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Expression of melatonin MT_1 and MT_2 receptors, and $ROR\alpha_1$ receptor in transplantable murine Colon 38 cancer

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Abstract

OBJECTIVES: There are some data suggesting that melatonin exerts oncostatic action through membrane as well as nuclear receptors. In previous studies we demonstrated the antiproliferative and proapoptotic action of melatonin on transplantable murine Colon 38 adenocarcinoma cells. Therefore, the aim of the present study was to determine whether the membrane melatonin receptors MT_1 and MT_2 as well as the nuclear receptor RZR/RORα are expressed in Colon 38 cells.

MATERIAL AND METHODS: Adult male B6D2F1 mice were used in this experiment. The induction of tumor was conducted by subcutaneous injection of 0.2 mL of a 33% suspension of Colon 38 cancer cells into axillary region. Expression of mRNA encoding MT_1 and MT_2 melatonin membrane receptors was studied by RT-PCR analysis, and expression of $ROR\alpha_1$ nuclear receptor protein was studied by Western blot analysis.

RESULTS: The expression of mRNA encoding both MT_1 and MT_2 melatonin receptors was demonstrated in Colon 38 cancer cells. Moreover, immunodetection revealed the expression of MT_1 and $ROR\alpha_1$ proteins in these cells.

CONCLUSIONS: Our studies on Colon 38 adenocarcinoma cells support the concept that both membrane and nuclear receptors are involved in the oncostatic action of melatonin.

CONCLUSIONS: On the basis of this preliminary open study it seems that melatonin administration may be beneficial for elderly subjects.

ORIGINAL ARTICLE

Introduction

There is substantial experimental evidence indicating inhibitory influence of pineal hormone melatonin on the malignant tumor formation and/or growth [1–4]. In the previous studies we demonstrated that in transplantable murine Colon 38 adenocarcinoma melatonin exerts inhibitory effect on the tumor cell proliferation and stimulatory effect on tumor cell apoptosis [5–9]. Moreover, we suggested that melatonin decreases the tumor proliferation acting through both membrane and nuclear receptors. On the other hand, the induction of apoptosis by melatonin seems to depend mainly on its action through RZR/RORα receptor [8, 9]. Therefore, the aim of the present study was to determine whether membrane melatonin receptors MT_1 and MT_2 as well as nuclear receptor RZR/RORα are expressed in Colon 38 cells.

Material and methods

Animals and tumor induction

Adult male B6D2F1 mice, weighing 30±4g were used in this experiment. B6D2F1 strain is the first generation of the crossbred between C57BL/6 and DBA/2 strains of mice. The animals were maintained under controlled temperature (22±2 °C) and 12/12 light/dark cycle (lights on from 08:00 h to 20:00 h) with free access to food and tap water. The study was performed in July. The induction of tumor was conducted by subcutaneous injection of 0.2 mL of a 33% suspension of Colon 38 cancer cells into axillary region. The Colon 38 is transplantable adenocarcinoma originally induced in the colon of C57BL/6 mice by 1,2-dimethylhydrazine [10].

MT1 and MT2 expression

mRNA was isolated by guanidium method [11]. Single stranded cDNA was then synthesized from tumors using the following method. 5 μg of RNA was preincubated with 1 μg of oligo$(T)_{15}$ in 20 μL RNase free H_2O at 85 °C for 10 min, and then rapidly chilled on ice. Then 1 μL RNasin (40 U/μL), 8 μL 5x RT buffer, 8 μL dithiothhreitol (100 mM), and 2 μL deoxyribonucleotides (dNTP; 10 mM of each) (all reagents from Promega, Madison, WI) were added and the mixture was incubated at 42 °C for 3 min. Finally, 1 μL Moloney murine leukemia virus reverse transcriptase (Mo-MuLVRT; 200 U/μL) (Promega, Madison, WI) was added to give a final volume of 40 μL, and the reaction was incubated at 42 °C for 60 min, then terminated by placing it on ice after deactivation at 95 °C for 5 min. After each extraction, one sample was run by RT-PCR without adding reverse transcriptase enzyme in order to check for possible DNA contamination.

