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Melatonin provokes cell death in human B-lymphoma cells by mitochondrial-dependent apoptotic pathway activation

Abstract: Apoptosis is an important cell suicide programme involved in physiological and pathological processes. Apoptosis can be induced in different ways depending on cell type and acquired signal. Melatonin, the major secretory product of the pineal gland, participates in many important physiological functions and displays a remarkable functional versatility exhibiting antioxidant, oncostatic, anti-aging, and immunomodulatory properties. Recently, it has been shown that, in addition to pineal gland, human lymphoid cells are an important physiological source of melatonin and that may be involved in the regulation of the immune system. In this work, we examine the effect of melatonin on RAMOS-1 human leukaemic cells. Cell growth and viability, DNA fragmentation and JC-1, and annexin V expression have been determined. To elucidate the mechanism of action of melatonin, Western blot analyses for Bcl-2 and caspase-3 expression, and cytochrome c release were carried out. The results suggest that the apoptotic effect of melatonin is associated with cell-cycle arrest, downregulation of Bcl-2, mitochondrial membrane depolarization, cytochrome c release and activation of caspase-3. The intrinsic (mitochondrial dependent) pathway of caspase activation is the 'point of no return' commitment to cell death. Taken together, our study indicates that melatonin may play a role as potential therapeutic drug in specific lymphoproliferative diseases.

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Introduction

Programmed cell death or apoptosis is a process whereby developmental or environmental stimuli activate a genetic programme to implement a specific series of events that culminate in cell death. Apoptosis is essential for normal development and the dysregulation of this process can lead to a variety of defects including embryonic lethality, tissue-specific perturbation, and high susceptibility to cancer. Although extensive biochemical and morphological studies have revealed several different mechanisms involved in the development of apoptosis, the key events underlying the induction of apoptosis remain largely unknown. Numerous genes that encode products regulating the apoptosis are highly conserved throughout evolution providing the evidence that the mechanism of apoptosis and its regulation is a fundamental property of eukaryotic cells in animal, plants and yeast.

In particular, apoptosis is carried out through the action of specific members of the caspase family [1]. In humans, 11 caspases have been identified. The caspases 2, 3, 6, 7, 8, 9 and 10 are involved in apoptosis, caspases 1 and 5 and probably caspase 4 are involved in inflammatory process while, caspase 14 is involved in skin differentiation. Caspase activation plays a central role in cell apoptosis and, depending on their moment of entry into the apoptotic pathway they are divided in two groups. Caspases 8, 9 and 10, initiator caspases, are the first to be activated. Subsequently, the initiator caspases activate the executioner caspases, including caspases 3, 6 and 7 that are responsible of cleavage of several cellular proteins [2]. In any case it is now clear that, in most models of apoptosis, the activation of such enzyme is the end step cell death process.

Recently, it has become clear that mitochondria are major players in the cell death decision process of mammalian cells. Specific apoptotic stimuli are known to alter mitochondrial membrane integrity and consequently to release cytochrome c into the cytosol [3]. In addition, recent evidence suggests that dysfunction of the mitochondria with a release of cytochrome c precedes activation of caspase and DNA fragmentation [4–7]. Therapeutics capable of regulating the apoptotic pathway provides new opportunities for the treatment of numerous diseases including cancer, viral pathogenesis, lymphoproliferation, inflammation and immunodeficiency. In situations such as cancer and lymphoproliferation, restoration of the apoptotic response by therapeutics would be advantageous.

Melatonin, the principal secretory product of the pineal gland, elicits potent anti-stress, antioxidant and oncostatic properties and influences various immunological and endocrinological functions [8–10]. Its biosynthesis from tryptophan involves four well-defined intracellular steps catalysed by tryptophan hydroxylase, aromatic amino acid decarboxylase, serotonin-N-acetyltransferase (NAT), and hydroxyindole-O-methyltransferase.

