Differential Expression of High-Affinity Melatonin Receptors (MT1) in Normal and Malignant Human Breast Tissue

Dionne C. Dillon,1* Samantha E. Easley, MS,1† Bonnie B. Asch, PhD,1,2 Richard T. Cheney, MD,3 Lena Brydon, PhD,4† Ralf Jockers, PhD,4 Janet S. Winston, MD,3 John S. Brooks, MD,3 Thelma Hurd, MD,5 and Harold L. Asch, PhD1,2

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**Abstract**

Melatonin is a pineal hormone that strongly inhibits the growth of breast cancer cells in vitro and in vivo. We report the first use of immunohistochemical analysis to determine the distribution of the high-affinity melatonin receptor subtype, MT1, in human breast tissue, the hypothalamic suprachiasmatic nucleus, and skin. The MT1 antibody, which is specific for the cytoplasmic portion of the receptor, produced cytoplasmic staining in normal-appearing breast epithelial cells and ductal carcinoma cells; stromal cells, myoepithelial cells, and adipocytes were nonreactive. The majority of nonneoplastic samples (13/19 [68%]) were negative to weakly positive, while moderate to strong reactivity was seen in most cancer samples (49/65 [75%]). Thus, although MT1 receptors were detectable in normal and malignant breast epithelium, high receptor levels occurred more frequently in tumor cells (P < .001), and tumors with moderate or strong reactivity were more likely to be high nuclear grade (P < .045). These findings may have implications for the use of melatonin in breast cancer therapy.

During the past few years, mounting evidence has implicated the pineal hormone melatonin (N-acetyl-5-methoxytryptamine) as having an important role in the regulation of multiple, diverse physiologic processes, including sleep, circadian rhythm, sexual maturation and reproduction, the immune response, and cancer. Melatonin is an indolamine synthesized from serotonin in the vertebrate pineal gland. Synthesis and release of melatonin are induced by darkness and inhibited by light via the retinohypothalamic pathway. To carry out its functions, melatonin binds to target cells via specific receptors. Two distinct types of membrane-bound, high-affinity melatonin receptors have been identified in humans: MT1 (Mel1a) and MT2 (Mel1b) receptors. These receptors are coupled to heterotrimeric guanine nucleotide binding proteins (G proteins) and are found at high concentrations in the pars tuberalis of the pituitary, the suprachiasmatic nucleus (SCN) of the hypothalamus, the retina, and ependymal cells of the choroid plexus.

Current evidence suggests that MT2 receptors in rats mediate the actions of melatonin on the retina and induce the phase shift of the circadian rhythm in the SCN. MT1 receptors seem to be involved in the acute inhibitory effect of melatonin on the SCN firing rate and also have been shown to be important for the effect of melatonin on MCF-7 breast cancer cells. Conflicting results have been reported concerning the existence of nuclear melatonin binding sites different from cloned melatonin receptors. Since melatonin easily crosses biologic membranes, the idea of intracellular melatonin binding sites has attracted considerable attention. However, convincing evidence for the existence of such a binding site is lacking.

The antitumorigenic properties of melatonin have garnered particular interest because of their potential clinical usefulness. Evidence for the oncostatic role of melatonin stems...
from numerous experimental and clinical studies. Addition of melatonin to culture medium slowed the growth of malignant melanoma cells.6 When added to the chemotherapeutic regimen of patients with advanced solid cancers, melatonin induced substantial tumor regression and prolonged overall survival.7 Melatonin also may help regulate normal functions of the human ovary and may retard or inhibit the growth of tumor cells of the colon and prostate.8-10

The antiproliferative effects of melatonin on neoplastic cells have been studied most extensively in breast carcinoma. In vivo studies in rodents show decreased incidence of mammary tumors after administration of melatonin; in vitro studies have shown that melatonin increases the estrogen receptor binding activity in MCF-7 human breast cancer cells and reduces their rate of proliferation.11 The mechanism of action by which melatonin exerts its protective effects and inhibits tumor cell proliferation is unresolved. However, melatonin is a highly efficient scavenger of hydroxyl and peroxyl radicals and may protect cells against the initiation of tumorigenesis.12 Furthermore, melatonin has been found to disrupt mitochondrial respiration in MCF-7 cells, an effect that was obliterated in the presence of the G protein melatonin receptor antagonist, luzindole. A 64% decrease in cellular adenosine triphosphate levels also was observed in melatonin-treated cells.13 Moreover, melatonin at physiologic concentrations increases the expression of both p53 and p21WAF1 proteins in MCF-7 cells, raising the possibility that induction of apoptosis by melatonin involves a p53-dependent pathway.12 Consistent with the hypothesis that melatonin may act to suppress breast cancer, decreased urinary excretion of melatonin has been reported in patients with breast cancer,14 and serum levels of melatonin were significantly depressed in patients with invasive breast cancer compared with those in healthy matched control subjects.15

