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Does melatonin induce apoptosis in MCF-7 human breast cancer cells in vitro?

Cos S, Mediavilla MD, Fernández R, González-Lamuño D, Sánchez-Barceló EJ. Does melatonin induce apoptosis in MCF-7 human breast cancer cells in vitro? J. Pineal Res. 2002; 32:90–96. © Munksgaard, 2002

Abstract: Melatonin inhibits proliferation of the estrogen-responsive MCF-7 human breast cancer cells. The objective of this work was to assess whether melatonin not only regulates MCF-7 cell proliferation but also induces apoptosis. In this experiment we used 1,25-dihydroxycholecalciferol (D_3) as a positive control because it inhibits MCF-7 cell proliferation and induces apoptosis. MCF-7 cells were cultured with either 1 nM melatonin, 100 nM D_3 or its diluent to determine their effects on cell proliferation, cell viability, cell-cycle phase distribution, population of apoptotic cells, and expression of p53, p21WAF1, bcl-2, bcl-X_L and bax proteins. After 24 or 48 hr of incubation, both melatonin and D_3-treatment significantly decreased the number of viable cells in relation to the controls, although no differences in cell viability were observed between the treatments. The incidence of apoptosis, measured as the population of cells falling in the sub-G_1 region of the DNA histogram, or by the TUNEL reaction, was similar in melatonin-treated and control cells whereas, as expected, apoptosis was higher among cells treated with D_3 than in controls. The expression of p53 and p21WAF1 proteins significantly increased after 24 or 48 hr of incubation with either melatonin or D_3. No significant changes in bcl-2, bcl-X_L and bax mRNAs were detected after treatment with melatonin whereas in D_3-treated cells, a significant drop in bcl-X_L was observed. These data support the hypothesis that melatonin reduces MCF-7 cell proliferation by modulating cell-cycle length through the control of the p53–p21 pathway, but without clearly inducing apoptosis.

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Introduction

The role of melatonin, the main pineal secretory product, on the development of mammary cancer has been widely studied (see reviews of Sánchez-Barceló et al., 1988, 1997; Blask, 1993, 2001; Cos and Sánchez-Barceló 2000a,b). Melatonin inhibits proliferation of the estrogen-responsive MCF-7 human breast cancer cells (Blask and Hill, 1986; Hill and Blask, 1988; Cos and Sánchez-Barceló, 1996a, 2001). These antiproliferative actions of melatonin are effective at physiological doses and depend on the rate of cell proliferation (Cos and Sánchez-Barceló, 1995), the pattern (pulsatile or continuous) of exposure to melatonin (Cos and Sánchez-Barceló, 1994), and the presence of serum in culture media (Blask and Hill, 1986). Melatonin also reduces the invasive and metastatic properties of MCF-7 cells (Cos et al., 1998). The mechanism involved in the oncostatic effects of melatonin remain unknown (Cos and Sánchez-Barceló, 2000a). Most efforts in understanding the oncostatic properties of melatonin have been centered on its antiproliferative effects. However, tumor growth should be considered as a balance between cell proliferation and cell death. The objective of the present work was to assess whether melatonin regulates mammary tumor growth by inducing apoptosis. In this experiment we used 1,25-dihydroxycholecalciferol (vitamin D_3), the active metabolite of vitamin D, as a positive control. Vitamin D_3 and its analogs exert antiproliferative effects on MCF-7 cells, in vitro (Chouvet et al., 1986; Abe et al., 1991; Colston et al., 1992; Saez et al., 1993; Welsh, 1994) and induce apoptosis (Welsh, 1994; Narvaez and Welsh, 1997; Simboli-Campbell et al., 1997; Van Weelden et al., 1998).

Materials and methods

Cells and culture conditions

MCF-7 human breast cancer cells, purchased from the American Tissue Culture Collection (Rockville, MD, USA), were maintained as monolayer cultures in 75 cm^2 plastic culture flasks in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (GIBCO, Cergy Pontoise, France), penicillin (20 units/mL) and streptomycin (20 μg/mL) (Sigma

Chemical Co., St Louis, MO, USA), at 37°C in a humid atmosphere containing 5% CO_2. Cells were subcultured every 3–4 days by suspension in 5 mM Na_2-EDTA in PBS (pH 7.4) at 37°C for 5 min.

