

Melatonin modulates aromatase activity and expression in endothelial cells

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Abstract. Melatonin is known to suppress the development of endocrine-responsive breast cancers by interacting with the estrogen signaling pathways. Paracrine interactions between malignant epithelial cells and proximal stromal cells are responsible for local estrogen biosynthesis. In human breast cancer cells and peritumoral adipose tissue, melatonin downregulates aromatase, which transforms androgens into estrogens. The presence of aromatase on endothelial cells indicates that endothelial cells may contribute to tumor growth by producing estrogens. Since human umbilical vein endothelial cells (HUVECs) express both aromatase and melatonin receptors, the aim of the present study was to evaluate the ability of melatonin to regulate the activity and expression of aromatase on endothelial cells, thus, modulating local estrogen biosynthesis. In the present study, we demonstrated that melatonin inhibits the growth of HUVECs and reduces the local biosynthesis of estrogens through the downregulation of aromatase. These results are supported by three lines of evidence. Firstly, 1 mM of melatonin counteracted the testosterone-induced cell proliferation of HUVECs, which is dependent on the local biosynthesis of estrogens from testosterone by the aromatase activity of the cells. Secondly, we found that 1 mM of melatonin reduced the aromatase activity of HUVECs. Finally, by real-time RT-PCR, we demonstrated that melatonin significantly downregulated the expression of aromatase as well as its endothelial-specific aromatase promoter region I.7. We conclude that melatonin inhibits aromatase activity and expression in HUVECs by regulating gene expression of specific aromatase promoter regions, thereby reducing the local production of estrogens.

Introduction

The pathogenesis and growth of breast cancer are linked to estrogens (1-3). High levels of estradiol found in some

breast tumors occur due to the uptake from the circulation or from *in situ* biosynthesis (4). One of the main pathways involved in the synthesis of estrogens in breast tumors is the aromatase pathway, which is responsible for the conversion of androgens into estrogens in extragonadal sites (5). The importance of the local biosynthesis of estrogens is highlighted by the high incidence of hormone-dependent breast cancer in postmenopausal women (6,7) and, consequently, aromatase inhibitors have become a successful treatment in this disease (8,9).

Melatonin, the main secretory product of the pineal gland, is widely known to reduce the growth and development of estrogen-responsive breast cancers (10-13). Melatonin exerts its oncostatic properties in hormone-dependent breast cancer by interfering at different levels with estrogen signaling pathways (14,15). One of the mechanisms through which this occurs is based on the regulation by melatonin of both expression and activity of several enzymes, particularly aromatase which is involved in the biosynthesis of estrogens in the peripheral tissues; thus, melatonin behaves as a selective estrogen enzyme modulator (16,17). In breast cancer cells which express aromatase (18) and the MT₁ melatonin receptor (19,20), melatonin inhibits aromatase activity and also downregulates aromatase expression at the transcriptional level (21). *In vivo* evidence of the modulator effect of melatonin on the enzyme aromatase has also been described in rats bearing dimethylbenzanthracene (DMBA)-induced mammary tumors (22).

Several lines of evidence highlight the contribution of the tumor microenvironment to tumor growth and maintenance. Adjacent adipose fibroblasts and vascular endothelial cells provide structural and biochemical support for tumor growth, mainly by increasing their estrogen biosynthesis in response to paracrine signals which are secreted by malignant breast epithelial cells in a phenomenon known as the desmoplastic reaction (23,24). Recently, our group demonstrated that melatonin interferes in the desmoplastic reaction by inhibiting adipocyte differentiation and decreasing both the aromatase activity and expression in adipose fibroblasts, thereby reducing the number of cells contributing to estrogen production in the tumor adjacent tissue (25,26). Adjacent adipose tissue surrounding malignant cells seems to account for the majority of aromatase expression in breast tumors (5,24). Endothelial cells surrounding tumor cells, may provide another source of estrogens since they also express aromatase (27,28). A