As breast epithelial cells must have melatonin receptors to respond to the indolamine, we hypothesized that these receptors should be detectable in tumor cells of breast cancers. Previous identification and analysis of melatonin receptors has been primarily through the use of radioligand binding receptor assays and autoradiography,2 although the latter has not been done on breast tissue. Radioligand assays do not identify the cell type(s) expressing the receptor in a tissue. Furthermore, most of the research on the effects of melatonin has been performed on 2 models, chemically induced mammary adenocarcinoma in rats and the human tumor cell line MCF-7.12

Brydon et al16 were the first to describe an antibody that was specific for the human MT1 receptor. To our knowledge, the present immunohistochemical analysis using this antibody is the first study on MT1 receptor distribution in normal human mammary tissue and breast carcinoma. The study revealed the cell types in benign and malignant paraffin-embedded breast tissues that express MT1 receptors and analyzed associations between the receptor expression and known prognostic clinicopathologic parameters.

### Materials and Methods

#### Patient Samples and Characteristics

Paraffin tissue blocks of 53 cases of invasive ductal carcinoma and 12 cases of ductal carcinoma in situ (DCIS) diagnosed between 1993 and 2001 and 19 samples of nonneoplastic breast tissue were obtained from archive files in the Department of Pathology, Roswell Park Cancer Institute, Buffalo, NY, according to the protocols established by the Roswell Park Cancer Institute Institutional Review Board (IRB No. CIC94-21). Tissue specimens had been fixed in 10% neutral buffered formalin and processed into paraffin wax by routine methods. Six of 19 nonneoplastic samples came from tissue adjacent to carcinoma. The patients, including 83 women and 1 man, were between 29 and 92 years of age. Pathology reports were reviewed, and the patients’ demographic and clinicopathologic characteristics were recorded. Table 1 and Table 2. Tissue sections were reviewed by a pathologist (R.T.C. or J.S.W.) to confirm the histopathologic diagnosis. Immunohistochemical staining with an MT1-specific antibody was scored independently by 2 observers (D.C.D. and S.E.E.) and corroborated by a pathologist (R.T.C.).

#### MT1 Polyclonal Antiserum

Development and characterization of a rabbit antiserum specific for the high-affinity MT1 receptor was carried out by Brydon et al.16 A peptide corresponding to the 19 C-terminal

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Breast Tissue Characteristics</th>
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<tbody>
<tr>
<td></td>
<td>Nonneoplastic (n = 19)</td>
</tr>
<tr>
<td>Needle biopsy</td>
<td>2</td>
</tr>
<tr>
<td>Excisional biopsy</td>
<td>2</td>
</tr>
<tr>
<td>Excision</td>
<td>8</td>
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<tr>
<td>Lumpectomy</td>
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<td>Mastectomy</td>
<td>4</td>
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<tr>
<td>Reconstruction</td>
<td>1</td>
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<tr>
<td>Reduction mammoplasty</td>
<td>2</td>
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<tr>
<td>Selected characteristics</td>
<td></td>
</tr>
<tr>
<td>Normal tissue adjacent to tumor</td>
<td>6</td>
</tr>
<tr>
<td>Age &lt; 40 y</td>
<td>3</td>
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<tr>
<td>Age 40-49 y</td>
<td>8</td>
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<tr>
<td>Age ≥ 50 y</td>
<td>5</td>
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<tr>
<td>Primary tumor</td>
<td>—</td>
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<td>Recurrence</td>
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<td>Estrogen receptor-positive</td>
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amino acids of the MT1 receptor was used as the immunogen. This sequence has little or no homology with the same region of other high-affinity melatonin receptors. The antiserum was used at a dilution of 1:40 in tissue immunostaining.