Before each experiment, stock subconfluent (80%) monolayers of MCF-7 cells were incubated with 5 mM Na_2-EDTA in PBS (pH 7.4) at 37°C for 5 min, resuspended in DMEM 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.

Cells were seeded at a density of 500,000 cells/plate (60 × 15 mm) in DMEM supplemented with 10% FBS and antibiotics and cultured for 24 hr at 37°C and 5% CO_2. After this period, media were aspirated and replaced by fresh media containing either 1 nM melatonin (Sigma Chemical Co., St Louis, MO, USA), 100 nM 1,25-dihydroxyvitamin D_3 (Sigma Chemical Co., St Louis, MO, USA) or the diluent of both hormones (ethanol, final concentration 0.0001%). At 24 or 48 hr of incubation culture media were aspirated and cells were harvested from plates and cell counts as well as cell viability were calculated. The dosage of vitamin D_3 (100 nM) was determined in a preliminary experiment as the amount which induced antiproliferative effects similar to those obtained with 1 nM melatonin, the dose of melatonin habitually used in these experiments.

Cell-cycle analysis

At 24 or 48 hr of treatment cells were harvested, centrifuged, washed with PBS, and resuspended in 3 mM citrate buffer containing propidium iodide (50 μg/mL) and RNase (37 μg/mL) (Sigma Chemical Co., UK) and incubated for at least 4 hr in darkness, at 4–5°C. The cells were analyzed on a FACSCAN flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) with excitation at 488 nm. At least 20,000 events per tube were acquired and DNA histograms were analyzed by using the MODFIT program (Verity Software) to determine the percentage of cells in each cell-cycle phase. There were at least triplicates for each analysis point and the data given are mean ± S.E.M. The experiment was repeated three times.

Detection of apoptosis

MCF-7 cells were plated in coverslips for 48 hr and processed for immunofluorescence analysis. An in situ cell death detection kit (Boehringer Mannheim, Indianapolis, IN, USA) was used to determine the population of apoptotic cells. In this kit, terminal deoxynucleotidyl transferase (TdT), which catalyzes the polimerization of nucleotides to free 3′-OH-DNA ends, is used to label DNA strands originating from the cleavage of DNA during apoptosis [TdT-dUTP nick end labeling (TUNEL)]. Quantitative analysis of apoptotic cells was carried out by double labeling with TUNEL and monoclonal anti-pan-histone (Boehringer Mannheim, Indianapolis, IN, USA). Cells were examined with a laser confocal microscope (Bio-Rad MRC-1024) by using argon (488 nm) and HeNe (543 nm).

DNA histograms of propidium iodide-stained cells were also used to evaluate apoptosis. Cells with hypodiploid DNA content (falling in the sub-G_1 region) were considered apoptotic (Ferlini et al., 1996). In order to avoid an overestimation of apoptosis because of the cellular debris accumulated in the sub-G_1 region, only cells with no more than 20 times less DNA content than cells in G_0–G_1 phase were considered apoptotic (Viora et al., 1997).

p53 and p21WAF1 proteins expression

p53 as well as p21WAF1 protein concentration in MCF-7 cells cultured for 24 or 48 hr were quantified by 'sandwich' enzyme immunoassays by using kits from Oncogene (Calbiochem, Cambridge, MA, USA) in two different experiments, following the protocols recommended by the manufacturer. Briefly, the samples to be assayed (the lysate from 400,000 cells from each culture plate) and the biotinylated detector antibody (mouse monoclonal) were placed (triplicate) in 96-well microtiter plates coated with either mouse monoclonal antibodies specific for both wild and mutant human p53 (MCF-7 cells express wild p53) or rabbit policlonal antibodies specific for the human p21WAF1, and incubated for 4 hr (p53) or 2 hr (p21WAF1) at room temperature. After removing unbound material by washing with PBS, horseradish peroxidase conjugated steptavidin was added to bind to the antibodies. The horseradish peroxidase catalyzes the conversion of a chromogenic substrate (tetramethylbenzidine) to a colored solution of a color intensity proportional to the amount of protein (p53 or p21WAF1) in the sample. The absorbance in each well was measured at 450/550 nm and the concentration of p53 and p21WAF1 was determined by interpolating from standard curves obtained with known concentrations of the protein. The results were indicated as nanograms of p53 protein/10^6 viable cells or units of p21WAF1/10^6 viable cells, and indicated as means ± S.E.M. of at least five plates. One p21WAF1 unit is the amount of protein originating from 2.7×10^5 MCF-7 cells, which is detected by the Oncogene WAF1 enzyme-linked immunosorbent assay kit, after the cells have been harvested from a confluent monolayer culture and resuspended in 1 mL of buffer.

