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Review

Melatonin and cell death: differential actions on apoptosis in normal and cancer cells

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Abstract. Melatonin is a natural compound synthesized by a variety of organs. It has been shown to function as a cell-protective agent. Since 1994, when the first paper was published documenting the role of melatonin in apoptosis, the number of reports in this area has increased rapidly. Much of the research conducted falls into three major categories: first, the role of melatonin in inhibiting apoptosis in immune cells; second, the role of melatonin in preventing neuronal apoptosis and finally, the role of melatonin in increasing apoptotic cell death in cancer cells. The mechanisms whereby melatonin influences apoptosis have not clarified, although a number of mechanistic options have been suggested. Apoptotic cell death is a physiological phenomenon related to homeostasis and proper functioning of tissues and organs; however, a failure in the apoptotic program is related to a number of diseases. The participation of melatonin in apoptosis in numerous cell types and its potential importance in a variety of diseases such as immunodeficiency, neurodegeneration and cancer is summarized in this review.

Key words. Melatonin; apoptosis; free radicals; neuroprotection; immune system; cancer cells.

Apoptosis: significance and molecular pathways

Equally as important as cell division and cell migration, a form of regulated cell death allows an organism to tightly control cell number and tissue size, and to protect itself from cells that threaten homeostasis [1]. First coined by Currie and colleagues in 1972 [2], apoptosis, also referred to as programmed cell death, is a physiological form of cell suicide that plays a critical role in embryogenesis, metamorphosis, cellular homeostasis and as a defensive mechanism to remove infected, mutated or damaged cells. During the last decade, there has been an explosion in apoptosis research, and the work has emphasized the importance of this type of cell death in a variety of pathologies. Excessive or insufficient apoptosis contributes to the pathogenesis of a wide variety of pathological processes including ischemia-reperfusion injury, neurodegeneration, autoimmunity, human immunodeficiency virus (HIV) infection and growth or regression in cancer.

Apoptosis clearly differs from accidental cell death, which occurs in response to severe stressful conditions. Apoptosis is morphologically characterized by cytoplasmic contraction, chromatin condensation, plasma membrane blebbing and DNA fragmentation into large and small oligosomes [3] (fig. 1). Most of the morphological changes observed in apoptotic cells are activated specifically by a set of cysteine proteases which are part of a large protein family known as the caspases. Thus, caspases can be considered the central executioners of the apoptotic pathway, and inhibition of their activities

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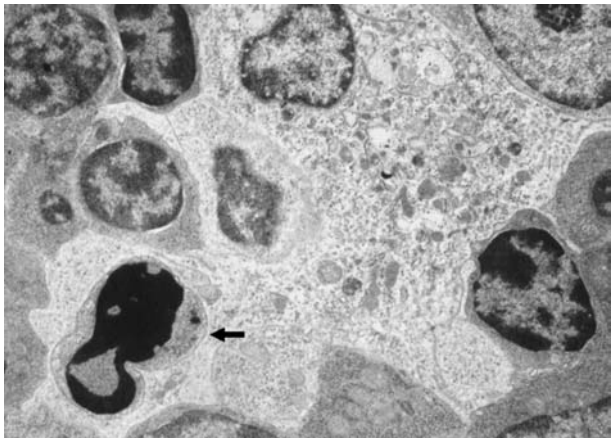

Figure 1. Electron microscopic image of the cortical area of the thymus of a 25-day-old rat. Apoptotic thymocytes are characterized by an extremely condensed chromatin (arrow). The plasma membrane is preserved and surrounds the cell until the formation of apoptotic bodies. Organelles also remain intact until the later steps of the apoptotic process. Magnification × 8000.

(chemically or through mutation) retards or even prevents apoptosis [4].

Two different apoptotic pathways have been described to date: the death receptor pathway and the mitochondrial pathway. At least in certain cell types both seem to be linked [5]. The biochemical steps of these pathways have been studied in detail. In both cases, activation of common effectors or executioners results in cleavage of a number of nuclear and cytoplasmic substrates [6] (fig. 2). In the death receptor pathway, death receptors (DRs) play a central role [5]. Ligands that activate these receptors are structurally related molecules that belong to the tumor necrosis factor (TNF) gene superfamily [7]. TNFα and other cytokines of the same group, such as FasL or TRAIL, activate a particularly broad spectrum of cellular responses but also trigger apoptosis via caspase activation [8, 9]. The most widely accepted pathway involves TNFR-associated death domain (TRADD), Fas-associated death domain protein (FADD) and caspase 8 [10]. DRs contain a homologous cytoplasmic sequence, the death domain (DD) that enables them to initiate a cascade of events that leads to apoptosis. Upon activation, through an induced proximity mechanism, the N-terminal prodomain of caspase 8 is cleaved and leads to activation of caspase 3, which in turn cleaves multiple cellu-

