Fas/Fas ligand regulation mediates cell death in human Ewing's sarcoma cells treated with melatonin

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BACKGROUND: Despite recent advances in cancer therapy, the 5-year survival rate for Ewing's sarcoma is still very low, and new therapeutic approaches are necessary. It was found previously that melatonin induces cell death in the Ewing's sarcoma cell line, SK-N-MC, by activating the extrinsic apoptotic pathway.

METHODS: Melatonin actions were analysed by metabolic viability/survival cell assays, flow cytometry, quantitative PCR for mRNA expression, western blot for protein activation/expression and electrophoretic mobility shift assay for transcription factor activation.

RESULTS: Melatonin increases the expression of Fas and its ligand Fas L, this increase being responsible for cell death induced by the indolamine. Melatonin also produces a transient increase in intracellular oxidants and activation of the redox-regulated transcription factor Nuclear factor-kappaB. Inhibition of such activation prevents cell death and Fas/Fas L upregulation. Cytotoxic effect and Fas/Fas L regulation occur in all Ewing's cell lines studied, and do not occur in the other tumour cell lines studied where melatonin does not induce cell death.

CONCLUSION: Our data offers new insights in the study of alternative therapeutic strategies in the treatment of Ewing's sarcoma. Further attention deserves to be given to the differences in the cellular biology of sensitive tumours that could explain the cytotoxic effect of melatonin and the increase in the level of free radicals caused by this molecule, in particular cancer types.

Keywords: apoptosis; ESFT cell lines; Fas/Fas L upregulation; melatonin

The Ewing's sarcoma family of tumours (ESFT) are aggressive neuroectodermal neoplasms of bone and soft tissues, with a peak incidence during childhood and adolescence. These types of tumours are characterised by the presence of a chromosomal translocation that produces a chimeric protein. Approximately 85% of these tumours carry the translocation t(11;22)(q24;q12), which produces the fusion protein EWS-FLI1 (Ewing's sarcoma type I) (de Alava and Gerald, 2000), although other less common translocations have been described (mostly Ewing's sarcoma types II and III). The fusion protein acts as an aberrant transcription factor and has the ability to modulate the transcription of specific genes involved in oncogenesis (Uren and Toretsky, 2005). Despite recent advances in cancer therapy, the 5-year survival rate for ESFT is still very low (Damron et al, 2007), and new therapeutic approaches are necessary.

The natural indolamine melatonin (N-acetyl-5-methoxytryptamine) has previously been shown to possess antitumoural properties that are generally mediated by the inhibition of tumour cell proliferation (Hill and Blask, 1988; Sainz et al, 2003; Reiter, 2004; Martin et al, 2006). There are, however, a few reports that show a cytotoxic effect of melatonin in some types of cancer cells (Trubiani et al, 2005; Buyukavci et al, 2006; Rubio et al, 2007; Martin-Renedo et al, 2008). Also, some authors found an improvement in chemotherapeutic regimes when melatonin treatment was included (Lissoni et al, 1999; Martin et al, 2010). Moreover, there are no publications reporting that melatonin could kill normal cells. Quite on the contrary, melatonin typically protects normal cells from a variety of insults (Antolín et al, 2002; Reiter et al, 2003; Bruck et al, 2004; Herrera et al, 2007). We had previously demonstrated that melatonin induces apoptotic cell death in the Ewing's sarcoma cell line, SK-N-MC, (Garcia-Santos et al, 2006) and presents synergism with vincristine and ifosfamide when administered to Ewing's cells in combination with such chemotherapeutic drugs (Casado-Zapico et al, 2010). The said synergism is the result of the potentiation of the extrinsic pathway of apoptosis. Combination of melatonin plus vincristine or ifosfamide potentiated the caspase 8 activation that is induced by each drug given alone (Casado-Zapico et al, 2010). The extrinsic pathway of apoptosis is activated after binding of death receptors to their ligands, followed by trimerisation of receptors and afterwards by recruitment of adaptor proteins. Such proteins in turn recruit caspase 8 to form the death-inducing signalling complex. The conversion of pro-caspase 8 to active caspase 8 by cleavage occurs at this membrane-bound complex. Active caspase
8 can cleave executioner caspases with scission of multiple targets that determine the typical features of apoptosis (Fulda and Debatin, 2006).