Changes in mRNA levels for bcl-2, bcl-X and bax

Differences in relative mRNA levels of bcl-2, bcl-X_L and bax genes between melatonin or vitamin D_3 treated and control MCF-7 cells were assessed by semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR). All samples were normalized for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the mRNA levels of which are neither influenced by melatonin nor vitamin D_3 treatments (Desprez et al., 1992). Briefly, total RNA samples were treated with RNase-free DNase I (Amersham Pharmacia Biotech, Barcelona, Spain) to remove any contaminating genomic DNA, quantified by spectrophotometry, and diluted as appropriate to equalize concentrations. Total RNA (1 μg) was reverse transcribed at 42°C for 1 hr in a reaction mixture (40 μL final volume) containing 10 mM Tris–HCl (pH 7.6), 12 mM KCl, 2 mM $MgCl_2$,

200 μM dNTPs, 0.5 μM random hexamers, 40 units RNAsin and 200 units avian myeloblastosis virus reverse transcriptase (Gibco). Ten per cent of the cDNA product was then subjected to a PCR (GeneAmp PCR system 2400, Perkin Elmer, Boston, MA, USA) in a total volume of 50 μL with 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 0.001% gelatin, 2 mM $MgCl_2$, 200 μM dNTPs, and 1 unit Taq polymerase (BioLine, London, UK) using the appropriate pair of primers (0.5 μM each), with the following protocol: 94°C, 5 min; 25 cycles of 94°C, 30 s; 72°C, 60 s; 72°C, 5 min. The primers used for gene amplifications were: 5′A-GATGTCCAGCCAGCTGCACCTGAC and 3′AGAT-AGGCACCAGGGTGAGCAAGCT for bcl-2; 5′CGGGCATTCAGTGACCTGAC and 3′TCAGGAAC-CAGCGGTTGAAG for bcl-X_L and bax; 5′GGTCTTACTCCTTGGAGGATGTG and 3′ACCTC ACTACATGGTTTACATGTT for GAPDH genes (used as controls).

Statistical analysis

Data are expressed as the mean ± S.E.M., with the number of replicates indicated in the figure legends. Differences in cell proliferation as well as p53 and WAF1 protein concentrations between controls and the different treatments were assessed by one-way analysis of variance. Differences between means were analyzed by using the Student–Newman–Keuls test.

Results

After 24 hr of incubation, no differences in the total number of MCF-7 cells were found among the controls and the experimental groups (Fig. 1). However, at 48 hr of culture the number of cells in the plates treated with melatonin (1 nM) or D_3 (100 nM) decreased in comparison with the controls (Fig. 2). Regarding the number of viable cells, it decreased significantly after 24 or 48 hr of incubation with melatonin or D_3 (Figs 1 and 2), although no differences in cell viability were observed between the two treatments.

The study of the cell cycle of cells treated for 48 hr with melatonin or vitamin D_3 did not show great differences between the proportion of cells in each of the cell-cycle phases depending on the treatment. Only the percentage of cells in G_2-M was significantly ($P < 0.05$) lower in cells treated with vitamin D (14.57 ± 0.26%) than in controls (15.91 ± 0.41%) or cells incubated with melatonin (16.04 ± 0.28%).

The incidence of apoptosis, measured as the population of cells falling in the sub-G_1 region of the DNA histogram, was similar in cells treated with melatonin and in those untreated ones (controls). However, among cells cultured with D_3 the proportion of apoptotic cell was significantly higher than among controls and melatonin treated cells (Fig. 3). When the incidence of apoptotic cells was estimated by the in situ labeling of DNA strands in MCF-7 cells (TUNEL reaction) and histone immunofluorescence (Fig. 4) we observed that, among cells treated with melatonin, the incidence of apoptosis tends to be an increase in relation to the controls, but this increase is not significant.