The following oligonucleotides (5'to 3') (Roche, Mannheim, Germany) were used as primers for RT-PCR:
mouse MT_1: `GTG GTG GAC ATT CTG GGC AAC CTG` (exon 1) and `GGT TGG GCA TGA TGG CGA TGA GTG` (exon 3), they amplified a single 374 bp band,
mouse MT_2: `TAC ATC AGC CTC GTC TGG CTC C` (sense) and `TTC CTC GTA GCC TTG GCC TTC C` (antisense), they amplified a single 239 bp band.

For the PCR reactions (1x PCR reaction buffer, 1.5 mM $MgCl_2$, 400 μM dNTPs), 3 mL of RT product was amplified after "hot start" procedure in a final volume of 25 μL using 2.5 U Taq-DNA-polymerase (Promega, Madison, WI). 35 PCR cycles were performed (94 °C, 1 min; 60 °C, 1 min; 72 °C, 1 min), followed by a final 10 min extension at 72 °C. Specific primers for mouse β-actin were used to test the efficiency of reverse transcription. The β-actin forward (exon 3) and reverse (exon 6) primers were (5' to 3') `TTG TAA CCA ACT GGG ACG ATA TGG` and `GAT CTT GAT CTT CAT GGT GCT AGG` (746-bp fragment), respectively.

Southern blotting

After amplification, 5 μl PCR reaction was electrophoresed in 2% agarose gel in 1X TAE buffer and visualized by staining with ethidium bromide and UV illumination using DNA molecular weight marker VI DIG-labelled (Roche, Mannheim, Germany) as size marker. The cDNA was transferred to a hy^+-nylon membrane (Amersham-Pharmacia Biotech, Uppsala, Sweden) using a vacuum blotting system (Hoeffer, San Francisco, Calif.), with 10X SSC as transfer solution and croos-linked to the nylon membrane using a calibrated UV light source. Blots were prehybridized at 68 °C for 1 hour in prehybridization buffer (5X SSC, 0.1% N-laurylsarcosyl, 0.02% SDS, 1% blocking reagent). The hybridization was performed at 60 °C overnight in the same prehybridization buffer containing 25 ng/mL of labelled probe with oligonucleotide tailing kit (Roche, Mannheim, Germany). Thereafter, blots were washed twice for 5 min in 2X SSC/0.1 % SDS at room temperature and twice for 5 min in 0.1X SSC/0.1% SDS at 60 °C. To detect the hybridization signal, the blots were incubated 30 min in 0.1 M maleic acid containing 0.15 M NaCl and 1% blocking reagent and 30 min with anti-DIG-AP (anti-digoxigenin conjugated to alkaline phosphatase). Finally, they were washed and incubated in CSPD. Blots were then exposed to Kodak X-OMAT AR film at room temperature.

The probes used in this study were:
MT_1 probe: `TGC CAC AGC TAA ACT CAC CAC AAA TAT ATT`,
MT_2 probe: `ACA AAG AAA TTG GGC ACC AAA GCC ACC AGA GT`,
both of them were directed against fragment amplified by PCR.

Immunodetection of MT1

Tumors were washed with ice-cold PBS. They were homogenized with a Polytron homogenizer (Kinematica, Switzerland) at 4 °C in HEPES 20 mM, pH 7.4 containing 0.02 % (w/v) bacitracin, 0.4 mM PMSF, 1 mM benzamidine, 1.5 μM pepstatin A, 0.1 mM TLCK, and 0.1 mM aprotinin. The tumor homogenates were then centrifuged for 10 min at 4000 xg, and supernatants were collected to assay the protein expression. The protein content of the supernatants was determined by the method of Bradford (Bradford). Samples were run into the SDS sample buffer on a 12% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Blots were blocked in 25 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl, 0.05% (v/v) Tween 20 (TBST buffer), and 5% (w/v) dry milk. Western blot analysis was carried out using a 1:500 dilution in block buffer of purified antisera against MT_1 for 2 hours at RT. After TBST washing procedure, the blots were incubated with 1:2000 peroxidase labelled anti-rabbit antibody (Amersham-Pharmacia Biotech, Uppsala, Sweden) in TBST for 1 hour at RT. The immunodetection was performed using the enhanced chemiluminescence ECL system (Amersham-Pharmacia Biotech, Uppsala, Sweden).