Recently, it has been show that both resting and stimulated human lymphocytes have the necessary machinery to synthesize and release large amount of melatonin [11]. The mechanism of most pharmacological effects of melatonin seems straightforward. It was found to be the most potent physiological free radical scavenger playing also a role in the regulation of apoptosis [12, 13]. Furthermore melatonin receptor gene expression in the thymus and spleen supports the notion of the immunomodulatory role of melatonin [14]. As melatonin has previously been reported to influence cell differentiation and growth in a number of in vitro systems, in this work we investigated the role of melatonin in cell growth, cell death, morphological and biochemical modification of RAMOS-1 human leukaemic cell line. The results indicate that melatonin induces cell growth inhibition and apoptosis by cytochrome c release and caspase 3 activation. Moreover, DNA fragmentation and Bcl-2 downregulation suggest that melatonin may be an important endogenous cell death modulator and a potential therapeutic agent.

Materials and methods

Cell cultures

RAMOS-1 lymphoblastoid cells (ATCC CRL 1596) were maintained in continuous suspension culture in RPMI-1640 medium supplemented with 10% foetal bovine serum, 4 mM l-Glutamine, 100 mM Na-pyruvate and 25 mM Hepes. Cells were grown at 2.5×10^5/mL, with more than 98% viability as determined by trypan blue exclusion test. During the log growth phase, the cells were treated with 2 mM of melatonin for a time ranging between 0 and 72 hr. Samples were immediately processed for morphological and biochemical analyses.

Cell cycle analysis

To evaluate the effect of melatonin treatment on the cell cycle, BrdU incorporation was analysed by flow cytometry as described [15]. Cell cycle analysis was performed by FACStar^{Plus} flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) equipped with an argon ion laser tuned at 488 nm, 200 mW output, for excitation of fluorescein isothiocyanate (FITC) and propidium iodine (PI). Data were analysed as the percentage of cells at different phases of the cell cycle (i.e. G_0/G1-S-G_2/M) and as the mean channel of FITC fluorescence of BrdU-positive cells. Both anti-BrdU and PI fluorescences were measured on a linear scale.

Propidium iodide staining

DNA fragmentation, as late feature of apoptosis, was determined by flow cytometry as percentage of nuclei with hypodiploid DNA content. Cells were incubated overnight with fluorescent dye propidium iodide PI (50 μg/mL in 0.1% sodium citrate containing 0.1% Triton X-100). Cell debris were gated out based on light scatter measurements before the single parameter histograms were drawn (ex. at 488 nm and em. at 620 nm).

Flow cytometric analysis of apoptotic cells

Cytofluorometric analysis was performed using an Epics XL, flow cytometer (Coulter Corporation, Miami, FL, USA) fitted with an air-cooled organ laser emitting at 488 nm. Typically, forward and orthogonal scatter signals were used to gate out live cells and in each experiment 10,000 events were collected using log amplification.

Annexin V binding

Cells (10^6/mL) were washed in annexin V binding buffer (10 mmol/L Hepes/NaOH, pH 7.4, 140 mmol/L NaCl, 2.5 mmol/L $CaCl_2$), resuspended in 195 μL of the same buffer and then incubated in the dark with 5 μL of FITC-conjugated annexin V (Alexis Corporation, Lausen, Switzerland) for 10 min at room temperature. After incubation cells were washed again in binding buffer, resuspended in phosphate-buffered saline (PBS) and counterstained with 1 μg/mL PI to identify dead cells. Percentages of annexin V-positive cells (ex. at 488 nm and em. at 525 nm) were calculated within the viable population of cells (i.e. PI negative).

Western blotting

The cells were resuspended in cold hypotonic lyses buffer (1 mM $NaHCO_3$, 5 mM $MgCl_2$, 100 μM PMSF, 10 μM leupeptin and 10 μg/mL soybean trypsin inhibitor). Protein concentration was determined by the Bio-Rad protein assay (Bio-Rad Laboratoires, Milan, Italy) and detected spectrophotometrically. Proteins (60 μg/sample) were separated using sodium dodecyl sulphate (SDS)-12% polyacrylamide gel, and then electrophoretically transferred to a 0.2-μm PVDF membrane for 1 hr. The transfer buffer contained 25 mM Tris–HCl, 192 mM glycine, 0.037% (w/v) SDS and 20% (v/v) methanol. The membranes were blocked by TBS (20 mM Tris–HCl, 150 mM NaCl, pH 7.5) containing 5% (w/v) skimmed milk and 0.1% Tween-20 for 1 hr. The membrane was then incubated overnight at 4°C with the appropriate dilutions of mouse monoclonal anti-Bcl-2 (sc-7382; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-caspase 3 (sc-7272; Santa Cruz Biotechnology) as primary antibodies. This was followed with a goat horseradish peroxidase-conjugated anti-mouse IgG (sc-2055; Santa Cruz Biotechnology) as secondary antibody at 1:1000 of dilution for 1 hr at room temperature. 3, 3′diaminobenzidine (DAB) reaction was used to visualize immune complex. Prestained standard size markers and β-actin control (data not shown) were used. For cytochrome c analysis cells were pelletted by centrifugation at 750 g for 10 min at 4°C. Then, cells were resuspended in 1.2 mL of cold lysis buffer (20 mM Hepes, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM PMSF) and incubated for 3 min on ice. Cells were homogenized with 10 shocks and centrifuged at 750 g for 15 min at 4°C. The supernatant (cytosolic fraction) was removed, and the mitochondrial pellet was resolved in 50 μL of lysis buffer. Supernatants were centrifuged at 100,000 g for 15 min at 4°C, and supernatants obtained were used for identification of cytosolic cytochrome c by immunoblotting. Proteins were loaded onto 15% SDS-