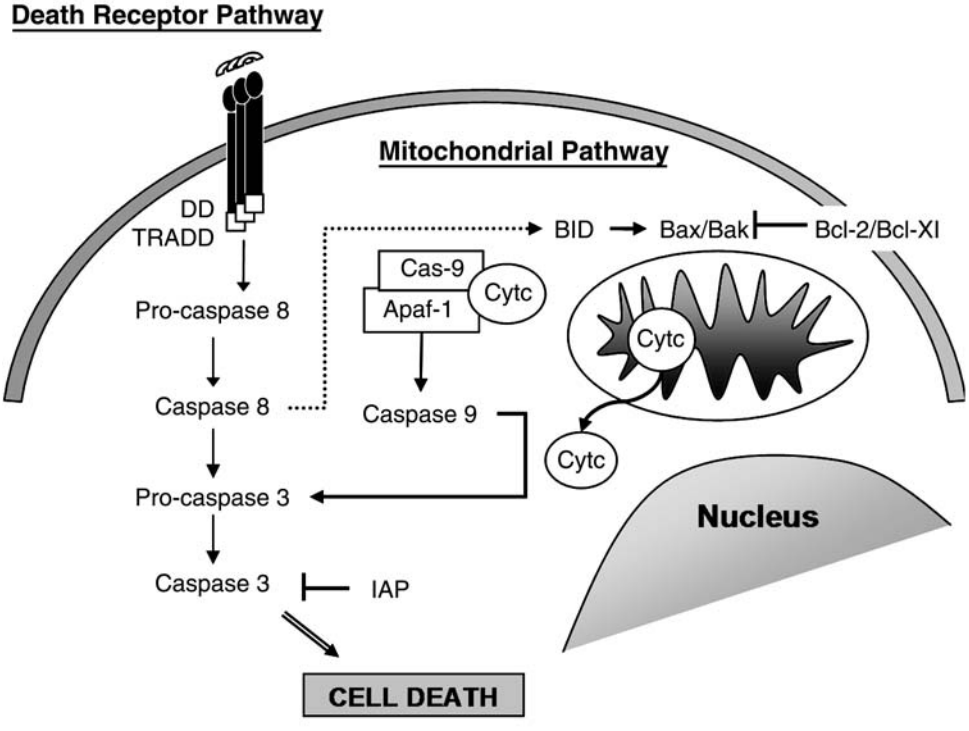

Figure 2. Death receptor and mitochondrial pathways in apoptosis. Caspases are common executors in both. Binding of death signals to the cellular surface initiates a cascade of phosphorylation events through death domains (DD) and caspase 3 that finally promote cellular death. After activation of caspase 8, BID sends the signal to the mitochondria, and cytochrome c (Cyt c) is released to the cytosol. Cyt c in addition to pro-caspase 9 and Apaf-1 forms the apoptosome. Activation of caspase 3 is necessary for the cell to commit suicide. Caspase 3 activation is prevented by inhibitors of apoptosis protein (IAP). Bcl-2 family proteins control the release of Cyt c from the inner membrane space of the mitochondria.

lar proteins, resulting in cell death [7]. This cell death is especially important in the immune system but also in tumor regression.

On the other hand, during the last decade, there has been an increased understanding of the critical role of mitochondria in cell death. There are a variety of events in apoptosis that involve mitochondria. Three general mechanisms are known, and their effects may be interrelated, including (i) disruption of electron transport, oxidative phosphorylation and ATP production; (ii) release of proteins that trigger activation of the caspase family of proteases and (iii) alterations in the cellular reduction-oxidation (redox) potential [11].

The disruption of electron transport chain has long been considered an early feature of cell death. As a direct consequence, a drop in ATP production takes place late in the apoptotic process [12]. In addition, one of the most common features in the apoptotic process is the release of cytochrome c from mitochondria [13]. The cytosolic form of cytochrome c then binds to the so-called apoptosome, which is composed, in addition to the cytochrome c, of the apoptotic protease-activating factor 1 (Apaf-1) and procaspase-9. As a consequence, procaspase-9 is converted to caspase-9, which then processes and activates other caspases to orchestrate the biochemical execution of cell death [14]. Cytochrome c release is usually accompanied by the loss of mitochondrial inner transmembrane potential ($\Delta\psi_m$) prior to classical morphological signs of apoptosis [15]. The mechanisms underlying the $\Delta\psi_m$ reduction are poorly understood. It is thought to be regulated by mitochondrial membrane permeabilization (MMP), implicating protein members of the Bcl-2 family and many other proteins. This family of proteins is composed of more than over 12 proteins with anti- or pro-apoptotic activity. Some of these proteins may be organized into a multi-protein ensemble, the permeability transition pore complex (PTPC) [16]. Pathways and intracellular factors implicated in apoptosis have been extensively studied; the reader can find excellent reviews on these subjects.

Many age-related diseases are characterized by apoptotic loss of post-mitotic cells, i.e. cardiomyocytes and neurons. Inappropriate apoptosis has been suggested to be involved in neuronal death in various human neurodegenerative diseases such as Alzheimer's disease, Huntington's disease, amyotrophic lateral sclerosis and spinal muscular atrophy [17, 18]. Thus, understanding the molecular basis of neuronal apoptotic pathways and searching for new anti-apoptotic agents could significantly improve the treatment of these disorders. On the contrary, since many tumors and cancer cell lines show resistance to the triggering of apoptosis, the search for agents which cause apoptosis specifically in tumor cells but not in normal cells has been the impetus for research by cancer biologists [19].

Reactive oxygen species and the cellular redox state in apoptosis

As the organelle that produces energy through the electron transport chain, mitochondria are one of the major sources of reactive oxygen species (ROS) in the cell. It is estimated that 1–5% of the electrons (e^-) in the respiratory chain are diverted and subsequently participate in the formation of superoxide anions [20]. Ubisemiquinone is generated in the course of the electron transport reactions in the respiratory chain. It sometimes donates an e^- directly to oxygen (O_2), providing a constant source of the superoxide anion radical ($O_2^{\cdot-}$) [21]. It is well known that superoxide anions are formed predominantly in complexes I and III [22]. Oxidative stress generated in the mitochondria by the overproduction of oxygen-based reactants seems to play an important role in the apoptotic process.

Oxidative stress has been defined as a disturbance in the prooxidant-antioxidant balance, resulting in potential cell damage [23]. It has been implicated in a number of biological and pathological processes in which the apoptotic program also takes place, suggesting that they may be functionally linked. Whether ROS production is central to the process of programmed cell death or is important only in some forms of apoptosis is still a matter of debate. There is a strong rationale for an association; first, direct treatment with oxidants such as hydrogen peroxide (H_2O_2) or ionizing radiation (which generates ROS) at low doses triggers apoptosis [24–26]; second, superoxides and lipid peroxides are increased during apoptosis induced by several stimuli [27]; third, several antioxidants such as catalase, *N*-acetylcysteine and trolox block apoptosis induced by a variety of agents [24, 28].

An increase of ROS is detected in the early stages of apoptosis caused by p53 activation, ceramide release, staurosporine and TNF [12], although in other models ROS generation during programmed cell death may be a relatively late event, occurring after caspase activation. Additionally, other extramitochondrial sites of $O_2^{\cdot-}$ production such as the arachidonic acid cascade (yielding prostaglandins and leukotrienes) or some cytochrome P-450 isoenzymes can also initiate apoptosis [29, 30].

The tripeptide glutathione (GSH) is a nonenzymatic water-soluble cellular defense against ROS. Reductions in GSH levels concomitant with the increase of ROS during apoptosis have been documented by many groups [31]. Furthermore, a decrease in GSH levels in non-prooxidant insults such as after Fas-induced cell death has also been reported [32]. Redistribution in the cellular GSH content can be another critical event in apoptosis. In fact, Bcl-2 overexpression can redirect GSH into the nucleus, suggesting that suppression of apoptosis may be linked to modulation of nuclear redox state [33]. Whether a drop in GSH content precedes intracellular ROS production dur-

ing apoptosis or vice versa requires additional study, but GSH depletion alone after treatments with buthione sulfoxamine (BSO) may not trigger apoptosis [34].