The next logical question on the cytotoxic effect of melatonin in Ewing’s sarcoma cells referred to the intracellular signalling pathways involved in the induction of such apoptosis by this indolamine. Besides the previous results with melatonin administration mentioned above, we also had previously found that this indolamine sensitises glioma cells to TRAIL treatment, probably due to the increase in the death receptor DR5 expression (Martin et al., 2010). On the other hand, there are reports of sensitivity to TRAIL of Ewing’s sarcoma cell lines (Kumar et al., 2001; Picarda et al., 2010). All these data together suggested the hypothesis that melatonin could be regulating the expression of a death receptor and/or its ligand. We demonstrate that this molecule increases the expression of Fas and its ligand Fas L. We also demonstrate that this increase is responsible for cell death induced by the indolamine in Ewing’s sarcoma cells and that this cytotoxic effect occurs in all Ewing’s cell lines studied. Increment of Fas/Fas L expression does not occur in other tumour cell lines where melatonin does not induce cell death.

MATERIALS AND METHODS

Cell culture and reagents

Culture flasks and dishes were obtained from Falcon (Becton Dickinson BioScience, Le Pont de Clai, France). Ewing’s sarcoma SK-N-MC cell line (EWS-FLI1 type 1) and neuroblastoma cell lines SK-N-SH, SK-N-SY5Y and SK-N-AS were purchased from the European Collection of Cell Cultures (Salisbury, United Kingdom). Ewing’s sarcoma cell lines TC-71 and A673 (type 1), SK-ES1 (type 2) A4573 (type 3) were a generous gift from Dr JA Toretsky (Departments of Oncology and Pediatrics, Georgetown University, Washington DC, USA). The presence of the Ewing’s sarcoma-specific transcription factor EWS-FLI1 (type 1 for SK-N-MC, TC-71 and A673; type 2 for SK-ES1; type 3 for A4573) was determined by PCR in these cell lines to confirm their origin. Caspase-8 inhibitor Z-IETD-FMK was purchased from Calbiochem (La Jolla, CA, USA). Anti-FAS antibody (clone ZB4) and anti-FAS-L antibody (clone NOK1) used in the neutralisation assays were obtained from Millipore (Billerica, MA, USA) and BD Pharmingen (Franklin Lakes, NJ, USA), respectively, and added to the cellular medium 4 h before treatment with melatonin. All other reagents were purchased from Sigma (Sigma-Aldrich, Milwaukee, WI, USA), unless otherwise indicated.

Cell viability assays

For the MTT assay, cells were plated in 96-well dishes. Assays were carried out as described by Casado-Zapico et al. (2010). Samples were measured in an automatic microplate reader (μQuant; Bio-Tek Instruments, Inc., Winooski, VT, USA) at the wavelength of 540 nm.

For the lactate dehydrogenase release assay, cells were seeded in 24-well plates. After treatment with melatonin or vehicle for the indicated time, determination of total and released LDH activity was accomplished following specifications of the In Vitro Toxicology assay kit (Sigma). Absorbance was determined using an automatic microplate reader (μQuant; Bio-Tek Instruments, Inc.) at 490 nm.

Caspase-3 activity

After treatment, activation of caspase-3 was determined using the fluorometric caspase-3 assay kit (Sigma Chemical Co., St Louis, MO, USA), following the recommendations of the manufacturer. After 2-hr incubation of the reaction mixture at room temperature in darkness, samples were analysed in a microplate fluorimeter FLX-800 (Bio-Tek Instruments, Inc.) at an excitation wavelength of 360 nm and an emission wavelength of 460 nm.

Flow cytometry analysis of intracellular peroxides

Intracellular production of peroxides was evaluated by using the fluorescent probe 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate as described by Casado-Zapico et al. (2010). The DCF fluorescence of 10,000 live cells per group was measured in a Beckman Coulter FC500 flow cytometer (Becton Dickinson).

Electrophoretic mobility shift assay (EMSA)

To determine nuclear factor kappaB (NF-κB) activation, EMSA was carried out. Nuclear extracts were obtained following the method described by Dignam et al. (1983). Oligonucleotide probes containing the consensus sequence for NF-κB (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were labelled with (α-32P)ATP (3000 Ci mmol⁻¹) using T4 polynucleotide kinase 5’-end labelling kit (Amersham Life Science, Pittsburgh, PA, USA). A 10-μg per sample of nuclear extracts was incubated on ice for 30 min, with 0.4 ng of the labelled oligonucleotide. The specificity of binding was determined by competition with the unlabelled oligonucleotide (100-fold excess). Protein–DNA complexes were resolved on 6% non-denaturing polyacrylamide gels at 250 V for 1.5 h in TBE. Gels were dried and exposed to Kodak Biomax X ray film (Amersham Life Science).