polyacrylamide gels. Monoclonal antibody against cytochrome c (sc-13560; Santa Cruz Biotechnology) was added in a final dilution of 1:500 in TBS-5% bovine albumin. The immunocomplex was then revealed as above. Quantitative analysis was carried out by laser densitometry (Bio-Rad). This optical system was associated with a digitizing pad (Matrix Vision GmbH) and a histometry software package with image capturing capabilities (Image-Pro Plus 4.5; Media Cybernetics Inc., Immagini & Computer Snc, Milan, Italy).

Analysis of mitochondrial membrane potential

$\Delta\Psi_m$ was measured by using the lipophilic cation probe 5,5′, 6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolylcarbocyanine iodine (JC-1; Molecular Probes, Eugene, OR, USA) [16]. The cells were incubated with JC-1 at a concentration of 10 μg/mL, for 15 min at 37°C and CO_2 5%. Subsequently the cells were washed and resuspended in 1 mL of PBS and then analysed with excitation of 488 nm. The emission was between 525 and 590 nm. JC-1 incorporates into mitochondria where it either forms monomers (green fluorescence, 525 nm) or, at high transmembrane potential, aggregates (orange-red fluorescence, 590 nm). Therefore a shift in the emission spectrum from red to green is interpreted as an indication of $\Delta\Psi_m$ dissipation.

Statistical analysis

Each experimental point was determined, at least, in quadruplicate. The results were analysed using Student's *t*-test for unpaired data. P values <0.05 were considered statistically significant.

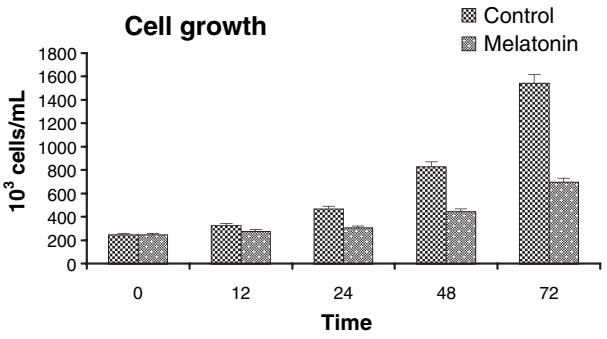

Fig. 1. Cell growth evaluated at different times of melatonin treatment and determined as described in Materials and methods. Mean values obtained from six different experiments. At 12 hr, melatonin versus control $P < 0.05$; at 24, 48 and 72 hr, melatonin versus control $P < 0.001$.

Results

The concentration of 2 mM of melatonin, which exerted the greatest pharmacological effect on RAMOS-1, was chosen after preliminary experiments (data not shown). This concentration had no toxic effects; in fact no quantitative evidence of acute cytotoxicity was detected as established by cell viability assay with trypan blue exclusion test. Melatonin treatment induced a cell growth downregulation (Fig. 1) detectable after 24 hr of incubation and increasing with the treatment time. The flow cytometry analysis of BrdU incorporation proves that cell growth reduction is due to a decrease of the cells entering in the S phase of the cell cycle (Table 1).