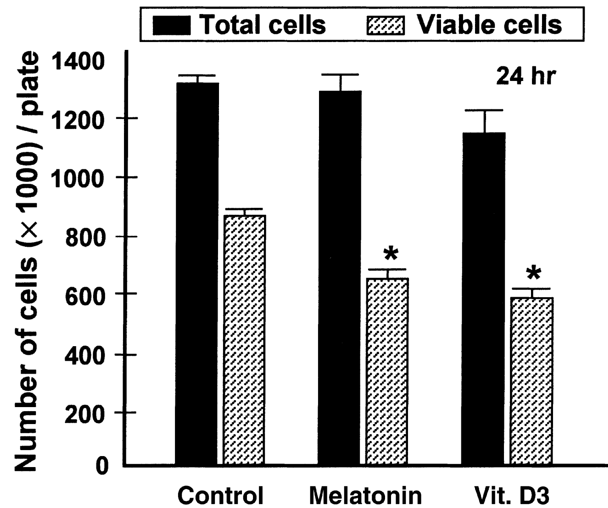


Fig. 1. Total number of MCF-7 cells and number of viable MCF-7 cells (determined by the trypan blue exclusion method) after 24 hr of incubation in presence of melatonin (1 nM), vitamin D3 (100 nM) or its diluent (ethanol, final concentration <0.01%) in culture media. (*) $P < 0.05$ versus controls.

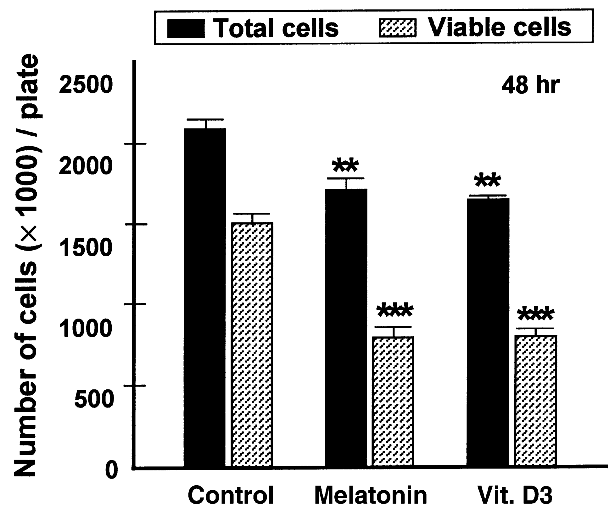


Fig. 2. Total number of MCF-7 cells and number of viable MCF-7 cells (determined by the trypan blue exclusion method) after 48 hr of incubation in presence of melatonin (1 nM), vitamin D3 (100 nM) or its diluent (ethanol, final concentration <0.01%) in culture media. (**) $P < 0.01$ (***) $P < 0.001$ versus controls.

The incidence of apoptosis (TUNEL reaction) among melatonin-treated cells was 1%, whereas among those treated with D_3 it was 8%.

The expression of p53 protein was significantly enhanced after 24 or 48 hr of incubation with either melatonin or vitamin D_3 (Fig. 5). No differences in the concentration of p53 were observed between vitamin D_3- or melatonin-treated cells.

After 24 or 48 hr of incubation with melatonin or vitamin D_3, concentration of p21WAF1 protein was significantly higher than in control cells (Fig. 6). The increase in the expression of p21 induced with vitamin D_3