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single gene encodes aromatase and its expression is directed by alternative promoter use in a complex and tissue-specific manner (29,30). Normal breast adipose tissue maintains low levels of aromatase expression, primarily via promoter I.4. However, in mammary cancer, aromatase levels are increased by the activation of promoters II and I.3 (31), both in malignant epithelial cells and adjacent adipose fibroblasts. Recent studies have led to the identification and characterization of the novel aromatase promoter I.7 which is upregulated in breast cancer tissue. This promoter contains endothelial cell-specific *cis*-acting elements and its activity has been demonstrated in an endothelial cell line (32).

In human breast cancer cells (MCF-7), melatonin inhibits aromatase expression by decreasing the activity of promoters I.3 and II (33). The binding of melatonin to the MT₁ receptor present in MCF-7 cells has been described as a first step in the antiaromatase action of melatonin (34). Since vascular endothelial cells express aromatase and melatonin receptors (MT₁ and MT₂) (35,36) and given the antiaromatase properties of melatonin, in the present study we explored the role of melatonin on the regulation of aromatase in the human umbilical vein endothelial cell line (HUVEC) and its contribution to local estrogen biosynthesis in the tumor microenvironment.

Materials and methods

Cells and culture conditions. Human umbilical vein endothelial cells (HUVECs) were purchased from the American Tissue Culture Collection (Rockville, MD, USA). They were maintained as monolayer cultures in 75-cm² plastic culture flasks in Vascular Cell Basal Medium (VCBM) (ATCC) supplemented with Endothelial Cell Growth Kit-BBE (ATCC) which consists of 2% fetal bovine serum (FBS), 0.2% bovine brain extract, 5 ng/ml rhEGF, 10 mM L-glutamine, 0.75 U/ml heparin sulfate, 1 µg/ml hydrocortisone hemisuccinate, 50 µg/ml ascorbic acid, penicillin (20 U/ml) and streptomycin (20 µg/ml) (Sigma-Aldrich, Madrid, Spain) at 37°C in a humid atmosphere containing 5% CO₂. To avoid genetic mutation and low viability, no more than six passages of HUVECs were used for the following experiments.

Measurement of cellular proliferation. HUVECs were seeded into 96-multi-well plates at a density of 7x10³ cells/well in VCBM supplemented with 2% FBS and incubated at 37°C. After 24 h of incubation, media were aspirated and replaced by fresh media supplemented with 5% charcoal-stripped FBS (sFBS) and containing either 1 mM, 10 µM, 100 nM, 1 nM melatonin (Sigma-Aldrich) and/or 10 nM estradiol (Sigma-Aldrich) and/or vehicle (ethanol at a final concentration <0.0001%). Cells were cultured for 3 days. Cell proliferation was measured by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method, reading the absorbance at 570 nm in a microplate reader (Labsystems Multiskan RC 351; Vienna, VA, USA). MTT was obtained from Molecular Probes Inc. (Eugene, OR, USA).

Indirect measurement of aromatase activity. Indirect measurement of aromatase activity of HUVECs was obtained by evaluating cell proliferation in estrogen-free media in the

presence of testosterone. Under these conditions, cell growth depends on the transformation of androgens to estrogens via the aromatase activity of the cells. To test this, HUVECs were seeded in 96-multi-well culture plates at a density of 7x10³ cells/well in VCBM supplemented with 2% FBS and incubated at 37°C. After 24 h of incubation, media were replaced by others supplemented with 2% sFBS containing either testosterone (Sigma-Aldrich) (1 µM), melatonin (1 mM), the aromatase inhibitor aminoglutethimide (Sigma-Aldrich) (100 µM) or vehicle (ethanol). Cell proliferation was assessed after 3 days of culture by using the MTT method.