In addition, caspases contain an active-site cysteine nucleophile which is prone to oxidation or thiol alkylation, thus suggesting that caspase activity is optimal under reducing environments. It clearly indicates that the redox status of a cell may influence its likelihood of undergoing apoptosis [28, 35]. Although these data are not definitive in linking ROS to apoptosis, there are a number of reports from independent groups showing oxidative stress-induced programmed cell death. The exact mechanisms of ROS induction in these cases remain to be established.

Intracellularly-generated oxidative stress can be also deleterious to the cell and cause apoptotic cell death. Nitric oxide ($NO^{\bullet}$) is a reactive free radical gas that crosses cell membranes easily. It is generated from L-arginine by at least three different isoforms of nitric oxide synthase (NOS) [36]. At low concentrations for short periods, $NO^{\bullet}$ functions as a neurotransmitter and regulates vasodilatation and platelet aggregation. However, high levels of $NO^{\bullet}$ produced by an inducible NOS (iNOS), mainly in macrophages or neutrophils, mediates cytotoxicity as the first line of host-defense against invading microorganisms or tumor cells. $NO^{\bullet}$ has been shown to induce apoptosis in macrophages, pancreatic cells and mouse thymocytes. Although the mechanisms are still controversial, it has been reported that $NO^{\bullet}$ induces cell death through caspase-3 activation by stimulation of the Fas pathway, increasing ceramide generation and induction of mitochondrial permeability transition [37].

The role of oxidative stress in apoptosis is also supported by several reports demonstrating that antioxidants protect efficiently against apoptosis both in vivo and in vitro. Oxidative stress-induced cell death is reduced by vitamin E or its hydrophilic analog, trolox [24, 38], ascorbate [39], thiol agents including GSH or its presursor *N*-acetylcysteine [40], melatonin [41] and resveratrol [42]. Furthermore, *N*-acetylcysteine [43–45] or melatonin [46] have been found protective in some non-prooxidant experimental insults such as growth factor deprivation, dexamethasone or TNF-induced apoptosis.

Antioxidant and cell protective activities of melatonin

Melatonin or *N*-acetyl-5-methoxytryptamine is a ubiquitous physiological mediator. Melatonin is mainly synthesized by the pineal gland but it is now known to be produced in a variety of other tissues as well [47]. The pineal gland synthesizes and releases melatonin into the circulation and into the cerebrospinal fluid, primarily during the dark phase of the light : dark cycle. Thus, melatonin levels in these fluids exhibit a distinct circadian rhythm in which highest concentrations are observed at night, while baseline levels are measured during the day [48]. Melatonin has been found in all organisms studied from bacteria to humans and in recent years, it has also been identified in plants [49–51]. Little is known to date about its functions in some of these organisms.

Functionally, melatonin has been classically related to adjustments in circadian rhythms, the mediation of seasonal reproductive events in photoperiodically dependent species and in the modulation of sleep regulation in at least diurnally active species [52]. In some species it influences the function of other endocrine organs [53], inhibits dopamine release in the retina [54] and potentiates endogenous and exogenous norepinephrine-mediated vasoconstriction [55]. In addition, melatonin stimulates immune function [56] and has a role in the control of tumor promotion and growth. It reduces tumor growth in experimental models in vivo [57–59] and proliferation and invasive properties of cancer cells in culture [60–62].

Some functions of melatonin are attributed to its interaction with specific receptors. Melatonin membrane receptors belong to a subfamily of receptors which consist of seven transmembrane domains coupled to an inhibitory G protein. The receptor subtypes, designated as MT_1, MT_2 or MT_3, suppress adenylyl cyclase activity via a pertussis toxin (PTX)-sensitive inhibitory G protein leading to a reduction in the intracellular level of cyclic AMP (cAMP), which then results in a change in the phosphorylation status of target proteins [63, 64]. In addition, coupling of high-affinity melatonin receptors to modulation of cyclic GMP (cGMP) formation has also been reported [65]. MT_1 receptors have been found in the pars tuberalis of the pituitary and the suprachiasmatic nuclei (SCN); they are presumably related to the circadian and reproductive functions of melatonin. MT_2, found in the retina, may be involved in the phase-shifting response of melatonin, while MT_3, which is only present in amphibians, was identified as the quinone reductase QR(2), and its main function has not been yet determined [66, 67].

The lipophilic nature of melatonin, in addition to immunohistochemical [68] and nuclear binding findings [69], also suggests that it may have intracellular binding sites. Thus, the indole has been reported to bind to a series of closely related nuclear receptors referred to as RZR/RORα and RZRβ, and direct effects of melatonin in the periphery may be mediated by these binding sites [70–72]. It has also been hypothesized that in some cases, RZR/RORα may be primary targets of the membrane melatonin receptors [73]. Finally, melatonin may act in the cytosol after binding to calmodulin [74] or directly at the mitochondrial level [75] (fig. 3).

Nonetheless, some melatonin functions are independent of receptor proteins. In 1993, Tan and colleagues [76] reported for the first time melatonin's ability to neutralize the highly toxic hydroxyl radical ($^{\bullet}OH$). Subsequently,

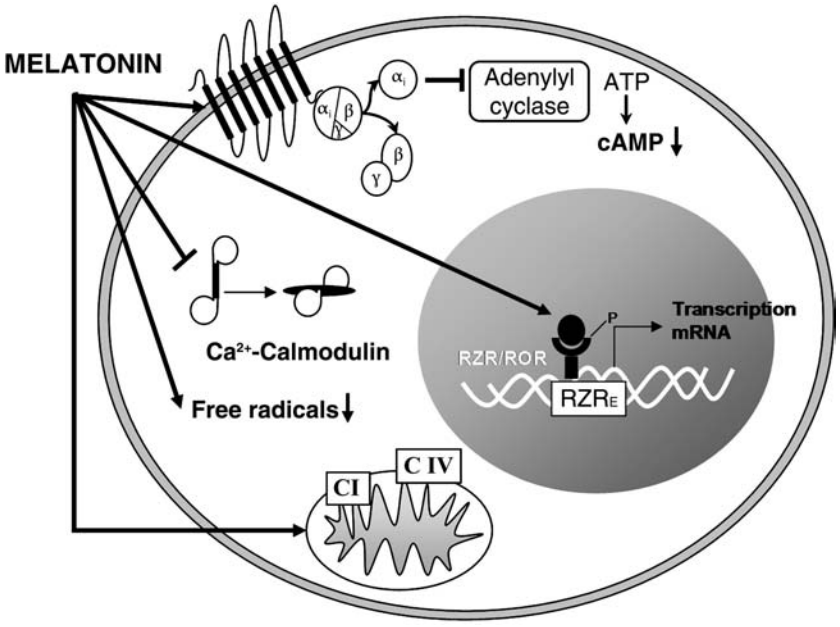

Figure 3. Melatonin's intracellular targets. A variety of intracellular pathways may be responsible for melatonin's biological actions. Melatonin membrane receptors exist in multiple tissues, and they are functionally related to circadian synchronization functions. In addition, the highly lipophilic nature of melatonin permits it to easily cross membranes and link to intracellular targets. Melatonin is an extremely effective scavenger of free radicals; it binds calcium-calmodulin complex, and it activates mitochondrial complex I and IV. Presumably, this combination of actions accounts for melatonin's protective effects against apoptosis in normal cells.