Cytofluorimetric detection of apoptosis-associated changes, other than an increase of PI positivity (Fig. 2), indicative of DNA damage, reveals, as shown in Fig. 3, the presence of hypodiploid peak indicative of apoptotic cell death starting 24 hr of incubation. These results are in agreement with the idea that the progression of the cell cycle and control of apoptosis are crucial and intimately linked processes that occur in all multicellular organism in response to different stimuli. As apoptosis is characterized by a variety of morphological features such as loss of membrane asymmetry and attachment and, as one of the earliest hallmarks of apoptosis is the translocation of the membrane phospholipids phosphatidylserine from the inner to the outer leaflet of the plasma membrane, we evaluated annexin V binding. The results obtained (Fig. 4) demonstrate an increase of labelling, starting at 2 hr and reaching the peak at 24 hr. These data proves that melatonin exerts an early redistribution of plasma membrane molecules.

Because *Bcl-2* gene was identified as a gene that prevents cells from undergoing apoptosis in response to a variety of stimuli by the reduction of cytosolic cytochrome c [17–19], we investigated whether these features are present in our experimental model and in which manner the melatonin may generate cell death studying the expression of Bcl-2 protein and mitochondrial membrane integrity. Western blot analysis reveal, after 24 hr of treatment (Fig. 5) the downregulation of Bcl-2 protein expression. To prove that the Bcl-2 regulation depends on mitochondrial homeostasis and as a biochemical hallmark of apoptosis is a change in mitochondrial potential and permeability [20], we tested these properties on melatonin-treated RAMOS-1. Incubation with lipophilic cation probe JC-1 of treated and

Table 1. Flow cytometric analysis of cell cycle found by BrdU incorporation

	0 hr		12 hr		24 hr		48 hr		72 hr	
Phase	C	T	C	T	C	T	C	T	C	T
G0 + G1	20 ± 0.5	20 ± 0.5	22 ± 0.5	19 ± 0.5**	24 ± 0.5	27 ± 1**	28 ± 1	33 ± 1.5**	30 ± 2	41 ± 2.5**
S	62 ± 1.5	62 ± 1.5*	60 ± 1	58 ± 1.5*	60 ± 1	52 ± 1.5**	56 ± 1	47 ± 1.5**	55 ± 2	38 ± 2.5**
G2 + M	18 ± 0.5	18 ± 1.5	18 ± 1.5	23 ± 0.5**	16 ± 1	21 ± 1.5**	16 ± 0.5	20 ± 1.5**	15 ± 2	21 ± 2**

The data report the percentage mean values of six separate experiments ± S.D. It is possible to note that melatonin provokes the arrest of S phase.
Melatonin treated (T) versus controls (C): *$P < 0.05$; **$P < 0.001$.

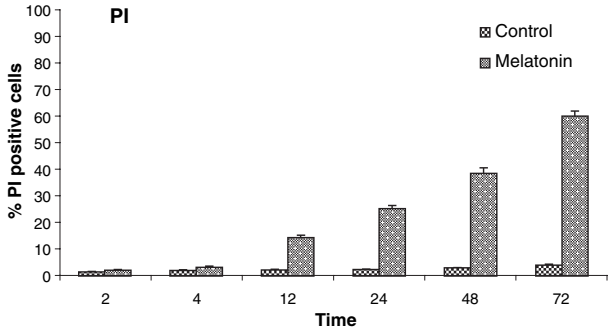

Fig. 2. PI incorporation in RAMOS-1-treated cells. Data show the percentage of stained cells at different time of incubation. Mean values obtained from six different experiments. At 12, 24, 48 and 72 hr, melatonin versus control $P < 0.001$.

untreated cells prove that melatonin directly affect the transmembrane potential and the depolarization initiate at 2 hr of treatment and go up with the treatment time (Fig. 6).

The modification of mitochondrial membrane polarization provokes the release of cytochrome c into the cytosol. In fact, the immunodetermination of cytochrome c in melatonin-stimulated cells shows a decrease of cytochrome c in the mitochondrial fraction (data not shown) with a corresponding increase of the protein in cytosolic fraction (Fig. 7). The upregulation of free cytoplasmic cytochrome c in treated cells follows the mitochondrial disorder. Given that cytochrome c release can potentially induce apoptosis activating in particular caspase-3, and that the caspase family is activated in response to many apoptogenic stimuli and are involved in both initiating and carrying out the apoptotic programme [7, 21, 22], we analysed whether melatonin-induced apoptosis involves this caspase. Western blotting performed at different time of melatonin exposure (Fig. 8), shows a decrease of full-length precursor form of caspase-3 (32 kDa) and the presence of the active fragment (17 kDa) recognizable at 12 hr of treatment. The activation of proteolytic cleavage of caspase-3 suggests that it is specifically involved in the apoptotic process of RAMOS-1 cells modulated by melatonin treatment.