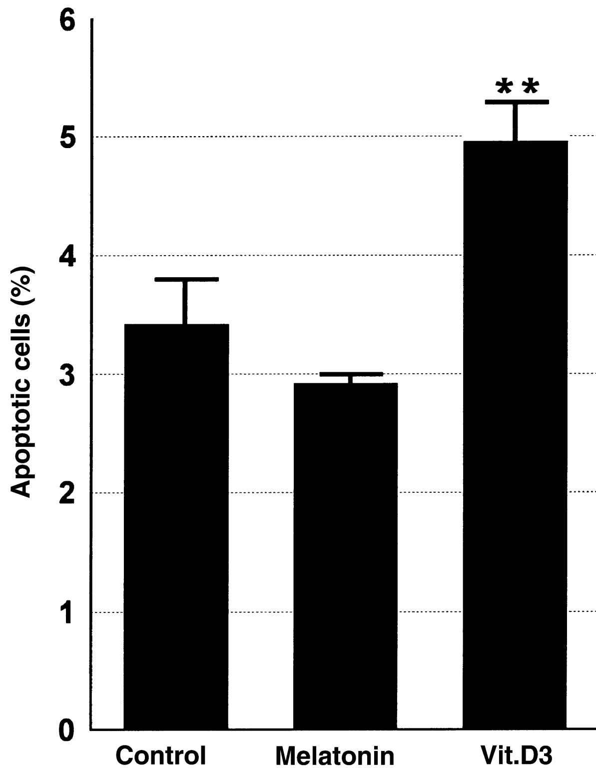


Fig. 3. Percentage of MCF-7 apoptotic cells determined by the DNA histograms of propidium iodide-stained cells as those with hypodiploid DNA content (falling in the sub-G_1 region). Cells had been incubated for 48 hr in media containing melatonin (1 nM), vitamin D_3 (100 nM) or its diluent. (**) $P < 0.01$ versus control.

was greater than that with melatonin, the differences being significant after 48 hr of treatment.

Cultured MCF-7 cell line express bcl-2, bcl-X_L and bax genes (Fig. 7). Changes in mRNA levels for bcl-2, bcl-X_L and bax were different depending on the treatment with melatonin or vitamin D_3. In vitamin D_3 treated cells, a dramatic descent in bcl-X_L (more than fourfold) was observed, whereas only a slight reduction and no significant changes were detected for bcl-2 and bax, respectively (Fig. 7). No significant changes in bcl-2, bcl-X_L and bax mRNAs were detected after treatment with melatonin (Fig. 7). Only for bcl-2 we observed a small decrease (less than twofold) in mRNA after the incubation with melatonin, although this change cannot, however, be considered as significant.

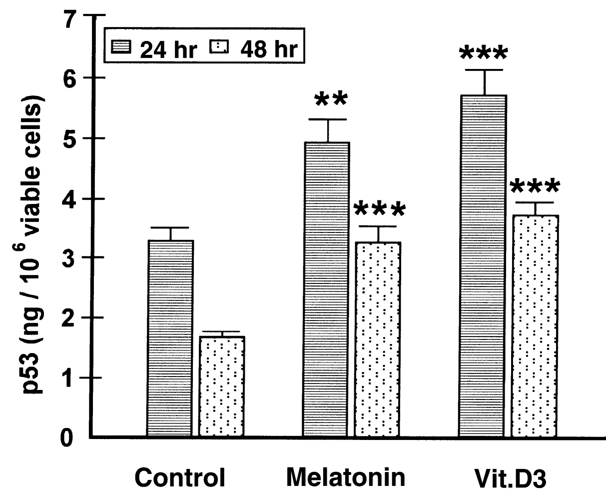


Fig. 5. Expression of p53 protein in MCF-7 cells after 24 or 48 hr of incubation in presence of melatonin (1 nM), vitamin D_3 (100 nM) or its diluent. (**) $P < 0.01$ (***) $P < 0.001$ versus controls.

Discussion

In this experiment we checked whether the inhibitory effects of melatonin on MCF-7 breast cancer cell growth could be explained, at least in part, on the basis of a melatonin-induced apoptotic process. In order to discard negative results related with the experimental procedures we carried out a parallel study of the effects of melatonin and vitamin D_3. It is well known that this latter hormone reduces MCF-7 cell growth by inhibiting cell proliferation and inducing apoptosis (Welsh, 1994).