**Histologic and Immunohistochemical Studies**

Formalin-fixed, paraffin-embedded tissue blocks were sectioned at 5-µm thicknesses and stored at 4°C until the time of processing. Tissue sections were deparaffinized in xylene, processed through a graded series of alcohols, and rehydrated in distilled water. Heat-induced antigen retrieval was performed using an H2500 Microwave Processor (Energy Beam Science, Agawam, MA) in citrate buffer (10-mmol/L concentration, pH 6) at 100°C for 15 minutes. The slides were cooled to room temperature for 30 minutes to complete antigen unmasking, and immunostaining was done according to standardized protocols. The Ventana 320 Automated Immunostainer and Basic DAB (3,3′ diaminobenzidine) detection kit (Ventana Medical Systems, Tucson, AZ) were used to perform the avidin-biotin complex immunoperoxidase technique for visualization of the primary antibody and color label. Samples were run with diaminobenzidine alone to assess nonspecific immunoreactivity of the secondary antibody and the effect of microwave antigen retrieval. To minimize nonspecific background reactivity, tissue sections were incubated with normal goat serum for 8 minutes before incubation with MT1 antiserum. Tissue sections then were incubated with 100 µL of diluted MT1 antiserum for 32 minutes. After completion of the immunostaining cycle, slides were counterstained in hematoxylin (Richard-Allan Scientific, Kalamazoo, MI), rinsed, dehydrated according to standard procedures, and coverslipped for microscopic evaluation.

**Evaluation of MT1 Staining**

A standardized scale was used to grade the intensity of immunoreactivity in each sample. The MT1 antiserum produced a consistently uniform, cytoplasmic staining pattern in all mammary epithelial cells of each sample. Breast tissue staining was graded as follows: negative, 0; weakly positive, 1+; moderately positive, 2+; or strongly positive, 3+, compared with brain tissue positive control samples.

Because of their known expression of MT1 receptors, sections of SCN and pars tuberalis with adjacent ependymal cells of benign brains obtained at autopsy were used as positive control samples. Brain and breast sections incubated with preimmune rabbit immunoglobulin (DAKO, Carpinteria, CA) or MT1 antiserum were used as negative and positive control samples, respectively. One negative control and 1 positive control sample were included in each staining run.

**Results**

**Expression of MT1 Receptor in Brain and Nonneoplastic Breast Tissue**

MT1 immunoreactivity in the SCN appeared as a granular cytoplasmic staining of neurons and adjacent paraventricular ependymal cells. Surrounding glial cells, nerve tissue, and endothelial blood vessels were nonreactive.

Immunohistochemical staining of nonneoplastic breast tissue is illustrated in Image 2A and Image 2B. Cytoplasmic immunoreactivity of luminal breast epithelial cells in ducts and acini with the MT1 antibody was observed in 16 (84%) of 19 specimens, 10 (53%) of which were weakly positive compared with the positive control sample. Five (26%) of 19 cases were moderately positive, and 1 case (5%) was strongly positive with intense cytoplasmic reactivity. Three (16%) of 19 cases were negative. All metaplastic apocrine cells were positive for MT1 staining, while stromal cells, myoepithelial cells, adipocytes, and nerve tissue were nonreactive. Skin epidermis stained uniformly 3+ for MT1 expression.
Expression of the MT1 Receptor in DCIS and Invasive Ductal Carcinoma

The results with the MT1 antibody in immunohistochemical staining of breast ductal carcinoma samples are illustrated in Image 2C through Image 2F. Some level (1+, 2+, or 3+) of cytoplasmic reactivity for MT1 was detected in nearly all (61/65 [94%]) of the ductal carcinoma cases (DCIS and invasive). Negative and weak staining (0 and 1+) was found in 16 (25%) of 65 DCIS and invasive tumors, with 12 (19%) of 65 cases demonstrating weakly positive reactivity. Moderate to strong reactivity (2+ and 3+) was present in 75% of both DCIS (9/12) and invasive tumors (40/53). Of 65 cases, 35 (54%) were moderately reactive with diffuse cytoplasmic staining. Strong MT1 reactivity comparable to the positive control (SCN) was observed in 14 (22%) of 65 cases. None of the 12 cases of DCIS was negative, while 4 (8%) of the 53 invasive tumors were negative. Interestingly, variable staining ranging from 0 to 3+ in intensity also occurred in infiltrating mononuclear inflammatory cells surrounding invasive tumor cells. The higher frequency of strong expression (2+ to 3+) of MT1 in tumor was significantly (P < .001, Fisher exact test) higher than for nonneoplastic tissues. Staining was not observed in other nonepithelial cells in these areas.