numerous reports have shown that melatonin directly neutralizes a number of toxic oxygen- and nitrogen-based species, including singlet oxygen, hydrogen peroxide, nitric oxide and peroxynitrite anion and/or its metabolites [77]. In addition to these actions, melatonin stimulates cellular antioxidant defense by increasing messenger RNA (mRNA) and protein levels of the major antioxidant enzymes [78–80] and reducing the activity of a prooxidative enzyme, i.e. iNOS [81, 82]. The role of melatonin in scavenging free radicals and stimulating cellular antioxidant defenses make this molecule of great interest in terms of altering the responses of cells to apoptotic stimuli. The potential use of melatonin in therapy has interested an increasing number of scientists given the relevance of free radicals to a number of diseases where cellular loss is a significant component of the condition.

Numerous reports have already shown that melatonin protects lipids, proteins and DNA from the harmful effects of free radicals [83]. These protective actions of melatonin are typically associated with preservation of cell viability. For example, it was demonstrated that pineal removal (which decreases blood melatonin levels) enhances cell death caused by ischemia-reperfusion in the central nervous system [84], while melatonin administration reduces the loss of CA1 pyramidal neurons in the hippocampus of male Wistar rats [85]. In peripheal tissues, melatonin protects liver [86] and the mucosa of the stomach from damaging effects of free radicals generated during ischemia-reperfusion [87], and parameters of myocardial dysfunction are markedly reduced in hearts perfused with melatonin suffering through an ischemic-reperfusion episode [88]. Numerous reports have also shown that melatonin protects against the damage caused by free-radical-generating agents such as ionizing radiation, lipopolysaccharide, zymosan, and a variety of genotoxic and chemoterapeutic agents including chromium, adriamycin, cisplatin and cytosine arabinoside (cytarabine) [89].

While the actions of melatonin are clearly diverse, collectively, neither melatonin's receptor signal transduction pathways nor its scavenging or antioxidant receptor-independent properties satisfactorily explain all the physiological and protective actions of the pineal indole. In the last 5 years, interest in melatonin has increased profoundly due to its influence on the processes of apoptosis. The degree to which melatonin acts on apoptosis in different tissues as well as the mechanisms whereby melatonin alters this process are yet unclear. Herein, we summarize what is currently known concerning the intracellular interactions of melatonin that may modify the apoptotic process.

Anti-apoptotic actions of melatonin

In 1994, a regulatory role of melatonin in apoptosis was first reported [90]. Currently, the number of papers published in this field is increasing rapidly, and there are now

in excess of 80 articles that demonstrate a role of melatonin in modulating experimentally induced apoptosis by a variety of agents. Much of the research carried out falls into three main categories: first, the role of melatonin in inhibiting apoptosis in immune cells; second, the role of melatonin in preventing neuronal cell death and finally, the role of melatonin in increasing apoptotic cell death in cancer cells. The mechanisms whereby melatonin regulates the apoptotic program and whether its role in apoptosis depends on the cellular lineage or on the cellular oxidative status are two major questions still to be answered.

Melatonin and apoptosis in immune cells

Cellularity of the immune system is tightly regulated, and homeostasis is undoubtedly important. Immunity is the first defense against harmful agents, and cellularity in the immune system is carefully controlled by apoptotic programs. It is well established that age-related loss of immature thymocytes during thymic involution is due to an increased rate of apoptotic cell death [91]. The delicate balance in the number of these cells is also highly controlled by exogenous factors and especially by a variety of hormones that influence apoptosis in primary lymphoid organs and in peripheral lymphocytes. For example, glucocorticoids in pharmacological doses induce apoptosis of immature thymocytes, Natural Killer (NK) cells and cytotoxic T lymphocytes [92, 93], and at physiological concentrations; they are mainly responsible for physiological cellular loss in the thymus [94]. Thus, thymic involution is a physiological phenomenon mainly mediated by the disappearance of cortical thymocytes through an apoptotic program and cultured thymocytes have been widely used to investigate apoptotic pathways and to test different drugs as potential inhibitors or promoters of apoptosis.

The pineal gland, via its main secretory product melatonin, enhances immune function [56, 95]. The administration of melatonin increases both thymus cellularity [96] and antibody responses [97]. Conversely, pinealectomy accelerates thymic involution [98] and depresses humoral and cell-mediated immune responses [99]. Considering this, a number of groups have tested whether melatonin modulates the apoptotic process in other organs of the immune system as it does in the thymus.

First, it was demonstrated that treatment of rats during their period of sexual maturation (from 25 days old through 65 days old) with physiological or pharmacological doses of melatonin diminished the number of apoptotic cells in the cortical area of the thymus and significantly reduced DNA laddering in these cells. Likewise, when physiological or pharmacological concentrations of melatonin were added to primary cultures of thymocytes, it partially prevented apoptotic cell death induced by pharmacological doses of the synthetic glucocorticoid, dexamethasone [46]. Soon thereafter, Provinciali and colleagues [100] showed that thymocytes or splenic lymphocytes isolated from old mice treated with melatonin were more resistant to apoptosis caused by serum deprivation than those obtained from old untreated or young mice. The authors claimed that indirect mechanisms mediated this anti-apoptotic action, and this was supported by the observations that plasma levels of both glucocorticoids and zinc were modified by melatonin; both are important modulators of thymocyte apoptosis.

Thymic involution plays an important role in the maturation of the immune system. Optimal functioning of the immune system depends on a properly timed disintegration of autoimmune cells due to apoptosis but also on their preservation against damaging agents that cause immunodepression. In general, immune cells are especially sensitive to a number of factors [101], and therefore there has been considerable interest in understanding the mechanisms of turnover of these cells, including the role of melatonin in this phenomenon.