Direct measurement of cellular aromatase activity. Aromatase activity in HUVECs was measured by the tritiated water release assay which is based on the formation of tritiated water during aromatization of a labeled androgenic substrate such as androst-4-ene-3,17-dione [³H(N)] (NEN Life Science Products, Boston, MA, USA) (37). HUVECs were seeded onto 75-cm² plastic culture flasks in VCBM supplemented with 2% FBS. When a homogenous monolayer of preconfluent HUVECs was reached, media were aspirated and replaced with serum-free media containing 300 nM of the labeled substrate androst-4-ene-3,17-dione [³H(N)] (75-80 Ci/nM) in the presence of melatonin (1 mM) or the diluent (ethanol). After 24 h of incubation, culture flasks were placed on ice for 15 min to condense any water vapor, and media were transferred to tubes containing 0.25 ml ice-cold 30% trichloroacetic acid (w/v), vortexed and centrifuged at 1700 x g for 20 min. The supernatants were extracted with chloroform, vortexed and centrifuged at 1700 x g for 20 min. The resulting aqueous supernatants were adsorbed with 10% dextran-coated charcoal (Sigma-Aldrich), vortexed, centrifuged at 1700 x g for 20 min, and the supernatants were added to vials with scintillation cocktail and counted in a beta counter (Beckman LS 6000 IC, Fullerton, CA, USA). The amount of radioactivity measured in [³H]-water 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 processing, utilizing parallel dishes containing medium plus known amounts of [³H]-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 consistently >85%.

Measurement of total aromatase mRNA and aromatase promoter region pI.7 gene expression. Analysis of the aromatase and aromatase promoter I.7 mRNA expression in HUVECs was carried out by real-time reverse transcription RT-PCR after incubation of cells with melatonin (1 mM) or vehicle (ethanol) for 4 h. The total cellular RNA was isolated from HUVECs and purified with the NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. Integrity of RNA was assessed by electrophoresis in ethidium bromide-stained 1% agarose-Tris-borate EDTA gels. The absorbance ratio A_{260 nm}/A_{280 nm} was >1.8. For cDNA synthesis, 1 µg of total RNA was denatured at 65°C for 10 min and reverse transcribed for 50 min at 45°C with a cDNA synthesis kit (Bioline, London, UK) in a final volume

Table I. Primers used for amplification of mRNA transcripts of human aromatase, aromatase promoter region pI.7 and s14 (control).

mRNA	Sequence	Product size (bp)	Primer concentration (nM)
hAromatase FW	5'-GTCGTGGACTTGGTCATGC-3'	109	100
hAromatase RV	5'-CGAGTCTGTGCATCCTTCC-3'		100
hpI.7 FW	5'-AACACTCAGCTTTTCCCAAC-3'	100	200
hpI.7 RV	5'-CTTGCTGATTCACCCCTTT-3'		200
hs14 FW	5'-TCCTGCGAGTGCTGTCAGAG-3'	159	200
hs14 RV	5'-TCACCGCCCTACACATCAAAC-3'		200

FW, sense (forward) primer; RV, antisense (reverse) primer.