The optical density (O.D.) of the bands was estimated using an HP scanjet 3670 scanner (HP, Madrid, Spain) and the Scion Image Alpha 4.0.3.2 free analysis software (Scion Corp., Frederick, MD, USA).

Western blot

After treatments, western blot analyses were done using standard methods (Casado-Zapico et al. 2010). Blots were incubated overnight at 4°C with appropriate antibodies: anti-Bcl-2, anti-Bcl-XL, anti-Bcl-xS, anti-Bax, anti-Bak and anti-caspase-8, which recognises the full and cleavage forms of the protein (Calbiochem), anti-Bid, anti-caspase-9, which also recognises the full and cleavage forms, and anti-FAS-L (Cell Signaling Technology, Beverly, MA, USA), anti-GAPDH and anti-FAS (Santa Cruz Biotechnology). Immunoreactive polypeptide was visualised using horseradish peroxidase-conjugated secondary antibodies anti-mouse and anti-rabbit (Calbiochem) and chemiluminescence detection reagents (Amersham Life Science), following the procedures supplied by the manufacturers. The O.D. of the bands was estimated as described above for EMSA.

Quantitative reverse transcription PCR (qRT–PCR)

The RNA extraction, reverse transcription and quantitative analysis of mRNA levels were carried out as described by Martin et al (2010). The sequences of the sense and antisense primers for human TNFR1, TNF-α, FAS-R, FAS-L, DR4, DR5, TRAIL and GADPH (housekeeping gene for normalisation) are described in Table 1. Each sample was tested in triplicate, and analyses of relative gene expression data were done using the 2−ΔCT method.

Flow cytometric analysis of FAS and FAS-L expression

For cell surface FAS or FAS-L protein measurement, tumour cells were stained with anti-FAS antibody (clone ZB4) and anti-FAS-L antibody (clone NOK1) (Billerica, MA, USA) or isotype-matched control for 1 h at 4°C, washed three times and then incubated with PE-conjugated secondary antibody (Invitrogen, Grand Island, NY, USA) for another 30 min. Fluorescence of 10,000 cells in each experimental group
RESULTS

Treatment with melatonin of the Ewing’s sarcoma cell line SK-N-MC induces an increase in Fas and Fas L expression that is responsible for cell death induction

Given that the first step usually required for activation of the extrinsic pathway of apoptosis is the binding to death receptors of their ligands, we first studied the expression of the death receptors TNFR1, Fas, DR4 and DR5, and their respective ligands TNF-α, Fas and DR4 and DR5, and their respective ligands. We incubated SK-N-MC cells with melatonin and analyzed the possible involvement of ROS in melatonin-induced apoptosis. We found that melatonin significantly increases intracellular ROS production in SK-N-MC, by the use of two well-known antioxidants. We incubated the production in the cytotoxic action of melatonin was confirmed after 4–6 h and falls after 8 h (Figure 3A). The involvement of ROS production in the cytotoxic action of melatonin was confirmed by the use of two well-known antioxidants. We incubated the SK-N-MC cell line with ascorbic acid (200 μM) or trolox (100 μM) in the presence or absence of melatonin 1 mM for 72 h, and found that both trolox and ascorbic acid prevent the cytotoxicity of melatonin (Figure 3B).

Activation of the transcription factor NF-κB is responsible for cell death and Fas/Fas L increase after the treatment with melatonin

Endogenous free radicals are involved in the induction and maintenance of signal transduction pathways regulating, among other processes, cell proliferation and cell death. One of the proteins directly regulated by the intracellular redox state is the transcription factor NF-κB, which is activated by oxidation and inactivated by reduction (Schoonbroodt and Piette, 2000) and has been shown to be regulated by melatonin in other experimental systems (Chuang et al., 1996; Post et al., 1998; Bruck et al., 2004). NF-κB activation has been shown indeed to be critical in the signalling pathway leading to Fas expression induced by TNF-α (Starace et al., 2005), and its role in the activation of Fas promoter has been previously reported (Hayden and Ghosh, 2004). It is also involved in the upregulation of Fas expression after the treatment with interleukin-12 in Ewing’s sarcoma cells (Chan et al., 1999). We found that melatonin increases the activation of NF-κB in SK-N-MC cells, reaching a peak after 8 h of incubation and decreasing thereafter (Figure 3C). Co-incubation with parthenolide, a well-known inhibitor of NF-κB activation, prevented both cell death (Figure 3D) and increase in Fas and Fas L expression (Figure 3E) induced by melatonin.
Effect on Fas and Fas L upregulation after melatonin treatment also occurs in other Ewing’s sarcoma cell lines but not in other cancer cell types that are non-sensitive to the cytotoxic effect of this molecule.

