT method, reading absorbance at 570 nm in a microplate reader.

Aromatase activity in vector-transfected or MT_1-transfected MCF-7 cells was also directly measured by the tritiated water release assay, based on the formation of tritiated water during aromatization of a labeled androgenic substrate such as [1ß-^{3}H(N)]-androst-4-ene-3,17-dione] (24). Cells were seeded onto 60x15-mm tissue culture dishes ($1.5x10^6$ cells/dish) in culture media supplemented with 5% FBS. When a homogeneous monolayer of preconfluent cells was reached on days 2-3 of the experiment, media were aspirated and replaced by fresh media (1 ml/plate) supplemented with 5% sFBS and containing 100 nM [1ß-^{3}H(N)]-androst-4-ene-3,17-dione] (NEN Life Science Products, Boston, MA, USA) (25-30 Ci/mM) in the presence of melatonin (10 μM or 1 nM) or the diluent (ethanol at a final concentration <0.0001%). At 24 h of incubation, the culture dishes were placed on ice for 15 min to condense any water vapor and the media were transferred to tubes containing 0.25 ml ice-cold 30% trichloroacetic acid (wt/vol), vortexed and centrifuged at 1700 x g for 20 min. The supernatants were extracted with chloroform, vortexed, set at room temperature for 10 min and then centrifuged at 1700 x g for 20 min. The resulting aqueous supernatants were adsorbed with 10% dextran-coated charcoal, vortexed, centrifuged at 1700 x g for 20 min and the supernatant added to vials with scintillation cocktail and counted in a beta counter. The amount of radioactivity in water [^{3}H] measured was corrected by subtracting the blank values from each sample, obtained by incubating dishes containing medium with the tritiated androgen but no cells. The values were also corrected by taking into account the fractional retention of tritium in medium water throughout the procedure of incubation and processing, utilizing parallel dishes containing medium plus known amounts of [^{3}H] water (NEN Life Science Products) through incubation and assay. The fractional retention of tritium in medium water throughout the incubation and processing of samples was always >87%.

Measurement of aromatase mRNA expression. Analysis of the aromatase mRNA was carried out by reverse transcription PCR (RT-PCR) in vector-transfected and MT_1-transfected MCF-7 cells. The total cellular RNA was purified with the Aurum Total RNA mini kit (Bio Rad Laboratories Inc.) following the manufacturer's instructions. Integrity of RNA was assessed by electrophoresis in ethidium bromide-stained 1.2% agarose-Tris-borate EDTA gels. The absorbance ratio A_{260nm}/A_{280nm} was >1.8. For cDNA synthesis, 0.5 μg of total RNA were denaturated at 70˚C for 10 min and reverse transcribed 50 min at 37˚C with M-MLV Reverse Transcriptase (Invitrogen) in a final volume of 20 μl in the presence of 500 ng of oligo (dT)12-18 primer.

PCR was performed using a set of human aromatase specific primers [5'-CAAGGTTATTTGATGCATGG (forward) and 5'-TTCTAAGGCTTTGCGCATGAC (reverse)] (Sigma Genosys Ltd.). The coding sequence between the two PCR primer sites is interrupted by three introns in the gene. As a control quantification, actin mRNA was also carried out by RT-PCR using the above described set of specific primers.

Real-time PCRs were performed for 40 cycles using the following temperature profile: 60˚C, 50 sec (annealing); 72˚C, 50 sec (extension); and 95˚C, 50 sec (denaturation).

Statistics. The data on cell proliferation, aromatase activity and aromatase expression are expressed as the mean ± standard errors of the mean (SEM). Statistical differences between groups were processed by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test.

Results

MT_1 mRNA expression. To confirm that MT_1-transfected MCF-7 cells had a higher MT_1 expression than vector-transfected MCF-7 we measured the expression of mRNA MT_1. As expected, MT_1 melatonin receptor was indeed overexpressed in MT_1-transfected MCF-7 in comparison to vector-transfected cells (Fig. 1).

Effects of MT_1 melatonin receptor overexpression on basal proliferation rate and aromatase activity of MCF-7. The functional significance of MT_1 overexpression in MCF-7 cells was studied by comparing the proliferation and the aromatase activity of vector-transfected and MT_1-transfected MCF-7 cells.

As shown in Fig. 2, the proliferation rate of cells over-expressing the MT_1 melatonin receptor was slightly lower than in vector transfected cells. However, transfection of the MT_1 melatonin receptor into MCF-7 cells significantly decreased aromatase activity this being 50% of vector-transfected cells ($p<0.01$).