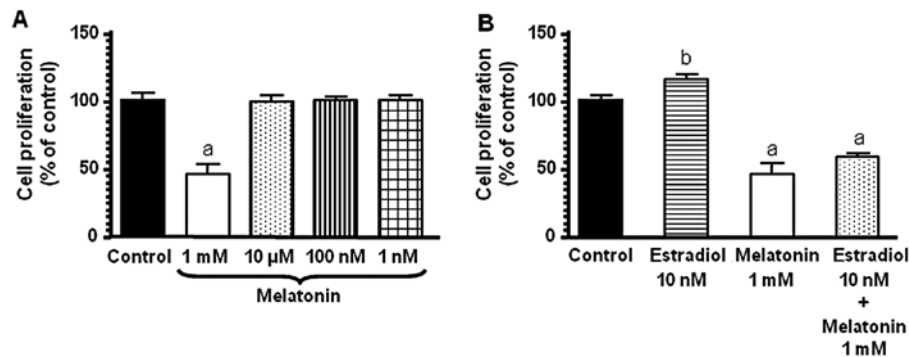


Figure 1. Effects of (A) melatonin (1 mM, 10 µM, 100 nM and 1 nM) and/or (B) 17β-oestradiol (10 nM) on HUVEC proliferation. Cells were seeded into 96-well culture plates (7,000 cells/well) in medium supplemented with 2% FBS for 24 h. Then media were aspirated and replaced by fresh media supplemented with 5% sFBS containing the indicated concentrations of melatonin and/or estradiol. After 3 days, cell proliferation was measured by the MTT method. Data are expressed as the percentage of the control group (mean ± SEM). ^aP<0.001 vs. control; ^bP<0.01 vs. control.

of 20 µl in the presence of 500 ng of oligo(dT)12-18 primers. Primers used for amplification of aromatase and aromatase promoter I.7 (Sigma Genosys Ltd., Cambridge, UK), using the housekeeping gene s14, are listed in Table I. Real-time PCRs were performed in an MX3000 (Stratagene, La Jolla, CA, USA) using Brilliant® SYBR® Green PCR Master Mix (Applied Biosystems, Madrid, Spain), following the manufacturer's instructions. Amplifications were performed for 40 cycles using the following temperature profile: 55°C for 45 sec (annealing); 72°C for 30 sec (extension) and 95°C for 30 sec (denaturation).

Statistical analysis. The data are expressed as the means ± standard errors of the mean (SEM) of six independent experiments. Statistical differences between groups were analyzed using one-way analysis of variance (ANOVA), followed by the Student-Newman-Keuls test. Results were considered as statistically significant at P<0.05.

Results

Effects of melatonin on HUVEC proliferation. To investigate the effect of melatonin on HUVEC growth, cells were cultured in VCBM supplemented with 5% sFBS in the presence of different concentrations of melatonin. Proliferation of HUVECs in the presence of melatonin (1 mM) was significantly

decreased while other concentrations of melatonin (10 µM, 100 nM and 1 nM) had no effects (Fig. 1A). The addition of 17β-estradiol (10 nM) into the media stimulated cell proliferation (Fig. 1B). This stimulatory effect of estradiol on cell proliferation was significantly counteracted by melatonin (1 mM).

Effects of melatonin on testosterone-induced HUVEC proliferation as an indirect measurement of aromatase activity. Indirect evidence of aromatase activity in HUVECs was obtained by evaluating cell proliferation in estrogen-free media in the presence of testosterone (Fig. 2). Testosterone (1 µM) significantly increased HUVEC proliferation (P<0.05) following culture for 3 days in media with sFBS. This stimulatory effect of testosterone was reduced (P<0.01) by the aromatase inhibitor aminoglutethimide (100 µM), thus, indicating that, at least in part, cell proliferation was dependent on the formation of estrogens from testosterone by the aromatase activity of the cells. Melatonin (1 mM) was also able to counteract the stimulatory effect of testosterone to values below those obtained by aminoglutethimide, which is indicative of an inhibitory effect on aromatase exerted by melatonin (Fig. 2).

Effects of melatonin on aromatase activity of HUVECs. The aromatase activity of HUVECs incubated for 24 h with tritiated