Sainz and colleagues [102] have shown that melatonin probably does not influence key regulatory pathways of the apoptotic program in thymic cells since the indole did not prevent apoptosis induced by agents other than glucocorticoids. The transcriptional regulation of genes, which encode inhibitory proteins implicated in the general machinery of apoptosis, also seemed not to be altered by melatonin. This group reported increased levels of *bcl-2* mRNA in the thymus of melatonin-treated rats, but no difference in cultured thymocytes that were treated with the indole. On the contrary, a reduction in glucocorticoid-receptor mRNA levels in the intact thymus as well as in cultured thymocytes treated with melatonin seem to be the most likely mechanism whereby melatonin lowered the apoptotic action of glucocorticoids in thymocytes.

Recently, a new thorough investigation has shown that chronic administration of melatonin not only prevents but also reverses age-related thymic involution. Thymuses from 8-month-old mice that had received melatonin for 40 consecutive days showed a rejuvenated appearance and architecture of the gland. The thymic cortex was reestablished and cellularity increased, indicating regrowth as a consequence of melatonin treatment. Interestingly, when thymocytes were preincubated with melatonin prior to the addition of the Fenton reagents ($FeSO_4/H_2O_2$), which generate $^{\bullet}OH$, the frequency of apoptosis in these cells was reduced from 60 to 20%. Generation of $^{\bullet}OH$ in these cells was associated with a rise in caspase-3 activity, a principal executor of apoptotic cell death; this rise reverted to control levels when cells were cultured in the presence of melatonin (fig. 4). Based on these findings, the authors surmised that the antioxidant capacity of melatonin is likely responsible for its anti-apoptotic action in the thymus. The reversal of thymic involution in old mice following melatonin administration was attributed to increments of thymic cellu-

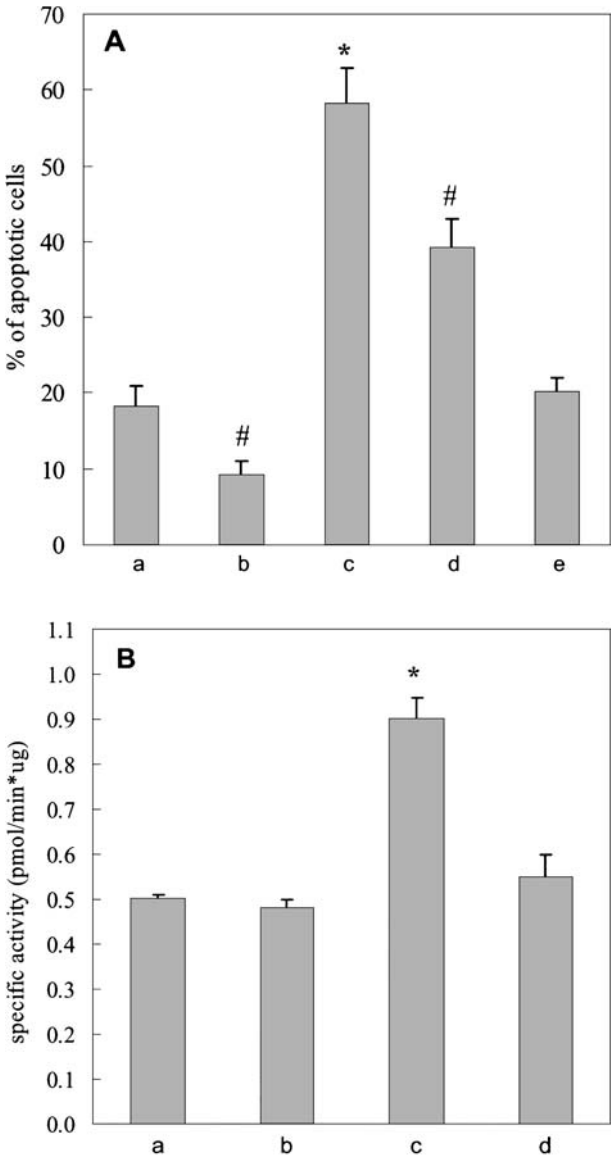

Figure 4. Melatonin effect on •OH-induced apoptosis in cultured thymocytes. Thymocytes from 3-week-old Balb/c mice were cultured with or without $FeSO_4/H_2O_2$ to induce apoptosis. (*A*) Percentage of apoptotic cells detected with flow cytometry: (a) control cells; (b) cells treated with 200 µM melatonin; (c) cells cultured for 4 h with $FeSO_4/H_2O_2$ (100 µM/50 µM); (d) cells pretreated for 1 h with 100 µM melatonin and then cultured with $FeSO_4/H_2O_2$ (100 µM/50 µM) for 4 h more; (e) cells pretreated for 1 h with 200 µM melatonin and then cultured with $FeSO_4/H_2O_2$ (100 µM/50 µM) for 4 h more. (*B*) Specific caspase-3 activity: (a) control cells; (b) cells treated with 200 µM melatonin; (c) cells cultured for 2 h with $FeSO_4/H_2O_2$ (100 µM/50 µM); (d) cells pretreated for 1 h with 200 µM melatonin and then cultured with $FeSO_4/H_2O_2$ (100 µM/50 µM) for 4 h more. Data are expressed as means ± S.D. of two separate experiments. # $P < 0.01$ vs. (a) and (e), * $P < 0.01$ vs. all groups. From [103].

larity caused by both the anti-apoptotic action and proliferation-augmenting effects of melatonin [103].

A critical role of free radicals has also been proposed in dexamethasone-induced apoptosis in T cells. Exogenous treatment of thymocytes with either low molecular weight antioxidants, antioxidant enzymes or metal chelators protects them from undergoing apoptosis [104, 105]; likewise, culturing thymocytes under hypoxic conditions, which lowers the generation of O_2-based reactants, generally protects them from dexamethasone-induced cell death [106]. Moreover, thymocytes selected for resistance to hydrogen peroxide exhibited resistance to dexamethasone-induced apoptosis and delayed cytochrome c release, supporting the idea that free radicals may mediate signaling pathways even in steroid-induced lymphocyte apoptosis [107]. As a result, it seems clear that the ability of melatonin to protect thymocytes from cell death may likely involve its antioxidant and/or scavenging properties. Since it is still difficult to clearly understand the role of free radicals in apoptosis, other possible mechanisms whereby melatonin modifies the process cannot be ignored. The MT_1 melatonin receptor has been found in thymic and splenic lymphocytes [108], and melatonin binding sites were also found in nuclei of immune cells [109], supporting the possibility that these molecules may participate in the physiology of primary lymphoid organs and peripheral lymphocytes.