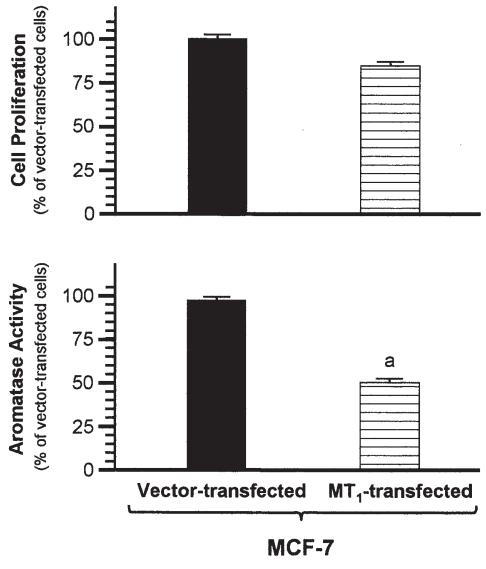

Figure 2. Cell proliferation and aromatase activity of vector-transfected and MT_1-transfected MCF-7 cells. Data are expressed as the percentage of the vector-transfected cells (mean ± SEM). [a]p<0.01 vs. vector-transfected MCF-7 cells.

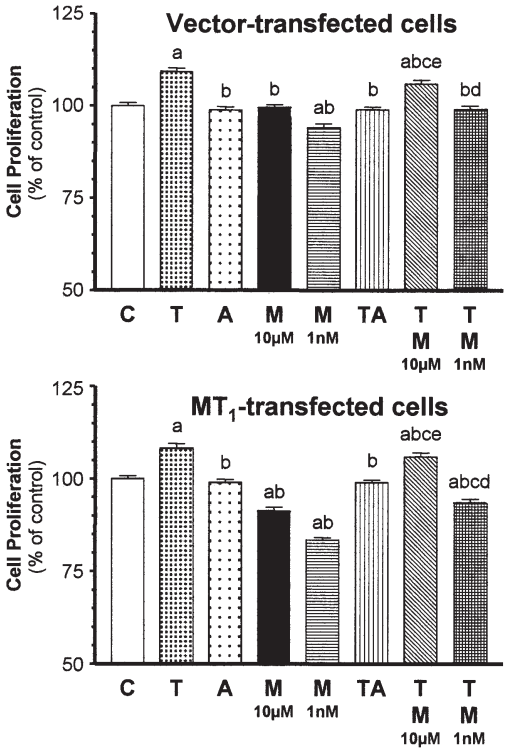

Figure 3. Effects of 1 μM testosterone (T), 10 μM-1 nM melatonin (M), 100 μM aminoglutethimide (A), or the diluent of these drugs (ethanol 0.0001%) (C), on cell proliferation of vector-transfected and MT_1-transfected MCF-7 cells. Cells were seeded into 96-well culture plates (8000 cells/well) in media supplemented with FBS for 48 h and subsequently for 5 days in media supplemented with sFBS containing the above mentioned drugs. Data are expressed as the percentage of the control group (mean ± SEM). [a]p<0.001 vs. C; [b]p<0.001 vs. T; [c]p<0.001 vs. A; [d]p<0.001 vs. M 1 nM; [e]p<0.001 vs. M 10 μM.

Effects of MT_1 melatonin receptor overexpression on melatonin-mediated inhibition of MCF-7 aromatase activity. To obtain an indirect evaluation of the possible influence of the MT_1 overexpression in the antiaromatase effects of melatonin, vector-transfected and MT_1-transfected MCF-7 cells were incubated for 5 days in estradiol-free media in the presence of testosterone and two different concentrations of melatonin (1 nM or 10 μM). As expected (Fig. 3), testosterone increased proliferation of MCF-7 cells. This stimulatory effect was reduced (p<0.001) by the aromatase inhibitor aminoglutethimide, thus indicating that, at least in part, this cell proliferation was dependent on the formation of estrogens from testosterone by the aromatase activity of the cells. Melatonin (1 nM and 10 μM) was able to counteract the stimulatory effect of testosterone in a similar manner than aminoglutethimide; thus, suggesting that it also exerts inhibitory effects on aromatase. The inhibitory effects of physiological doses of melatonin (1 nM) on the testosterone-induced cell proliferation were higher in MT_1-transfected cells (20%) than in the vector-transfected ones (10%) (Fig. 3).

In a second set of experiments, we analyzed the effect of the MT_1 overexpression in the melatonin's antiaromatase activity (measuring aromatase activity by tritiated water release assay). Vector-transfected and MT_1-transfected MCF-7 cells were exposed to physiological (1 nM) or pharmacological (10 μM) doses of melatonin in the presence of a tritiated androgenic substrate for aromatase (^{3}H-androstenedione). In MT_1-transfected cells, melatonin significantly reduced the aromatase activity (20%, p<0.001 at 1 nM; 40%, p<0.001 at 10 μM) whereas it was ineffective in vector-transfected cells (Fig. 4).