Immunohistochemical staining results for the invasive carcinomas were grouped into 2 phenotypically identifiable categories: scores 0 and 1+ (negative or reduced immunoreactivity) vs scores 2+ and 3+ (moderate or strong immunoreactivity), for testing associations with clinicopathologic parameters by Fisher exact test. No significant association was found for patient age, tumor size, number of resected or positive lymph nodes, estrogen receptor, progesterone receptor, HER2/neu, or TNM stage. However, a significant correlation (P = .045; odds ratio, 4.1) was detected between MT1 receptor staining and nuclear grade. A tumor that had moderate or strong reactivity for MT1 receptor was 4 times as likely to be of high nuclear grade (grade 3) as a tumor expressing low levels of MT1.
Expression of MT1 in nonneoplastic and neoplastic breast tissues. A, Normal ductules, weak (1+) reactivity (×20). B, Apocrine metaplasia, uniform cytoplasmic reactivity (2+), and apical membrane reactivity (3+) (×20). C-F, Invasive ductal carcinoma, 0+, 1+, 2+, and 3+ reactivity, respectively (×20). All images were stained with MT1 antibody and counterstained with hematoxylin.
Discussion

To our knowledge, the present study is the first to determine the expression pattern of the high-affinity receptor, MT1, for melatonin in specific cell types of human breast tissue. Normal and malignant breast tissues are very heterogeneous in cellular composition. Luminal epithelial cells lining ducts and acini are subtended by a layer of myoepithelial cells, and the underlying stroma contains fibroblasts, adipocytes, macrophages, endothelial cells, and leukocytes. Previous research used radioligand binding, in vitro autoradiography, and biochemical detection techniques to investigate melatonin receptors. In clinical studies, results of these techniques were used to analyze correlations among serum, tissue, and urinary levels of melatonin and patient variables; other experiments examined cultured cells and various tissues such as retina, ovary, skin, prostate, colon, brain, and breast. In these studies, the specific cell type expressing MT1 receptors in the various tissues (excluding brain) was not identified, except for the report on immunostaining of cerebrovascular MT1 in human brain sections.

In the present study, we demonstrated that in mammary gland tissue, expression of this receptor subtype occurs almost exclusively in epithelial cells. In addition, while several studies have reported MT1 receptors in the SCN of hamsters and mice, the present study is the first to find MT1 reactivity in the SCN of the human hypothalamus.

In our study, neoplastic and nonneoplastic breast tissues were immunostained with an antiserum to the high-affinity MT1 receptor to identify the expression of this receptor in individual cells. The data indicate that although MT1 receptors can be detected in normal breast tissue (16/19 [84%]) and in ductal carcinoma (61/65 [94%]), there is a quantitative difference between nonneoplastic breast tissue and carcinoma. Thus, most carcinomas showed moderate to strong expression, whereas most normal epithelium displayed weak or no reactivity. The antiserum displayed uniform, fine granular, cytoplasmic staining of normal breast epithelium and carcinoma cells. Nuclear or plasma membrane reactivity was not seen. The cytoplasmic reactivity is consistent with the location of the targeted antigen (peptide 536), since this peptide corresponds to the 19 amino acid sequence of the C-terminal region of the receptor.

The specificity of the antiserum is supported by the lack of reactivity with the surrounding stromal cells in nonneoplastic breast tissue. In one interesting exception, cytoplasmic staining also was observed in mononuclear inflammatory cells surrounding invasive tumor cells. Melatonin has been shown to have a role in the immune response, and T-helper lymphocytes may have melatonin receptors that mediate increased production of certain lymphokines.

To our knowledge, this is also the first report of MT1 reactivity in human skin. This observation is consistent with the identification of high-affinity melatonin receptor expression in human malignant melanoma cells. In fact, the indolamine was first identified in bovine pineal extracts owing to its ability to aggregate melanin granules, thus lightening the color of frog skin.

Weakly positive reactivity (1+) was characteristic of half (52%) of the nonneoplastic breast tissue samples, whereas weak expression in carcinomas was less common (DCIS, 3/12 [25%]; invasive carcinoma, 9/53 [17%]). The overall significant difference (P < .001) in staining between normal and malignant breast tissue samples suggests that melatonin may...
have different effects in nonneoplastic than in malignant cells. The mechanism of action of this hormone on normal breast epithelium may be 2-fold. First, normal cells may have a constitutive basal level of MT1 receptor expression for mediating the normal physiologic function of melatonin. Such a population may be represented by the 53% of nonneoplastic cases (10/19) that exhibited weak expression of MT1 receptors. Second, the level of MT1 receptor expression may be up-regulated in normal epithelium in response to various stimuli such as injury or alteration in the hormonal environment. This population of cells may be represented by the 26% of cases (5/19) that exhibited moderate reactivity and the 1 case (5%) that was strongly reactive. Heterogeneity of MT1 receptor expression in normal tissue also may reflect the multiple factors that affect the cyclic hormonal milieu regulating breast epithelium. The mechanism and impact of melatonin on normal breast epithelial cells remain largely unknown.