B lymphopoiesis and apoptosis in bone marrow cells are also influenced by melatonin [110]. In vitro, melatonin counteracts apoptosis in bone marrow cells incubated with the chemotherapeutic agent etoposide, while monoclonal antibodies against anti-granulocyte macrophage-colony stimulating factor neutralized its effect [90]. The total number of nucleated cells recovered from the bone marrow of mice given dietary melatonin (14 µg/mouse/day) for 16 days was not significantly different from that of age- and sex-matched control mice. However, the incidence of apoptotic cells among the B cell lineage was lower in melatonin-treated mice than in the control animals. Thus, orally administered melatonin inhibited B cell apoptosis in mouse bone marrow, with this effect being restricted mainly to the large pre-B-cell stage in contrast to the unrestricted stage effect of melatonin on T cell lineage observed in the thymus [110]. Direct and indirect mechanisms were shown to be involved in melatonin's hematopoietic protection. Thus, melatonin may have protected the cells by acting via specific receptors, thereby increasing levels of a variety of cytokines or neuropeptides [111] or through granulocyte macrophage-colony stimulating factor and T-cell-dependent interactions between local stromal cells and hematopoietic cells [90]. These data are consistent with the results obtained 1 year earlier by Tan and colleagues [112], who found extremely high levels of melatonin in bone marrow cells of rats. Indeed, melatonin concentrations in these cells are two or-

ders of magnitude higher than in the circulation and, remarkably, 8 months after the pineal removal melatonin levels in bone marrow were still highly elevated. Shortly thereafter, Conti and colleagues [113] showed that bone marrow cells actually synthesize melatonin. Given the high sensitivity of bone marrow cells to different oxidative agents such as anticancer drugs [114], the presence of high melatonin concentrations in bone marrow cells may be important in preserving cell viability.

While several mechanisms have been proposed to explain melatonin's anti-apoptotic actions in immune cells, none has been definitively proven. It is clear that melatonin plays a role as an antioxidant in immune cells, but to date, no direct evidence has been found justifying the conclusion that its antioxidant properties are exclusively responsible for its anti-apoptotic function in thymus or bone marrow cells. The extreme lability of immature immune cells renders them highly sensitive to the destructive actions of oxidizing agents. A role for melatonin in terms of immune function has been known for years, but recent discoveries concerning its relationship to apoptosis suggest an even stronger association. On the bases of these data, Lissioni and colleagues [115] performed a phase II pilot study with patients suffering from persistent thrombocytopenia. After melatonin administration (20 mg/day in the evening for 2 months) normalization of platelet number was achieved in 8 of 14 individuals (57%), and mean platelet number significantly increased with melatonin therapy. This preliminary study suggests that melatonin may be effective in the treatment of thrombocytopenia for which no effective standard therapy is available. Consequently, the role of melatonin in apoptosis is not only of academic interest but may also be useful as a therapeutic approach in disease.

It is known that lymphocytes readily undergo apoptosis in patients receiving anticancer drugs or treatment with ionizing radiation [116, 117]. It is well documented that melatonin protects normal cells against the harmful effects of anticancer drugs and treatments [118]. Also, a prophylactic action of melatonin has been shown in a single whole-body exposure to 815 cGy radiation in mice [119] or under in vitro conditions reduced gamma radiation-induced chromosomal damage [120]. Although its anti-apoptotic actions were not specifically examined, it is likely that melatonin reduced radiation-induced cell loss.

Melatonin and apoptosis in the central nervous system

The death of central neurons is widely recognized as a normal feature of vertebrate development. One function of neuronal programmed cell death is to remove neuronal precursors that fail to establish appropriate synaptic connections [121]. Inappropriate apoptosis has been suggested to be involved in pathological neuronal death in various human neurodegenerative diseases such as Alzheimer's disease, Huntington's disease, amyotrophic lateral sclerosis and spinal muscular atrophy. Additionally, ROS have been implicated as mediators of excitotoxic and apoptotic nerve cell death [122]. Taking this into account, the role of melatonin in apoptosis in several neurodegenerative models has come under investigation, and the results have focused interest on melatonin as a neuroprotective agent.

Initially, it was shown that melatonin reduced the number of TUNEL (in situ terminal deoxynucleotidyl transferase assay)-positive neurons in primary cultures of rat cerebellar granule neurons, when they were treated with singlet oxygen ($^1O_2$) [123]. The ability of melatonin to quench $^1O_2$ in a pure chemical system had been previously suggested [124]. The antioxidant action of melatonin was found to be operative in protecting neurons from excitotoxicity and apoptotic cell death after kainate acid administration. In vivo, kainate, which triggers convulsions and brain damage via free radical processes, has been used as a pharmacological model of epilepsy. Hippocampal injury induced by kainate is accompanied by DNA fragmentation, which is commonly detected in situ by the TUNEL assay. Systemic administration of melatonin reduced the neurotoxicity of kainate, and although the precise mechanism of melatonin's neuroprotection was not elucidated, its antioxidant activity was considered the underlying mechanism [125]. Later, melatonin's mechanisms of protection against kainate-induced excitotoxicity were found to involve at least some antioxidative processes. Thus, melatonin reduced cell death and also restored critical components of the intracellular antioxidative defense system, i.e. GSH concentrations and glutathione peroxidase (GPx) activity, in the striatum and neighboring cortical areas damaged after kainate treatment [126].

It is known that melatonin is synthesized in the pineal gland but also in other tissues, including the neural retina. Melatonin's function in the retina seems to be as a physiological regulator of dopamine release [127]. In addition, melatonin receptors are present on the photoreceptors and on retinal pigment epithelial (RPE) cells [128, 129]. When RPE cells are cultured under confluent conditions and then subjected to experimental ischemia, a severe oxidative stress, they die by apoptosis [130]. In this model, the anti-apoptotic ability of melatonin was obvious as shown by the fact that ischemia-induced apoptosis was significantly reduced by the indole [131]. Even though there was a correlation between the efficacy of melatonin in reducing free radical damage, no differences where found in Bcl-2 protein levels. In addition, luzindole, a melatonin membrane receptor antagonist [132], did not blunt the protective effect of melatonin, suggesting that melatonin receptors associated with retinal pigmented epithelial cells were not involved in the protective actions

of melatonin [131]. In humans with retinitis pigmentosa (RP), an inherited retinal degenerative disease, photoreceptors undergo apoptosis. Rds/rds mice, a model of RP, in which a similar retinal degeneration occurs, melatonin significantly delayed cellular loss and reduced the number of apoptotic photoreceptors, suggesting it may have utility in the treatment of an inherited type of retinal degeneration [133].