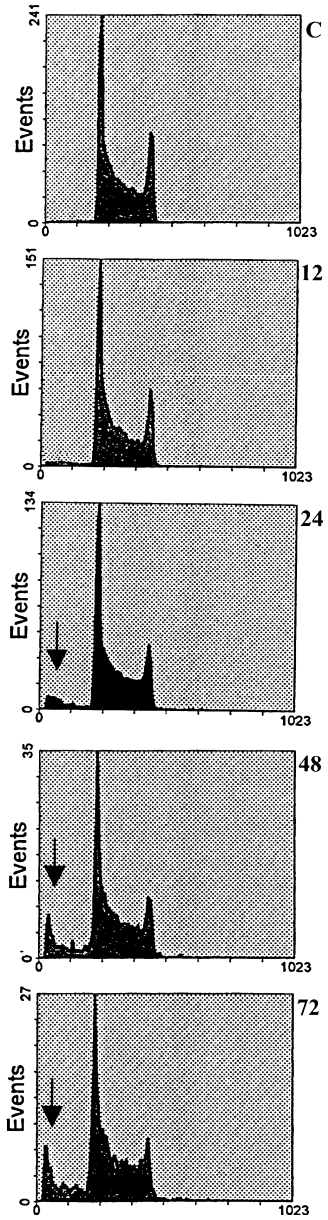

Fig. 3. Cytofluorimetric detection of apoptosis-associated hypodiploic peak (arrow) recognizable after 24 hr of melatonin treatment. Data represent one of four independent experiments. C: untreated cells. 12, 24, 48 and 72: hours of treatment.

Discussion

It is well recognized that melatonin is involved in many important physiological functions and its role has been extensively examined in vivo and in vitro. In vivo models to test the immunomodulatory role of melatonin have been widely used. Most authors agree that pinealectomy and in vivo models of melatonin administration clearly document the immunoenhancing properties of the melatonin.

Concerning the influence of melatonin on programmed cell death although a number of mechanistic options have been suggested, the exact mechanism whereby melatonin influences apoptosis have not yet clarified as melatonin has both pro- and anti-apoptotic actions [23–31]. Sainz et al. [25] concerning the role of melatonin in apoptosis have provided a potential explanation for some of the apparent inconsistencies suggesting also that much of the research conducted falls into three major categories: first, melatonin inhibits apoptosis in immune cells; secondly, melatonin prevents neuronal apoptosis, and finally melatonin increases apoptotic cell death in cancer cells. In any case Harms et al. [26] assessed the neuroprotective potential of melatonin in apoptotic neuronal cell death, and suggested that the neuroprotective effect of melatonin depends on the model of neuronal cell death applied. As demonstrated in three different models of neuronal apoptosis, the progression of the apoptotic type of neuronal cell death is not prevented but rather exaggerated by melatonin; in contrast to its beneficial effect in the necrotic type of cell death. Using human leukaemic Jurkat cells melatonin promotes Fas-

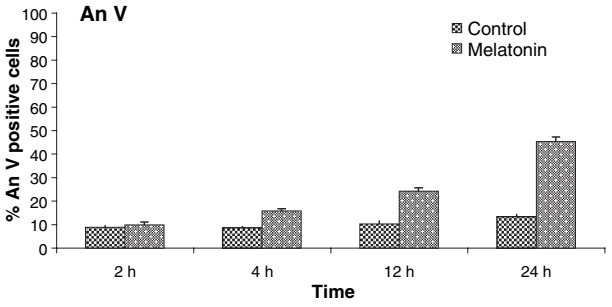

Fig. 4. Cytofluorimetric analysis of annexin V labelling. The values represent the percentage of positive cells ranging from 0 to 24 hr of melatonin treatment. Mean values obtained from four different experiments. At 2, 12 and 24 hr, melatonin versus control $P < 0.001$.