Effects of MT_1 melatonin receptor overexpression on melatonin-mediated down-regulation aromatase steady-state mRNA expression. To determine whether MT_1 melatonin receptor overexpression modifies the melatonin-induced inhibition of aromatase steady-state mRNA expression, we incubated vector-transfected cells and MT_1-transfected cells with either 1 nM or 10 μM melatonin or vehicle for 90 min, and total RNA was isolated to perform real-time RT-PCR with specific primers for human aromatase. As expected, melatonin treatment (1 nM and 10 μM) down-regulated aromatase mRNA expression in vector-transfected cells (Fig. 5). Aromatase mRNA expression of MCF-7 cells was significantly affected by MT_1 overexpression (Fig. 5). MT_1 melatonin receptor transfection induced a significant 55% inhibition of aromatase steady-state mRNA expression in comparison to vector-transfected MCF-7 cells (p<0.001). In addition, in MT_1-transfected cells melatonin treatment inhibited aromatase mRNA expression and 1 nM melatonin induced a higher and significant down-regulation of aromatase mRNA expression (p<0.05) than in vector-transfected cells.

Discussion

Melatonin displays pleiotropic physiological functions. At present, the validity of melatonin as an oncostatic agent, more especially in hormone-dependent mammary tumors, is well established (2-6,24-26). The direct oncostatic effect of melatonin might occur via specific receptors in the cell

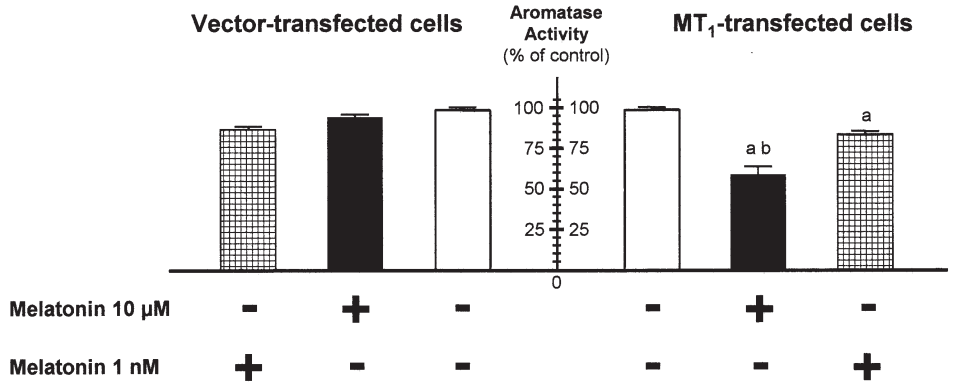

Figure 4. Effects of melatonin (10 μM or 1 nM) or the diluent (ethanol at 0.0001% final concentration) on aromatase activity of vector-transfected and MT_1-transfected MCF-7 cells. Cells were seeded onto 60x15-mm dishes (1.5x10^6 cells/dish) in media supplemented with 5% FBS for 2-3 days. Then, media were aspirated and replaced by fresh media supplemented with 5% sFBS and containing tritiated androstenedione and the indicated concentrations of melatonin. Aromatase activity was determined after 24 h of incubation, as described in Materials and methods. Data are expressed as the percentage of the control group, relative to diluent-treated control for each cell line (mean ± SEM). [a]p<0.001 vs. control; [b]p<0.001 vs. melatonin 1 nM.