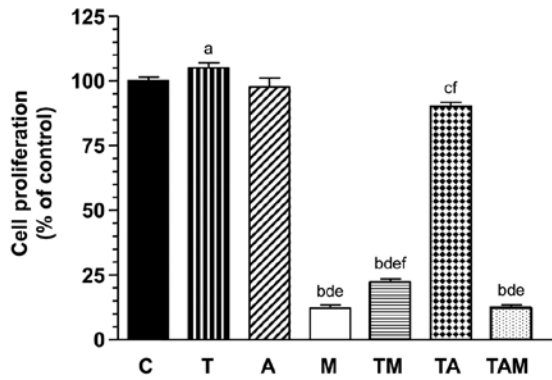


Figure 2. Indirect measurement of aromatase activity. Effects of 1 μ M testosterone (T), 1 mM melatonin (M), 100 μ M aminoglutethimide (A), or vehicle (ethanol 0.0001%) (C), either alone or in combination on HUVEC cell proliferation. Cells were seeded into 96-well culture plates (7,000 cells/well) in medium supplemented with 2% FBS for 24 h and subsequently for 3 days in medium supplemented with 2% sFBS containing the above mentioned drugs. Data are expressed as the percentage of the control group (means \pm SEM). ^aP<0.05 vs. C; ^bP<0.001 vs. C; ^cP<0.01 vs. T; ^dP<0.001 vs. T; ^eP<0.001 vs. A; ^fP<0.05 vs. M.

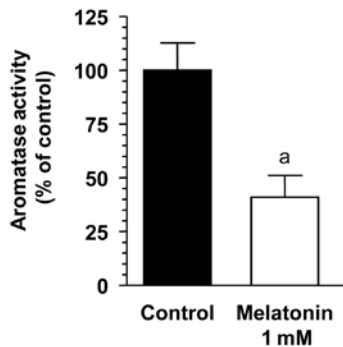


Figure 3. Effects of melatonin (1 mM) or the diluent (ethanol 0.0001%) on the aromatase activity of HUVECs. Cells were seeded onto 75-cm² plastic culture flasks in VCBM supplemented with 2% FBS. When a homogeneous monolayer of confluent cells was reached, media were aspirated and replaced by serum-free media containing tritiated androstenedione in the presence of melatonin (1 mM) or vehicle. 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 (means \pm SEM). ^aP<0.01 vs control.

androstenedione was estimated by the formation of tritiated water. Melatonin at pharmacological concentrations (1 mM) significantly decreased the aromatase activity of HUVECs (P<0.01) (Fig. 3). Melatonin (1 mM) induced a significant 60% inhibition of the aromatase activity of the HUVECs.

Effects of melatonin on total aromatase mRNA and aromatase promoter region pI.7 expression. With the aim of determining whether the inhibitory effect of melatonin on aromatase activity is due to the downregulation of aromatase expression, we incubated HUVECs with either melatonin (1 mM) or vehicle for 4 h, and total RNA was isolated to perform real-time quantitative PCR with specific primers for human aromatase. As a control, the same samples were subjected to real-time quantitative PCR with specific primers for the housekeeping gene s14, a ribosomal protein component of the 40S subunit.

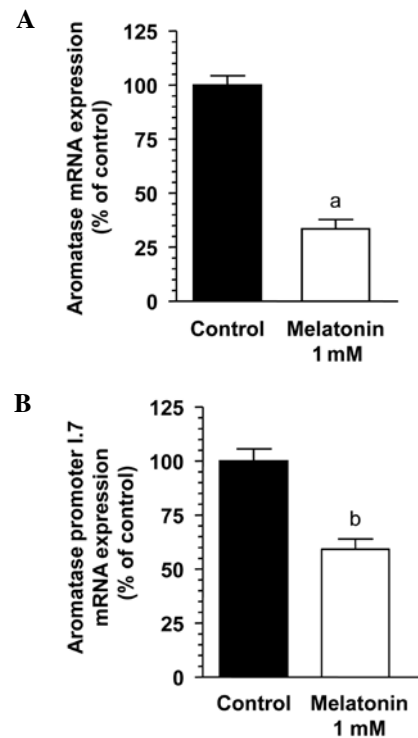


Figure 4. Effects of melatonin on (A) total aromatase mRNA and (B) aromatase promoter I.7 mRNA expression in HUVECs. Cells were incubated with melatonin (1 mM) or ethanol (0.0001%) for 4 h. Total mRNA was extracted from HUVECs and reverse transcribed. cDNA was subjected to PCR using specific primers for human aromatase, aromatase promoter I.7 or s14. Data are expressed as the percentage of the control group (means \pm SEM). ^aP<0.001 vs. control; ^bP<0.01 vs. control.