Immunodetection of RORα

Tumors were washed with ice-cold PBS. They were homogenized with a Polytron homogenizer (Kinematica, Switzerland) at 4 °C in HEPES 20 mM, pH 7.4 containing 0.02 % (w/v) bacitracin, 0.4 mM PMSF, 1 mM benzamidine, 1.5 μM pepstatin A, 0.1 mM TLCK, and 0.1 mM aprotinin. The tumor homogenates were then centrifuged for 10 min at 4000 xg, and supernatants were collected to assay the protein expression. The protein content of the supernatants was determined by the method of Bradford (Bradford). Samples were run into the SDS sample buffer on a 8% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Blots were blocked in 25 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl, 0.05% (v/v) Tween 20 (TBST buffer), and 5% (w/v) dry milk. Western analysis was carried out using a 1:500 dilution in block buffer of specific polyclonal antibody against RORα (RORα$_1$, sc-6062, Santa Cruz Biotechnology, CA, USA) for 4 hours at RT. After TBST washing procedure, the blots were incubated with 1:1000 peroxidase labelled anti-goat antibody (DAKO, Denmark) in TBST for 1 hour at RT. The immunodetection was performed using the enhanced chemiluminescence ECL system (Amersham-Pharmacia Biotech, Uppsala, Sweden).

Results

Presence of membrane melatonin receptors in Colon 38 cancer cells

PCR primers specific for MT$_1$ and MT$_2$ receptors were used in a RT- PCR reaction with RNA isolated from mice Colon 38 cancer cells to determine which of these mouse melatonin receptor subtypes are expressed in these tumor cells. The expression of mRNA encoding MT$_1$ and MT$_2$, is presented in Figures 1 and 2. Agarose gel electrophoresis of both PCR products showed a single DNA band of the expected size (MT$_1$: 374 bp; MT$_2$: 239 bp). No specific band was obtained from reaction in which cDNA was omitted. The β-actin primers amplified the expected 746-mer product, indicating the absence of any DNA contamination in analysed samples.

The Southern blot analysis performed with DIG-labelled MT$_1$ (Fig. 1) and MT$_2$ (Fig. 2) melatonin receptor-specific probes confirmed the identity of DNA fragments.

Finally, we used specific rabbit antisera against MT$_1$ melatonin receptor (obtained by Dr. Guerrero group) to determine whether these cancer cells expressed MT$_1$ protein. The Western blot analysis revealed a positive result; acrylamide gel electrophoresis showed a band of the expected weight (37 KDa). Mouse brain used as positive control also gave a band of the same weight (Fig. 1).

Immunodetection of melatonin nuclear receptor in Colon 38 cancer cells

During Western blot analysis, the presence of RORα$_1$ receptor was demonstrated at the protein level in Colon