The next step was to ensure that cytotoxicity and upregulation of Fas and FasL are general effects of melatonin in Ewing’s sarcoma, and not particular effects on SK-N-MC cells. We first studied cell death induction by this indolamine in four other Ewing’s sarcoma cell lines (TC-71, A673, -type I-, SK-ES1 –type II- and A4573 –type III). All of them were sensitive to melatonin, showing a decrease in cell viability (Figure 4A), which paralleled an increase in cell death (Figures 4B and C) and caspase 3 activity (Figure 4D). To ensure that Fas and Fas L regulation also took place after melatonin treatment in these cells, we measured mRNAs for both proteins by qRT–PCR, and found upregulation of both of them.

Figure 1  Fas/Fas L expression after treatment with melatonin. (A) SK-N-MC cells were treated with or without 1 mM melatonin for 8 or 24 h, and TNFRI, TNF-α, Fas, Fas-L, DR4, DR5 and TRAIL expression were determined by quantitative PCR to assess mRNA expression levels. GAPDH was used as a housekeeping gene. Relative gene expressions are represented as the n-fold increase compared with basal level (vehicle-treated cells: dotted line). *P<0.05 vs vehicle-treated cells. (B) Expression of Fas and Fas L proteins was determined by western blot after treatment with 1 mM melatonin for the indicated times. GAPDH was used as housekeeping gene. A representative blot is shown and densitometric analysis of the immunoblots of three independent experiments is represented below. *P<0.05 vs vehicle-treated group (0 h). (C) Flow cytometric analysis (FACS) of SK-N-MC cells treated with 1 mM melatonin for 48 h. Cells were stained with anti-FAS (left panel) or anti-FAS-L (right panel) and then PE-conjugated secondary antibody. The values obtained are shown above the graphs. *P<0.05 vs control (vehicle-treated group). (D) Overexpression of FAS/FAS-L mediates melatonin cytotoxic effect. SK-N-MC cells were incubated with 1 μg/ml of neutralising antibody against FAS (ZB4), FAS-L (NOK1) or both, 4 h prior to the addition of melatonin 1 mM. Cell death was evaluated by the release of lactate dehydrogenase (LDH) after 72 h. *P<0.05 vs vehicle-treated cells; #P<0.05 vs melatonin 1 mM-treated group. (E) Evaluation of cell death by the release of LDH after 72 h of incubation with melatonin plus 0.1 μM of nordihydroguaiaretic acid (NDGA); *P<0.05 vs vehicle-treated group; #P<0.05 vs melatonin 1 mM-treated group.
Despite the advances made in cancer therapy over the last decade, the 5-year survival rate for Ewing’s sarcoma patients remains very low. This emphasises the need for new therapeutic approaches. The main challenge in cancer therapy is to find treatments that specifically target and kill tumoural cells, while being harmless to normally dividing cells. We had previously communicated a pro-apoptotic effect of melatonin in the Ewing’s sarcoma cell line SK-N-MC (García-Santos et al., 2006) that is mediated by an activation of the extrinsic pathway of apoptosis and which presents a synergistic effect with classical chemotherapeutic drugs such as vincristine or ifosfamide (Casado-Zapico et al., 2010). In the present manuscript, we address the expression and functional relevance of the Fas/Fas L system in the cell death induced by melatonin in Ewing’s sarcoma. We report the upregulation by melatonin of the death receptor Fas and its ligand Fas L without effect on any other death receptors or ligands. The finding of Fas- and Fas L-neutralising antibodies preventing cell death induced by melatonin is in agreement with the role of such regulation in the cytotoxic effect of this indolamine. Cell death induction and Fas/Fas L upregulation extends to four other Ewing’s sarcoma cell lines. Such upregulation does not occur in other non-Ewing’s sarcoma cancer cell lines where melatonin does not induce cell death.