Melatonin treatment (1 mM) exhibited a significant and potent inhibition (65%) in aromatase mRNA expression in HUVECs (Fig. 4A).

To determine whether aromatase promoter I.7, an aromatase promoter mainly active in vascular endothelial cells in breast cancer, is involved in the melatonin regulation of aromatase expression, we used real-time quantitative PCR to amplify the aromatase endothelial-specific promoter I.7 transcripts from RNA extracted from HUVECs. As shown in Fig. 4B, melatonin (1 mM) significantly (P<0.01) downregulated the gene expression of the aromatase promoter I.7.

Discussion

The relevance of estrogens in the growth and pathogenesis of hormone-dependent breast cancer is supported by extensive experimental and epidemiological evidence (1-3). In premenopausal non-pregnant women, estrogen synthesis occurs mainly in the ovary. However, in postmenopausal women local estrogen biosynthesis in extraovarian sites has great relevance. Extraovarian estrogen biosynthesis is directed by different enzymes (aromatase, sulfatase) which transform androgens into estrogens as well as compounds of weak estrogenic activity into more active forms. All of these locally produced steroid hormones contribute to the genesis and development of breast tumors as well as other types of tumors such as endometrial and ovary carcinoma (24,38). Targeting aromatase has proven beneficial in treating breast cancer, and aromatase

inhibitors are being used clinically as chemotherapeutic agents for hormone-dependent breast cancer (9,39).

Melatonin, the main pineal hormone, is well known for its oncostatic properties (10,11,40). This indoleamine exerts its oncostatic effects on estrogen-dependent breast tumors mainly by two mechanisms: interfering with the estrogen signaling pathways at the estrogen receptor level (41,42) and regulating both the activity and expression of the enzymes involved in local estrogen biosynthesis. In relation with the modulator effect of melatonin on the aromatase enzyme, it is known that melatonin inhibits aromatase activity and expression *in vitro* in human breast cancer cells (21), as well as *in vivo* in rats bearing DMBA-induced mammary tumors (22). Since in breast cancer tissue aromatase expression in stromal cells adjacent to tumor cells seems to account for the majority of aromatase expression in breast tumors, and because it is known that melatonin decreases the aromatase activity and expression in adipose fibroblasts surrounding malignant cells (25,26), in this study, we investigated whether melatonin is able to regulate the production of estrogens in endothelial cells. To accomplish this, we used HUVECs which are known to express aromatase (27,28) and melatonin receptors (MT₁ and MT₂) (36).

According to previous findings (35), melatonin (1 mM) added to the culture decreased HUVEC growth. This melatonin concentration reduced HUVEC proliferation by 50% after 3 days of culture. Interestingly, the addition of estradiol (10 nM) significantly increased HUVEC growth and melatonin (1 mM) counteracted this stimulatory effect. This finding is consistent with the proangiogenic effects of estradiol that have been described by other authors both *in vivo* and *in vitro* (43) and is a consequence of the presence of estrogen receptors in endothelial cells, which enables the response to this hormone. The present study demonstrated that melatonin at a 1-mM concentration reduced the synthesis of estrogens in HUVECs through the inhibition of aromatase, the enzyme that catalyzes the rate-limiting step in the conversion of androgens to estrogens (18). These results are supported by three types of experiments. In the first experimental series, we demonstrated that melatonin (1 mM) counteracted the stimulatory effects of testosterone on HUVEC growth cultured in estrogen-free media. Under these conditions, the proliferative effects of testosterone depend on the local biosynthesis of estrogens from testosterone carried out by the aromatase activity of the cells. Thus, the inhibitory effects of melatonin could be due to its antiaromatase activity. In a second series of experiments, we directly measured the aromatase activity of HUVECs by the tritiated water release assay, finding that this was reduced by melatonin at a 1-mM concentration, which is the same concentration that gives the highest antiproliferative effects in HUVECs. Finally, we evaluated the possible role of melatonin on the modulation of aromatase at the transcriptional level in HUVECs and we demonstrated that 1 mM of melatonin induced a marked decrease in the expression of mRNA aromatase in the HUVECs.