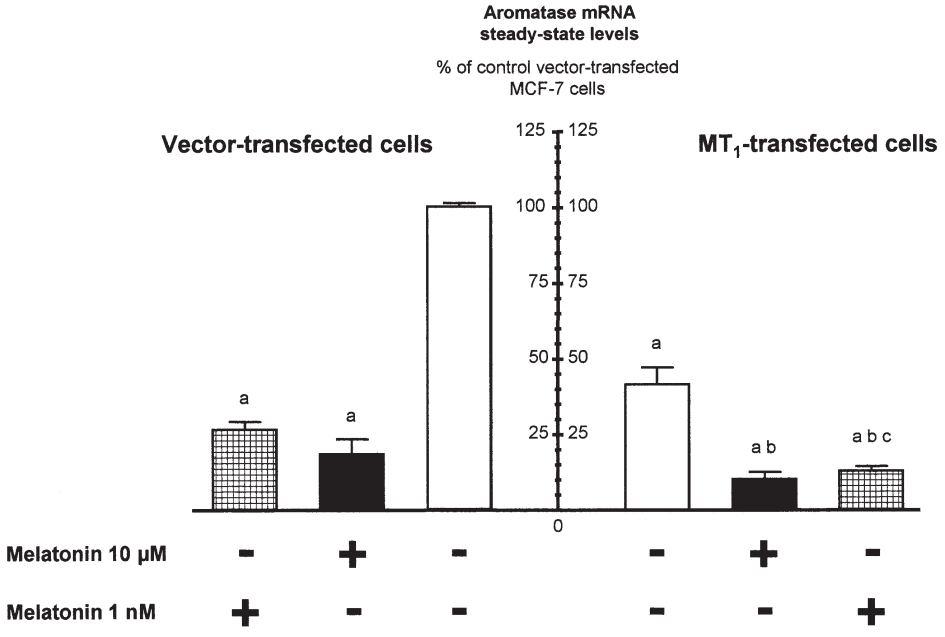

Figure 5. Effects of melatonin (10 μM or 1 nM) on aromatase mRNA steady-state levels in vector and MT_1-transfected MCF-7 cell lines. Cells were incubated with melatonin (10 μM or 1 nM) or ethanol (0.0001%) (control) for 90 min. Total mRNA was isolated from cells and reverse transcribed. cDNA was subjected to PCR using specific primers for P450 aromatase or GADPH. Data are expressed as the percentage of the control vector-transfected cells (mean ± SEM). [a]p<0.001 vs. control vector-transfected cells; [b]p<0.001 vs. control MT_1-transfected; [c]p<0.05 vs. vector-transfected cells treated with melatonin 1 nM.

membrane. Two subtypes of mammalian melatonin receptors (MT_1 and MT_2), that are members of the seven-transmembrane G protein-coupled receptor family, have been cloned (13). MT_1 receptor is coupled to various Gi-proteins that mediate adenylyl cyclase inhibition. MCF-7 human breast cancer cells are estrogen receptor-positive cells that express the MT_1 receptor and are sensitive to the antiproliferative and antiaromatase effect of melatonin (11,19). Overexpression of MT_1 melatonin receptor in human breast cancer cells both decreases the basal *in vitro* proliferative rate and enhances the sensitivity of MCF-7 cells to melatonin (19). Athymic nude mice implanted with MT_1-overexpressing MCF-7 cells develop significantly fewer palpable tumors than mice receiving vector-transfected MCF-7 cells when treated with melatonin (21).

MCF-7 cells also express aromatase gene (7) and are able to synthesize estrogens from androgens. Since we previously demonstrated that melatonin modifies aromatase activity and expression (11), we wanted to assess the effect of MT_1 over-expression on the ability of melatonin to modulate aromatase activity and expression.

Our results corroborate the role of the MT_1 melatonin receptor in melatonin's growth-inhibitory and gene-modulatory effects in MCF-7 human breast cancer cells (19). It is possible

to find in the literature additional evidence on the role of MT_1 receptor as mediator of the melatonin effects in breast cancer. Thus, even though ERα-positive MCF-7 cells express low levels of the MT_1 receptor, they exhibit a growth suppressive response to melatonin whereas the ERα-negative MDA-231 cells, which express even lower levels of the receptor are insensitive to melatonin (4,27). Furthermore, overexpression of MT_1 enhances the growth-inhibitory and gene-modulatory effects of melatonin in the ERα-positive MCF-7 cells (19).

As previously reported (19), even in the absence of melatonin in culture media (basal conditions), the proliferation rate of MCF-7 cells overexpressing the MT_1 receptor is lower than in vector-transfected and parental cells, although it was statistically significant only in comparison to parental cells. The presence of melatonin in the fetal bovine serum used in the experiments, could activate the MT_1 receptor and be the explanation for these observations (19). Furthermore, overexpression of the MT_1 melatonin receptor may enhance the level of constitutively active receptors, leading to a higher inhibition of cellular proliferation (19,28). We found a similar effect concerning aromatase activity and expression, both were significantly lower in cells overexpressing the MT_1 receptor than in the vector-transfected cells, and similar reasons as described above may explain the differences in the proliferation rate between vector and MT_1-transfected cells.

In our experiments, MT_1 overexpression enhanced the cell's sensitivity to the melatonin inhibitory effects of aromatase activity, measured either indirectly (inhibition of testosterone-induced proliferation in an estradiol-free medium), or directly (release of tritiated water assay). This enhanced inhibition of breast cancer cell aromatase activity and expression by melatonin indicates the involvement of the MT_1 receptors in these responses.

The findings presented herein point to the role of MT_1 melatonin receptor in mediating the oncostatic action of melatonin in MCF-7 human breast cancer cells and they confirm MT_1 melatonin receptor as a key mediator in the melatonin signalling pathway in breast cancer. MT_1 melatonin receptor overexpression leads to an enhanced cell responsiveness of estrogen-dependent human breast cancer cells to melatonin. Since drugs that interfere with the synthesis of steroid hormones by inhibiting the enzymes controlling the interconversion from androgenic precursors, are a pharmacological strategy currently employed to selectively neutralize the effects of estrogens on the breast, the development of endocrine therapies that up-regulate the expression of MT_1 melatonin receptor may enhance melatonin's antiaromatase effects on estrogen-responsive mammary tumors.

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