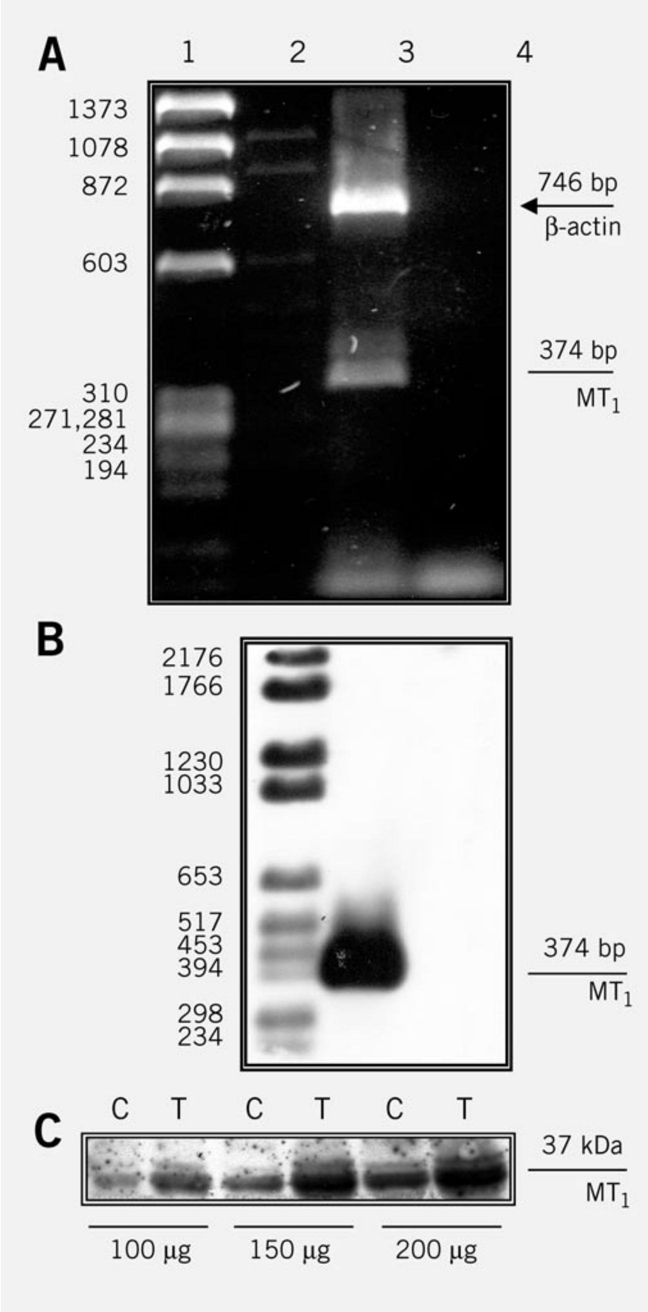

Fig. 1. MT$_1$ expression. **A** - RT-PCR analysis of melatonin receptor MT$_1$ mRNA from Colon 38 tumor. Lanes 1 and 2 show PCR molecular size marker (ϕX174/Hae III) and Southern molecular size marker (DNA molecular weight marker VI DIG-labelled), respectively. Lane 3 shows the MT$_1$ (374 bp) and β-actin (746 bp). Lane 4 shows PCR reaction without cDNA substrate (PCR control). **B** - Southern blot hybridization of the PCR products shown in panel A with the DIG-labelled melatonin receptor-specific probe. **C** - Western blot analysis of MT$_1$. Different content proteins (from 100 to 200 μg) from Colon 38 tumor were separated by 12% SDS-PAGE, and then transferred to nitrocellulose and immunoblotted with specific purified rabbit antisera against MT$_1$. T - Colon 38 tumor; C - brain used as positive control.