We observed that melatonin as well as vitamin D_3 decreased cell proliferation and increased the expression of p53 and p21 proteins in MCF-7 cells. These effects of melatonin had been described previously (Mediavilla et al., 1999) and, with regard to D_3, our results agree with previous studies showing that this active form of vitamin D inhibits breast cancer cell proliferation by interacting with specific nuclear receptors (Davoodi et al., 1995), and that the incubation of MCF-7 cells for 6 days with vitamin D_3 increased p53 as well as p21 protein expression (James

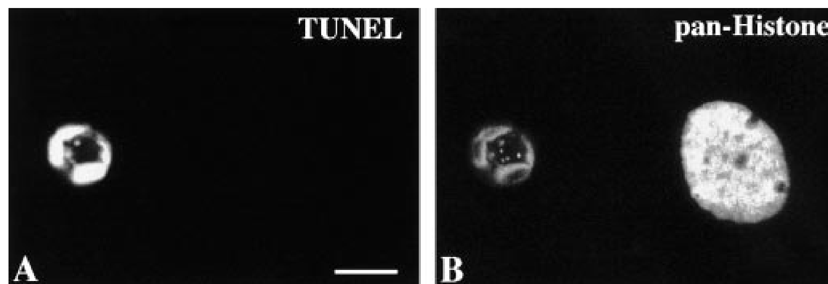


Fig. 4. Confocal microscopy images of MCF-7 cells after two days of incubation with 1 nM melatonin. The cells were double stained with the TUNEL procedure and monoclonal anti pan-histone. (A) MCF-7 cell in an advanced stage of apoptosis; nuclear shrinkage as well as positive TUNEL labelling can be observed. The apoptotic MCF-7 cell (B, left) exhibits a weak peripheric histone staining. A normal MCF-7 cell stained for histone is also shown (B right). Scale bar = 10 μm.

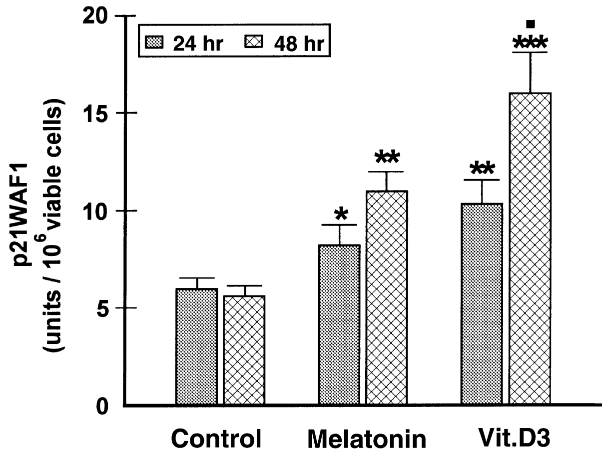
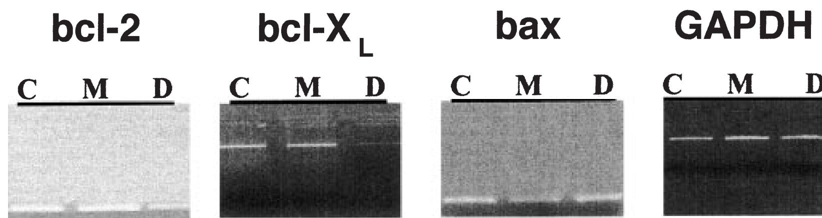


Fig. 6. Expression of p21WAF1 protein in MCF-7 cells after 24 or 48 hr of incubation in presence of melatonin (1 nM), vitamin D_3 (100 nM) or its diluent. (*) $P < 0.05$ (**) $P < 0.01$ (***) $P < 0.001$ versus controls; (■) $P < 0.05$ versus melatonin.