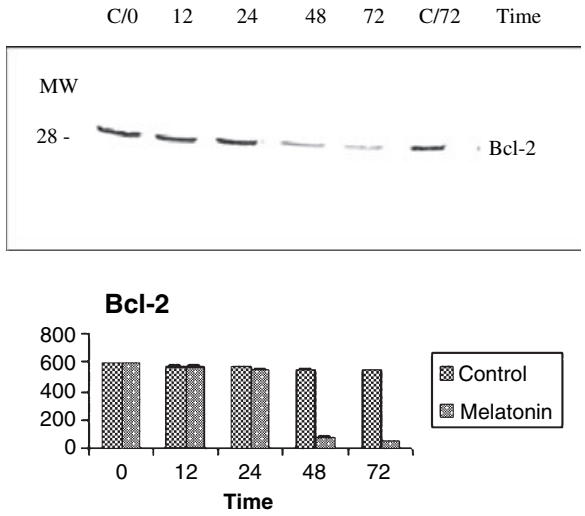

Fig. 5. Immunoblot (upper level) and densitometric analysis of Bcl-2 expression evaluated as reported in Materials and methods. Melatonin treatment induces the downregulation of Bcl-2. C/0 and C/72 represent untreated samples analysed at 0 and at 72 hr of culture, while 12, 24, 48 and 72 are the time of melatonin treatment. Note that incubation in the presence of medium alone has no effect on Bcl-2 expression. The reported data are representative of four different experiments. At 24 hr, melatonin versus control $P < 0.05$; at 48 and 72 hr, melatonin versus control $P < 0.001$.

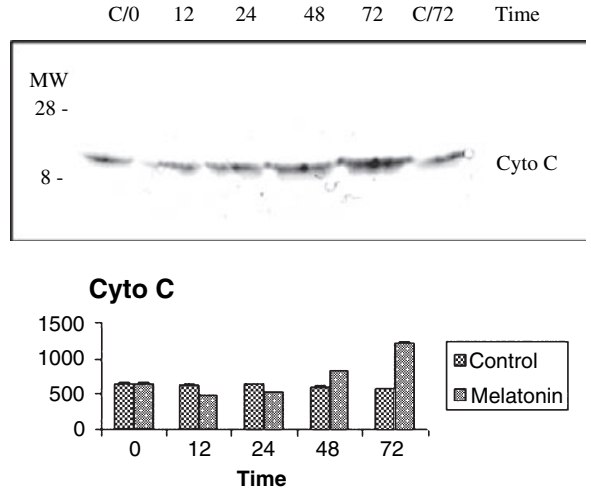

Fig. 6. Flow cytometry of $\Delta\Psi_m$ dissipation. The effect of 2 mM of melatonin on the mitochondrial membrane potential is detected by incubation of JC-1 probe. Mean values obtained from four different experiments. At 4, 12, 24, 48 and 72 hr, melatonin versus control $P < 0.001$.

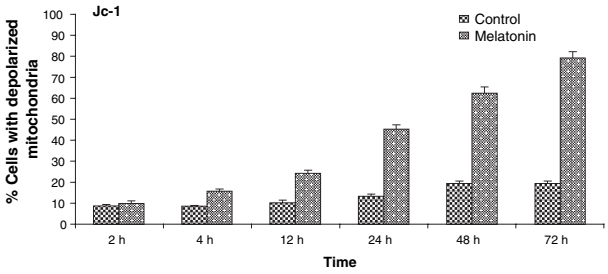

Fig. 7. Immunoblot (upper level) and densitometric analysis of cytochrome c expression evaluated as reported in Materials and methods. Melatonin treatment induces the upregulation of cytosolic cytochrome c. C/0 and C/72 represent untreated samples analysed at 0 and at 72 hr of culture, while 12, 24, 48 and 72 are the time of melatonin treatment. Note that incubation in the presence of medium alone has no effect on cytochrome c expression. The reported data are representative of four different experiments. At 12, 24, 48 and 72 hr, melatonin versus control $P < 0.001$.