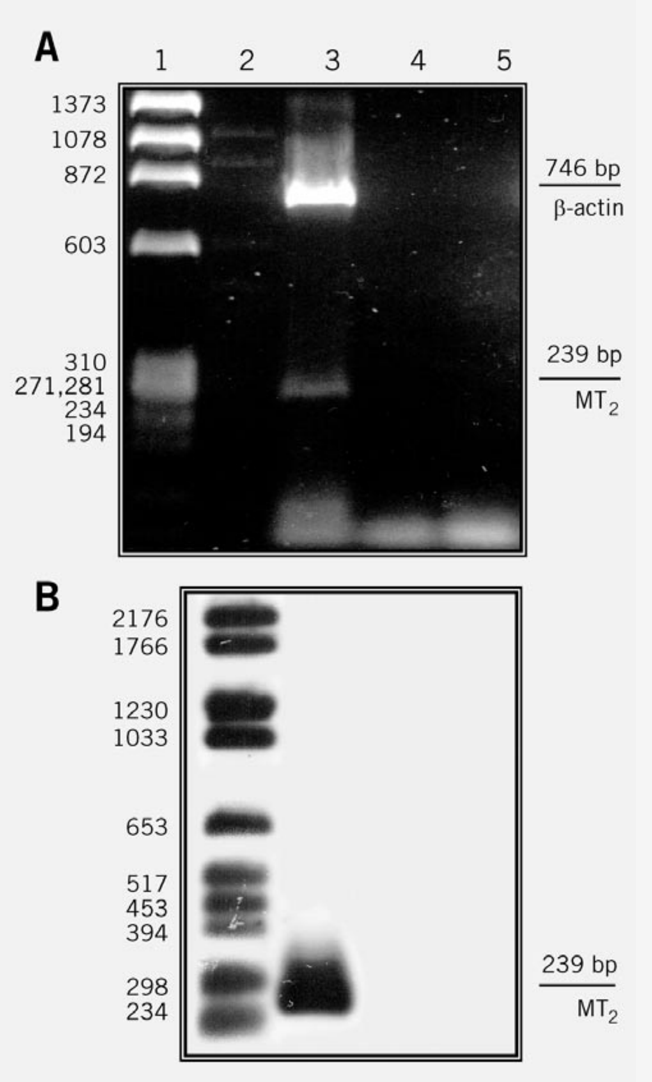

Fig. 2. MT_2 expression. **A** - RT-PCR analysis of melatonin receptor MT_2 mRNA from Colon 38 tumor. Lanes 1 and 2 show PCR molecular size marker (ϕX174/Hae III) and Southern molecular size marker (DNA molecular weight marker VI DIG-labelled), respectively. Lane 3 shows the MT_2 (239 bp) and β-actin (746 bp). Lane 4 shows mRNA from tumor processed in absence of MuLV retrotranscriptase to avoid DNA contamination. Lane 5 shows PCR reaction without cDNA substrate (PCR control). **B** - Southern blot hybridization of the PCR products shown in panel A with the DIG-labelled melatonin receptor-specific probe.

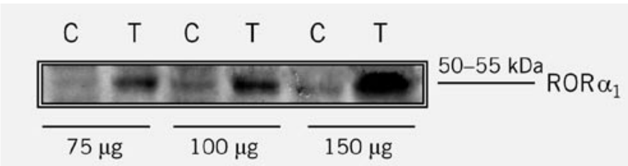

Fig. 3. $ROR\alpha_1$ expression. Western blot analysis of $ROR\alpha_1$. Different content proteins (from 75 to 150 μg) from Colon 38 tumor were separated by 8% SDS-PAGE, and then transferred to nitrocellulose and immunoblotted with specific polyclonal antibody against $ROR\alpha_1$.
T - Colon 38 tumor; C - brain used as positive control.

38 cancer cells (Fig. 3). We detected a protein with a weight between 50 and 55 KDa, which is in agreement with previous studies [12]. Mouse brain was used as positive control.

Discussion

Melatonin has been shown to regulate many physiological, endocrine, and non-endocrine functions [13]. Like many other hormones, it acts through the activation of specific, seven transmembrane domain G protein-coupled receptors in target cells [14]. Three melatonin membrane receptors have been cloned [14]. Those with well defined functional pharmacology in target tissues as well as known molecular structure and chromosomal gene localization are termed MT_1 and MT_2 receptors, whereas putative melatonin receptor with characterized pharmacology in target tissues but without known molecular structure is named MT_3 receptor [15, 16].