et al., 1996). As an important tumor suppressor gene, p53 is involved in cell-cycle regulation (Mercer, 1992). One of the functions of the wild-type p53 protein is to cause G_1 arrest, thus permitting efficient repair of damaged DNA before the initiation of the cell DNA synthesis (G_1 arrest) thus avoiding the propagation of mutagenic lesions and possible neoplastic transformation. The wild-type p53 regulates cycle progression by the induction of its downstream target gene WAF1/CIP (El-Deiry et al., 1993; Xiong et al., 1993). The 21 kDa product of the WAF1 gene is considered a potent universal inhibitor of cell-cycle progression at G_1 check-point (El-Deiry et al., 1994, 1995) by inhibiting the cyclin D1-CDK4-6 responsible for the activation of the retinoblastoma protein (pRb). Furthermore, p21WAF1 also inhibits the activity of each member of the cyclin/CDK family, thus regulating the whole cycle and not only the G_1 transition (Xiong et al., 1993; El-Deiry et al., 1994, 1995). Under these premises, from our results it is possible to conclude that melatonin and vitamin D_3 act in a similar way on MCF-7 cells by retarding the progression of the cell cycle by activating the p53-p21 pathway. These results agree with previous reports indicating that both melatonin (Cos et al., 1991) and vitamin D_3 (Eisman et al., 1989a,b; Simboli-Campbell and Welsh, 1994) caused a G_1-S transition delay in MCF-7 cells with the subsequent accumulation of cells in the G_0/G_1 phase of cell cycle. We did not observe the previously referred changes in cell-cycle phase distribution after melatonin treatment, possibly because of the short time of incubation of 48 hr compared with 5 days of the experiment of Cos et al. (1991). Nevertheless, as p21WAF1 is a universal inhibitor of cyclin kinases, a possibility of melatonin-induced delay in the progression of cell cycle without an arrest in a specific phase of the cycle is evident.

Concerning the main objective of the study, we did not observe, with either of the methods used (TUNEL reaction and DNA histogram), a significant increase in the population of apoptotic cells among those treated with melatonin in comparison with the controls. However, as expected, the treatment with vitamin D_3 resulted in apoptosis of MCF-7 cells. This different behavior of melatonin and vitamin D_3, despite their similar effect on p53 and p21 expression, may be explained by their different modulation of the expression of genes of the bcl-2 family. Despite its role in the control of cell-cycle progression, p53 is also involved in the induction of apoptosis, probably by down-regulating bcl-2 expression



	Control	Melatonin	Vit. D_3
GAPDH	+++	+++	+++
bcl-2	++++	++++	+++
bcl-X_L	++++	++++	+
bax	+++	++	+++

Fig. 7. Differences in relative mRNA levels of bcl-2, bcl-X_L and bax genes between MCF-7 cells treated for 48 hr with melatonin (1 nM), vitamin D_3 (100 nM) or its diluents (control), assessed by semiquantitative RT-PCR. All samples were normalized for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). A significant descent in bcl-X_L (more than four fold) is observable in cells treated with vitamin D_3.

and up-regulating bax expression (King and Cidlowsky, 1998). In our experiments, vitamin D_3-treated cells showed a significant descent in bcl-X_L, the antiapoptotic gene, without significant changes for bcl-2 and bax, the inductors of apoptosis, whereas no significant changes in bcl-2, bcl-X_L and bax mRNAs were detected after treatment with melatonin. The only previous studies on the possible apoptotic effects of melatonin on breast cancer cells are those of Eck et al. (1998, 2000). These authors have reported that the sequential exposure of MCF-7 cells to melatonin and all-trans retinoic acid induced apoptosis, whereas melatonin alone did not. The pathway through which melatonin acts in this regimen of association of drugs to induce apoptosis seems to be down-regulation of the expression of the death suppressor gene bcl-2 and up-regulation of death promoter bax without changing the levels of p53 (Eck et al., 2000). We also observed a diminution of bcl-2 expression in our cells after melatonin treatment, although in our semiquantitative method the difference cannot be considered as significant.

It is important to clarify that we have been studying whether melatonin modifies the incidence of apoptotic cell death under specific experimental conditions: cells growing in a serum containing culture media. Under these conditions, we conclude that melatonin does not induce MCF-7 cells to fall into apoptosis. However, as we know that melatonin actions on MCF-7 cells are dependent on the culture conditions (Cos and Sánchez-Barceló, 2000a) we cannot ignore the possibility that, under different experimental conditions, i.e. serum deprived cells or cells exposed to agents inducing apoptosis, the effects of melatonin could be different. We have observed in a preliminary study (unpublished data) that melatonin increases apoptosis in MCF-7 cells which have been cultured for 5 days in a serum-free medium previous to their exposure to melatonin. This time the serum-free medium appears to up-regulate the cells' melatonin response pathway.

Considered as a whole, these data support the hypothesis that melatonin reduces MCF-7 cell proliferation by modulating cell-cycle length (Cos et al., 1996b) through the control of the p53–p21 pathway, but without clearly inducing apoptosis.

Acknowledgements

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