To our knowledge, this is the first time that the effects of melatonin on aromatase activity and expression in endothelial cells have been established. However, the antiaromatase properties of melatonin have been solidly described in several other cell lines. Melatonin modulates the transformation of androgens to estrogens in breast cancer cells and adjacent

adipose fibroblasts as well as in glioma cells through the inhibition of aromatase activity and expression (26,44,45). The aromatase enzyme is encoded by one single gene and its tissue-specific expression is regulated by alternative splicing mechanisms (29). In the disease-free breast, aromatase is expressed in the mammary adipose tissue mainly by the relatively weak promoter I.4. However, in breast cancer tissue, both in malignant epithelial cells and surrounding fibroblasts, aromatase expression is mainly driven by the activation of promoters I.3 and II (31). In human breast cancer cells (MCF-7), melatonin inhibits aromatase expression by decreasing the activity of promoters I.3 and II (33). In vascular endothelial cells of breast cancer, promoter I.7 has been identified, which is a novel breast cancer-associated aromatase promoter mainly active in vascular endothelial cells in breast cancer. Promoter I.7 contains a crucial GATA-binding site characteristic of a promoter of endothelial cell origin (32) even though it is also present in adipose tissue adjacent to breast tumors (43). Excessive aromatase expression via promoters I.3, II and I.7 and the consequent increase in estrogen biosynthesis in malignant epithelial cells, undifferentiated adipose fibroblasts and adjacent endothelial cells contribute to the development and progression of breast cancer. This study shows that melatonin-dependent regulation of aromatase gene expression occurs mainly through downregulation of gene expression of the specific aromatase promoter region I.7 in HUVECs. Extensive angiogenesis, which consists of the formation of new blood vessels from existing endothelium, is a hallmark of aggressive tumor growth (46). The ability of estrogens to stimulate angiogenesis has been well-established (43). Overexpression of aromatase from aromatase promoter I.7 in vascular endothelial tissue surrounding breast tumors may contribute to the growth of breast cancer by two mechanisms. Firstly, the elevated *in situ* aromatase activity may increase the local estrogen concentration thus directly affecting tumoral growth. Secondly, the high estrogen concentration would presumably promote angiogenesis.

The effects of melatonin on cancer cells are dependent on melatonin concentrations, duration of exposure and the characteristics of cells being tested. In the present study, only pharmacological concentrations of melatonin were effective. In previous studies (10,11,16,21), a strong inhibitory effect induced by nanomolar concentrations of melatonin on human breast cancer cells was described. However, high concentrations of melatonin are necessary to obtain oncostatic actions in other types of normal and cancer cells (25,26,35,36,47,48). Melatonin is a highly lipid-soluble indoleamine which may easily cross the blood-brain barrier, and there is evidence that the melatonin concentration in the cerebrospinal fluid is higher than that in blood (49). Melatonin is also 3-fold more concentrated in neoplastic and adipose tissue of the breast (50). These high concentrations of melatonin in certain tissues may contribute towards the explanation of why high levels of melatonin are necessary to obtain various oncostatic actions of melatonin.

The findings present herein point to a role of melatonin in mediating the aromatase activity and expression in human endothelial cells by regulating the gene expression of specific aromatase promoter regions. Since the enzymes responsible for estrogen biosynthesis in tumoral tissue constitute a

pharmacological target by which to selectively reduce the effects of estrogens in hormone-dependent cancers, the development of drugs that inhibit these enzymes is currently a major strategy for treating and preventing carcinogenesis. Antiaromatase properties of the pineal hormone make melatonin an attractive compound to be tested for its possible therapeutic value in breast cancer.

Acknowledgements

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