As noted above, the critical role of apoptosis in the loss of neurons in neurodegenerative disorders stimulated several groups to examine the function of melatonin in reducing apoptosis induced by neurotoxin exposure. Neuronal apoptosis is generally believed to contribute to neurodegeneration in Alzheimer's disease (AD). The accumulation of the amyloid β protein (Aβ) in the brain is a central pathological feature in patients with AD. Increased levels of Aβ are known to induce apoptosis both in vivo and in vitro [134], downregulating the anti-apoptotic protein Bcl-2 and up-regulating the pro-apoptotic Bax [135]. Melatonin has been shown to be remarkably effective against Aβ-induced neuroblastoma cell death as well as preventing oxidative damage and intracellular Ca^{2+} increases induced by exposure to the cytotoxic Aβ peptide 25–35 (Aβ_{25-35}) [41]. Confocal scanning images of annexin/propidium iodide fluorescence showed a marked increase of apoptotic cells after 24 h of treatment (70 ± 25%) with Aβ_{25-35} with this effect being reduced (to 20 ± 10%) by simultaneous addition of melatonin to the culture medium. It is generally agreed that the neurotoxicity of Aβ is mediated by oxygen-based free radicals. Hence, cells exposed to Aβ generate higher levels of H_2O_2, which, in the presence of transition metals, promotes $^{\bullet}OH$ generation [136]. Furthermore, Aβ binds to cell surface receptors which impair Ca^{2+} membrane pumps, resulting in activation of calmodulin-dependent NOS, thereby increasing NO which in turn reacts with $O_2^{\bullet-}$ to form $ONOO^-$ which is also highly destructive [137]. The ability of melatonin to scavenge each of these reactants was proposed as the key mechanism of neuroprotection. Additionally, melatonin's protection may also include significantly more complex processes; however, the participation of melatonin membrane receptors on neurons is not involved in the protective actions of melatonin [138]. Using different analogs of the melatonin receptor which do not possess antioxidant activity, neuroblastoma and hippocampal cells were not protected from the toxicity caused by Aβ. In addition, phenyl-*N*-*t*-butyl nitrone (PBN), a radical scavenger with no structural relation to melatonin, mimicked its effect in protecting these neurons. Another receptor-independent mechanism of melatonin's protection against Aβ is possibly implicated and involves the ability of the indole to inhibit spontaneous formation of toxic β sheets and amyloid fibrils [139]. Melatonin's beneficial actions against Aβ was also documented by Shen and colleagues [140], who found similar results using primary cultures of hippocampal neurons.

Parkinson's disease (PD) is a neuropathological condition characterized by a progressive loss of dopaminergic neurons in the substantia nigra (SN). These cells participate in a neuronal circuit that controls voluntary movements. Some of the dopaminergic neuronal loss in Parkinson's disease has been postulated to be via apoptosis, given the morphological characteristics exhibited by what appear as dying cells in postmortem brains of Parkinsonism patients [141]. In vivo experimental models of PD have strongly suggested a role for apoptosis in the pathology of this human disease. In rodents, systemic administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a commonly used neurotoxin which induces PD-like signs, causes selective degeneration of dopaminergic neurons in the SN, concomitant with DNA fragmentation and caspase-3 activation [142]. Melatonin administration protects against MPTP-induced neurodegeneration in C57Bl mice [143]. As part of the response, melatonin rescues the drop of tyrosine hydroxylase immunoreactivity in the striatum and in the SN after MPTP injection; this is a key regulatory enzyme in dopamine synthesis. Also melatonin reduces markers of cellular damage, lowers the number of TUNEL-positive neurons and decreases DNA fragmentation in the brain of MPTP-treated mice to those in controls [144, 145].

In vitro experimental models of PD have clarified possible pathways involved in this disease. MPP^+, the active metabolite of MPTP, causes apoptosis in dopaminergic PC12 cells and in cultured primary midbrain mesencephalic neurons. Furthermore, either dopamine itself or 6-hydroxy-dopamine (6-OHDA), an inhibitor of mitochondrial complex I which is frequently used to model PD-like cell loss, induced cell death in PC12 cells with the morphological and biochemical characteristics of apoptosis [146, 147]. PC12 cells are a dopaminergic cell line derived from a pheochromocytoma, a tumor which differentiates into neuron-like cells in the presence of neuronal growth factor (NGF). PC12 cells are highly sensitive to 6-OHDA, and therefore they are commonly used to investigate mechanisms of cell death in PD. TUNEL-positive cells and DNA fragmentation caused by 6-OHDA treatment are also prevented by melatonin [148]. Although drugs which generate oxidative stress are generally considered to promote accidental cell death or necrosis, low doses of such drugs given over long periods of time enhance the frequency of apoptosis. It was established that not only does melatonin inhibit apoptosis caused by low doses of 6-OHDA, but also cellular necrosis induced by high doses of 6-OHDA in PC12 is likewise inhibited by melatonin [149] (fig. 5). Both effects of 6-OHDA seem to be mediated by increased levels of oxidative stress within cells. For this reason, the antioxidant properties of melatonin presumably account for its ability