**DISCUSSION**

Despite the advances made in cancer therapy over the last decade, the 5-year survival rate for Ewing’s sarcoma patients remains very low. This emphasises the need for new therapeutic approaches. The main challenge in cancer therapy is to find treatments that specifically target and kill tumoural cells, while being harmless to normally dividing cells. We had previously communicated a pro-apoptotic effect of melatonin in the Ewing’s sarcoma cell line SK-N-MC (García-Santos et al., 2006) that is mediated by an activation of the extrinsic pathway of apoptosis and which presents a synergistic effect with classical chemotherapeutic drugs such as vincristine or ifosfamide (Casado-Zapico et al., 2010). In the present manuscript, we address the expression and functional relevance of the Fas/Fas L system in the cell death induced by melatonin in Ewing’s sarcoma. We report the upregulation by melatonin of the death receptor Fas and its ligand Fas L without effect on any other death receptors or ligands. The finding of Fas- and Fas L-neutralising antibodies preventing cell death induced by melatonin is in agreement with the role of such regulation in the cytotoxic effect of this indolamine. Cell death induction and Fas/Fas L upregulation extends to four other Ewing’s sarcoma cell lines. Such upregulation does not occur in other non-Ewing’s sarcoma cancer cell lines where melatonin does not induce cell death.

The main route for the activation of the extrinsic pathway of apoptosis is the binding to death receptors of their ligands, although some drugs can induce apoptosis through the extrinsic pathway in a Fas L-independent manner (Reis-Sobreiro et al., 2009). Such binding is one of the mechanisms used by the host immune system to fight aberrant proliferating tumour cells. In addition, several chemotherapeutic drugs induce cell death in found in TC-71 are higher than in SK-N-MC (a seven-fold increase over the non-treated cells). In the case of A4573 there was an ~3-fold increase over the control after 8 h of treatment and a markedly higher increase after 24 h of treatment (6- to 7-fold greater than the control).

Subsequently, the effect of melatonin on levels of mRNA for death receptors and their ligands was evaluated in one neuroblastoma cell line. Neuroblastoma is especially interesting as it is closely related to Ewing’s sarcoma in origin, but it does not present the fusion protein characteristic of the EFST. In this case, melatonin failed to induce cell death (Figure 5A). The fact that there was no variation in mRNAs for Fas and Fas L, and neither for any other death receptors or ligands (Figure 5B) indicates that this indolamine does not regulate such death proteins in a general manner, but it does so very specifically in some tumour cells, where it is able to induce cell death.
tumour cells by activating this pathway (Friesen et al, 1996; Micheau et al, 1997; Fulda et al, 2000). However, not all tumour cells are sensitive to such activation, and some of them show resistance to this pathway by means of several mechanisms, and therefore manage to evade the host immune system, hence becoming resistant to chemotherapy. Such mechanisms can be, among others, a downregulation of death receptors; a down-regulation of caspase-8 expression or an upregulation of inhibitors of apoptosis such as the cellular FLICE inhibitory protein (c-FLIP). The c-FLIP expression by Ewing’s sarcoma cells does not interfere with the induction of the extrinsic pathway of apoptosis (Kontny et al, 2001; Mitsiades et al, 2001). In the present work, we demonstrate that melatonin raises Fas and Fas L expression in Ewing’s sarcoma cells at mRNA and protein levels, including the transmembrane forms, which were also measured. After years of controversy due to the immune privilege of tumours expressing soluble Fas L, it has been shown recently that transmembrane Fas L is the form necessary for apoptosis induction (O’Reilly et al, 2009). The increased expression of a death receptor ligand, either alone or accompanied by the increase in its receptor, has been previously shown to induce cell death in Ewing’s sarcoma cells. Abadie et al (2004) reported that a combination of cytokines (TNF-α with interferon γ or interferon α with interferon γ) induces cell death in SK-N-MC cells, and that this is mediated by the increased expression of TRAIL. A collaboration of TRAIL and interferon γ was also observed in vivo in a xenograft model of Ewing’s sarcoma (Merchant et al, 2004). Similarly, inhibitors of metalloproteinases induce apoptosis in Ewing’s sarcoma cell lines.
by avoiding the cleavage of transmembrane Fas L, thus increasing the level of transmembrane Fas L, as well as that of its receptor Fas (Mitsiades et al., 1999). In the present study, we also show that the rise in expression of Fas/Fas L by melatonin is responsible for the cell death induced by this molecule in SK-N-MC cells. This is supported by the finding of inhibition of melatonin-induced cell death by Fas- and Fas L-neutralising antibodies as well as by the lipoxygenase inhibitor NDGA, which has been associated with the inhibition of cell death mediated by Fas. There is abundant literature showing that the increase in death receptor expression potentiates cell death induced by the administration of the corresponding ligands. This is the particular case for melatonin, which sensitises glioma cells to TRAIL. Glioma cells are non-responsive to administration of TRAIL, whereas the antitumoural effect of melatonin when administered alone in glioma cells is due to its antiproliferative effect (Martin et al., 2006). However, melatonin increases the expression of the TRAIL receptor DR5, so the administration of TRAIL and melatonin in combination results in glioma cell death (Martin et al., 2010). Additionally, substances that increase expression of a death receptor and its ligand may induce cell death even when administered alone. This is the case of arachidonic acid in human leukaemia cells (Liu and Chang, 2009), apicidin in promyelocytic leukaemia cells (Kwon et al., 2002) or the inhibitors of metalloproteinases in Ewing’s sarcoma cells; these substances are able to increase the expression of Fas, as well as Fas L, by avoiding transmembrane Fas L cleavage (Mitsiades et al., 1999). As already mentioned, treatment of Ewing’s sarcoma cells with melatonin also has the ability to raise both Fas and Fas L (including transmembrane Fas L) expression, and so induce cytotoxicity.