induced cell death [30] as well as in malignant cells, such as human breast MCF-7 cell line, micromolar concentrations of melatonin have been reported to induce apoptosis [27]. Furthermore, melatonin was reported to enhance programmed cell death in a murine colon cancer model in vivo [28] and in epatocarcinoma cells [31]. The reported results are then indicative of melatonin action may depend on cell type, functional state of the cell or some other factor. The results of our experiments demonstrate that melatonin induces apoptosis of human RAMOS-1 lymphoblastoid cells by the activation of the intrinsic apoptotic pathway. In fact, the mechanism underlying the destructive effect of melatonin appears to involve destabilization of mitochondria, supporting the release of the mitochondrial protein cytochrome c into the cytosol. Cytosolic cytochrome c has been shown to be necessary to activate the apoptosome complex leading to caspase activation and apoptosis induction [20]. Activation of the caspases cascade, the final common effector proteases mediating apoptosis signalling, has been demonstrated to play a central role in lymphoid cell apoptosis. Thus, the extrinsic death pathway is used for the deletion of unwanted cells during development and diseases and it is known for the downstream executioner caspases 3 and 7 that the activation event is proteolytic cleavage. In fact the activity of pro-caspase 3 is increased >10,000-fold by cleavage [32].

Recent evidence now suggests that dysfunction of the mitochondria with a release of cytochrome c precedes activation of caspases and DNA fragmentation. The findings of the present study demonstrate that melatonin stimulation induces the release of mitochondrial cytochrome c into the cytosol in RAMOS-1 lymphoblastoid cells, which is essential for activation of caspase-3, the central executioner of apoptosis that exists in the cyto-

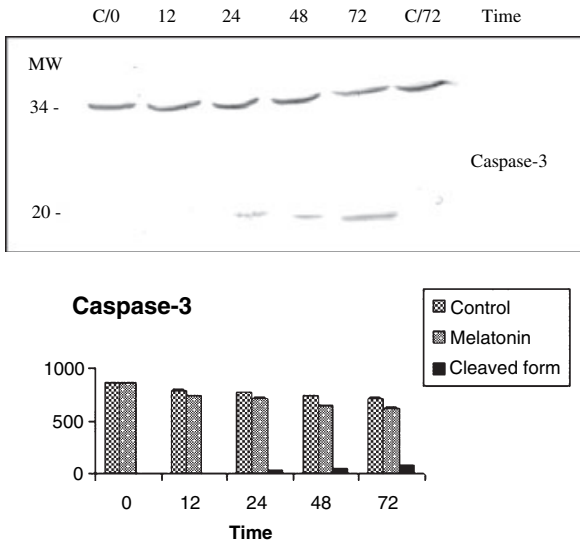

Fig. 8. Immunoblot (upper level) and densitometric determination of caspase-3 expression evaluated as reported in Materials and methods. Melatonin treatment induces the decrease of full-length precursor form of caspase-3 (32 kDa) and the presence of its the active fragment. C/0 and C/72 represent untreated samples analysed at 0 and at 72 hr of culture, while 12, 24, 48 and 72 are the time of melatonin treatment. Note that incubation in the presence of medium alone has no effect on caspase-3 expression. The reported data are representative of four different experiments. At 12, 24, 48 and 72 hr, melatonin versus control $P < 0.001$.

plasm. Active procaspase-3 becomes proteolytically activated by multiple cleavages as a result of melatonin treatment of its 32 kDa precursor to generate the 17 or 15 kDa form which is active in the apoptotic process [1, 33, 34].

This concept is in accordance with previous studies that demonstrate that melatonin potently induces the reduction of the mitochondrial transmembrane potential [35] that may trigger mitochondrial transition pore opening affecting then the apoptotic cascade [36]. Furthermore, the decrease of the protooncogene *Bcl-2*, implicated as a component of the molecular processes that decide whether some cells live or die and detected to the inner mitochondrial membrane, suggests the fundamental metabolic functions of the inner mitochondrial membrane, including oxidative phosphorylation and electron and metabolite transport mechanisms implicated in the cell survival.

The statistically significant data contained in this paper are compatible with the hypothesis that melatonin, other than cell growth inhibition and cell cycle perturbation, triggers apoptosis in RAMOS-1 lymphoblastic cells via a specific effect on mitochondrial membrane potential, leading to cytochrome c release and caspase-3 activation. The possibility to manipulate the apoptotic cell death of RAMOS-1 human lymphoblastoid cells provides an opportunity to consider this indoleamine as an important pro-apoptotic pharmacological molecule in specific type cancer cells.

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