At the level of statistical power the present data allowed, statistically significant associations were not detected between MT1 immunoreactivity and patient age, tumor size, number of resected or positive lymph nodes, estrogen receptor, progesterone or Herceptin receptor (HER2/neu), or TNM stage (data not shown). However, a significant correlation (P = .045; odds ratio, 4.1) was detected between MT1 receptor staining and nuclear grade. Thus, a tumor that had moderate or strong reactivity for MT1 was 4 times as likely to be of high nuclear grade (grade 3) as a tumor expressing low levels (score 0 or 1+) of the receptor. This supports the inverse relationship between serum melatonin levels and nuclear grade reported elsewhere.28 Nuclear grade has been used as a prognostic factor in breast cancer.29 Our data are consistent with those from a study indicating that MT1 receptor expression is correlated inversely with melatonin concentrations and that absence of melatonin increases expression of the MT1 receptor.30 We speculate that MT1 receptor levels in tissues, particularly carcinoma of high nuclear grade, might be up-regulated in the presence of low serum melatonin levels. Thus, high nuclear grade, low serum melatonin levels, and high MT1 receptor expression in tumor cells might represent a constellation of features that are characteristic of aggressive breast cancer.

With regard to MT1 receptor expression among tumors of different histologic types, 2 of the 53 cases of invasive carcinoma examined in our study were of mixed histologic types composed of ductal carcinoma with lobular differentiation. These 2 compartments exhibited differential staining, with the lobular component having significantly less immunoreactivity than the areas of ductal carcinoma (data not shown). The difference in staining intensity observed may be due to the patterns of invasion that characterize these 2 entities.31,32 For instance, lobular and ductal carcinoma have different patterns of invasion, a morphologic feature that aids in distinguishing between these 2 types of breast carcinoma. One important characteristic of both invasive lobular and ductal carcinoma is altered expression of adhesion molecules. Virtually all well-differentiated lobular carcinomas lack cell-cell adhesion molecules, while only half of invasive ductal carcinomas exhibit this phenotype.32 As a result, lobular carcinoma tends to infiltrate breast tissue in a single-file pattern, while ductal carcinoma invades as groups of cells. The differential expression of melatonin receptors in invasive lobular carcinoma and invasive ductal carcinoma may be related to the effects of melatonin on these cell types: either down-regulating or up-regulating the expression of adhesion molecules via its G protein–coupled cascade.33,34

The difference in MT1 receptor immunoreactivity between DCIS and invasive ductal carcinoma is consistent with a possible role for melatonin in the progression of DCIS to invasive carcinoma. The natural history of tumor progression includes a multistep malignant transformation process, growth, and invasion.31 Progression to malignancy in breast cancer cells is accompanied by altered expression of proteins such as E-cadherin and beta1-integrin that facilitate or mediate cell-cell interactions.33,34 Melatonin decreased the invasiveness of MCF-7 cells by causing a reduction in cell attachment and motility, accompanied by increased expression of these 2 proteins.34 It would be worthwhile to determine whether a pattern exists between MT1 expression and expression of relevant cadherins and integrins in breast tissue and whether the melatonin receptors present in breast cancers have been altered in structure, function, or number during carcinogenesis.

It is clear that enhanced expression of the high-affinity MT1 receptor is associated with breast cancers. The nature of any putative causal relationship to development and progression of this malignant neoplasm remains to be elucidated. Our findings will be useful to understanding the biologic bases for supporting, refining, or refuting the current use of melatonin in therapy and prevention of this disease.

From the 1State University of New York at Buffalo School of Medicine; the 2Division of Experimental Pathology and Departments of 3Pathology and Laboratory Medicine and 5Surgery, Roswell Park Cancer Institute, Buffalo, NY; and the 4Department of Cell Biology, Institut Cochin, Paris, France.

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* These authors contributed equally to the project and share the position of first author.
† Dr Brydon is currently with the Psychobiology Group, Department of Epidemiology and Public Health, University College London, England.
Dillon et al / MT1 RECEPTORS IN BREAST TISSUE

Address reprint requests to Ms Dillon c/o Dr Asch: Dept of Pathology and Laboratory Medicine, Roswell Park Cancer Institute, Elm and Carlton Sts, Buffalo, NY 14263.

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