Figure 4  Melatonin specifically induces cell death in a panel of Ewing’s sarcoma cell lines. (A) TC-71, A673 (EWS-FLI1 type 1), SK-ES1 (EWS-FLI1 type 2) and A4573 (EWS-FLI1 type 3) cell number, determined by MTT assay decreased after 72 h of treatment with melatonin in a dose-dependent manner; *P<0.05 vs vehicle-treated group. There are also statistical differences in the effect of melatonin between the EWS-FLI1 type 1 cells and EWS-FLI1 type 2, 3 cells (**P<0.05). (B) Decrease in the number of cells in Ewing’s sarcoma (TC-71, SK-ES1 and A4573). A rise in cellular debris is observed under phase contrast microscopy after 72 h of melatonin treatment Bars: 50 µm. (C) Increase of cell death in Ewing’s cells was evaluated by the release of LDH into the extracellular medium, after 72 h of incubation with melatonin; *P<0.05 vs vehicle-treated cells; **P<0.05 vs EWS-FLI1 type 1 cells. (D) Increase in caspase-3 activity in a panel of Ewing’s cell lines determined after 48 h of incubation with melatonin; *P<0.05 vs control groups. (E) Fas and Fas L mRNA expression was evaluated in the same cells by quantitative PCR. GAPDH was used as a housekeeping gene. Relative gene expression is represented as the n-fold increase compared with basal level (dotted lines represent vehicle-treated cells). *P<0.05 vs vehicle-treated cells.
It has been reported that there is an increase in free radicals in the apoptosis induced by the activation of death receptors and that antioxidants block such apoptosis (Lee et al., 2002; Devadas et al., 2003; Sato et al., 2004; Shen and Pervez, 2006). Furthermore, some pro-oxidant effects of melatonin have been recently reported in a few human leukaemia and hepatoma cell lines, where this molecule exerts cytotoxic effects (and only in these kinds of cells) (Osseni et al., 2000; Wolffert et al., 2001). In the present report, we also find an early and transient increase in intracellular oxidants, which is accompanied by an activation of the transcription factor NF-κB. We show indeed that addition of antioxidants prevents the cytotoxicity of melatonin in Ewing’s sarcoma cells, supporting the involvement of an increase in such oxidants in melatonin-induced cell death. Nuclear factor-κB is one of the proteins directly regulated by the intracellular redox state, and it has been shown to be regulated by melatonin in other experimental systems (Chuang et al., 1996; Schoonbroodt and Piette, 2000). Nuclear factor-κB activation is essential in activation-dependent Fas promoter induction (Chan et al., 1999). This role has been reported particularly in Fas expression induced by TNF-α (Starace et al., 2005). The fact that the addition of parthenolide, a well-known inhibitor of NF-κB activation, prevents both Fas/Fas L regulation and induction of cell death by melatonin in these cells supports the implication of NF-κB in melatonin-induced apoptosis. NF-κB is a transcription factor that has been shown to be involved in the regulation of apoptosis. It is also known to be involved in the regulation of several pro-apoptotic pathways, such as the Fas/Fas L pathway.

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