Besides well-known actions via membrane receptors melatonin may also exert its biological action through nuclear signalling involving RZR/ROR receptors [17, 18]. The RZR/ROR receptors belong to a novel subclass of orphan nuclear receptors with three subtypes (α, β, and γ) and four splicing variants of α-subtype. They have been cloned simultaneously by two different groups and obtained the names retinoid Z receptor (RZR) [19] and retinoid acid receptor-related orphan receptor (ROR) [21]. RZR/RORα is expressed nearly ubiquitously in all tissues [19], whereas RZR/RORβ is restricted to the brain [21]. RZR/RORγ is expressed preferentially in skeletal muscle but also in the thymus, testis, prostate, pancreas, liver, and heart [22].

In the present study Colon 38 cancer cells were shown to express MT_1 and MT_2 receptors mRNA by RT-PCR and Southern blot analyses, and MT_1 and $ROR\alpha_1$ receptors proteins by Western blot analysis.

Although melatonin has been shown to exert direct antiproliferative and proapoptotic effects on tumor cells, the mechanisms of oncostatic action of melatonin seem to be complex and are only partially clarified. It seems that melatonin may exert its oncostatic activity through modulation of the endocrine and immune systems, antioxidative activity, antiangiogenic activity but also through the direct antiproliferative action [2, 4].

Both membrane and nuclear melatonin receptors seem to play a role in the regulation of immune functions [23]. MT_1 melatonin receptors are expressed in thymus and spleen as well as in all the lymphocyte subpopulations ($CD4^+$, $CD8^+$, doubled negative, doubled positive, and B cells) from the thymus [24]. Moreover, a fundamental role of RZR/RORα receptor activation in the modulation of both IL-2 and IL-6 production in human lymphocytic (Jurkat) and monocytic (U937) cell lines is suggested by Garcia-Mauriño et al. [25].

A question arises what type of melatonin receptors is involved in the direct oncostatic effects of this hormone. Both membrane and nuclear melatonin receptors have been identified in some tumors.

Many data suggest the involvement of the membrane melatonin receptors in oncostatic action of melatonin. It has been shown that melatonin-induced suppression of hepatoma growth is mediated via G-protein connected membrane receptors and involves the fall of cyclic AMP formation [26]. Melatonin and 6-chloromelatonin (membrane receptor agonist) but not CGP-52608 (the putative nuclear receptor agonist) inhibit the growth of human uveal melanoma cells in vitro [27]. Moreover, melatonin inhibit the proliferation of JAr and JEG-3 human choriocarcinoma cell lines expressing MT_2 receptors but not in a 3A-Sub-E transformed trophoblast cell line devoid of melatonin membrane receptors [28, 29]. Involvement of melatonin membrane receptors in antiproliferative and proapoptotic effects of melatonin on Colon 38 cells was also suggested [9]. Expression of MT_1 receptors has been found in MCF-7 human breast cancer cells [30, 31], in DU-145 and LNCaP prostate cancer cell lines [32, 33], in PC12 rat pheochromocytoma cell line [30], and in N1E-115 mouse neuroblastoma cell line [34].

On the other hand, some data support the involvement of the nuclear RZR/ROR receptors in antiproliferative and proapoptotic action of melatonin on tumor cells. Melatonin and CGP 52608, the RZR/ROR receptor ligand, similarly inhibited the proliferation of Colon 38 cancer cells *in vitro* and *in vivo* [5, 7], ovarian cancer cell line [35], and prostate cancer cell lines DU-145 and LNCaP *in vitro* [36, 37]. Both melatonin and CGP 52608 induced apoptosis in Colon 38 cells [5, 7] and the proapoptotic action of melatonin was blocked by CGP 54644 assumed as RZR/ROR receptor antagonist (Winczyk, Pawlikowski, Guerrero, Karasek, unpublished data). Moreover, in MCF-7 cells RORα transcripts have been identified [21, 30, 38]. RORα receptors were expressed also in DU-145 prostate cancer cells [36].

Summing up, our studies on Colon 38 adenocarcinoma cells support the concept that both membrane and nuclear receptors are involved in the oncostatic action of melatonin.

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