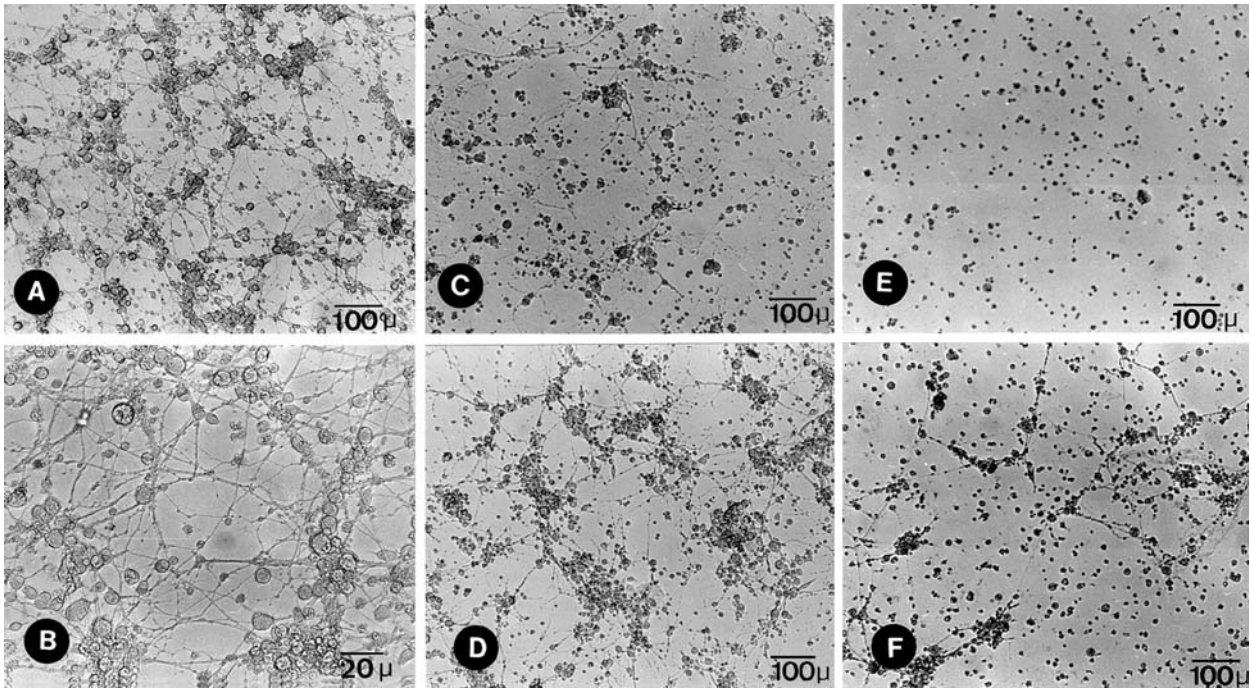

Figure 5. Melatonin protection in differentiated PC12 neurons. Cells were cultured in type I collagen-coated dishes for 14 days with 100 ng/ml NGF. Thereafter, cells were pretreated with melatonin for 24 h followed by the addition of 6-hydroxydopamine (6-OHDA). (*A*) and (*B*) Control groups; (*C*) 50 µM 6-OHDA; (*D*) 50 µM 6-OHDA plus 100 nM melatonin; (*E*) 100 µM 6-OHDA; (*F*) 100 µM 6-OHDA plus 100 nM melatonin. NGF promote the formation of neurites and neuronal-like morphology (*A, B*). After treatment with 6-OHDA, neurons lose neurites and appear damaged (*C, E*); on the other hand, low concentrations of melatonin preserved neuronal morphology even with the highest doses of 6-OHDA used (*D, F*). From [148].

to suppress both necrosis and apoptosis. Specifically, the authors reported that melatonin prevented the reduction in mRNA levels for antioxidant enzymes, i. e. CuZn SOD, MnSOD and GPx, which normally occur several hours after cells are treated with 6-OHDA.

More recently, Yoo and colleagues [150] proposed a possible mechanism by which melatonin inhibits apoptosis in PGT-β cells. PGT-β is a cell line derived from a pineal tumor which expresses tryptophan hydroxylase, the rate-limiting enzyme in serotonin biosynthesis, and serotonin *N*-acetyltransferase (NAT), which is involved in the conversion of serotonin to melatonin. These cells die through an apoptotic program in the presence of *S*-nitroso-*N*-acetylpenicillamine (SNAP), which raises intracellular NO levels. Melatonin not only prevented cell death and DNA laddering but also decreased caspase-3 activation and Bcl-2 protein accumulation measured by Western blot. Thus, Yoo and colleagues [150] claimed that melatonin, through Bcl-2 induction, abolished cytochrome c release from the mitochondria, preventing activation of caspase-3 and poly(ADP-ribose) polymerase (PARP) (fig. 6).

In a few instances the ability of melatonin to inhibit apoptosis has been compared with that of other neuroprotective agents, including some well-known spin-trapping agents or neurotrophins. ROS are essential mediators of cell death caused by withdrawal of trophic concentrations of K^+ or by depletion of intracellular GSH stores [151]. Neurotrophins, members of nerve growth factor family, such us brain-derived neurotrophic factor (BDNF) or neurotrophin-4/5 (NT-4/5) protected in a concentration-dependent manner against neuronal death [152]. However, neither nerve growth factor (NGF), neurotrophin-3 (NT-3), human ciliary neurotrophic factor (CNTF) nor glial cell line derived neurotrophin factor (GDNF) (up to 1 µg/ml) significantly reduced granule neuron degeneration. Addition of either melatonin (0.5 mM) or PBN (0.1 mM) raised cerebelar granule neuron survival up to 88 ± 3 and 87 ± 2%, respectively, compared with 40 ± 9% for low-K^+ cells or 37 ± 7% for glutamate-treated cells. Neurotrophins promote cell survival and differentiation through the TrK family of receptor tyrosine kinases, activating several intracellular signaling pathways. Of these, the phosphatidylinositol 3-kinase (PI 3-kinase) and mitogen-activated protein (MAP) kinase pathways have been suggested to be involved in neuronal survival [153, 154]. Addition of the PI 3-kinase inhibitors, LY294002, wortmannin or PD98059, a specific inhibitor of MEK (MAP/extracellular signal-regulated kinase), significantly reversed the ability of the neurotrophins to suppress neuronal loss. However, neither LY294002 nor PD98059 blocked melatonin-mediated survival in this

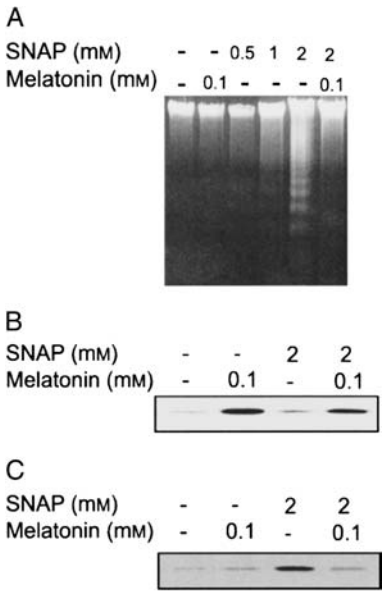

Figure 6. Protective effect of melatonin against *S*-nitroso-*N*-acetylpenicillamine (SNAP) induced apoptosis in PGT-β cells. PGT-β cells were incubated with or without melatonin 10 h prior to the addition of SNAP for 24 h. (*A*) DNA fragmentation determined by agarose gel electrophoresis; (*B*) Western-blot analysis of Bcl-2 and (*D*) cytochrome C. From [150].

model [152]. Even though the effect of melatonin could be explained in terms of GSH recovery, measurements of this intracellular constituent did not show it to be elevated in cells treated with melatonin. The authors claimed that although neurotrophins and related growth factors may play important roles in neuronal apoptosis, where free radicals are an essential component, endogenous small molecules with antioxidant/neuroprotective properties such as melatonin may also represent an important facet of the body's defense against cerebrovascular pathology. Thus, combined trophic factor-antioxidant therapy may be a greater benefit than either agent alone.

Essentially the same results have been obtained by Iacovitti and colleagues [155] using striatal neurons which were plated at low density in serum-free media and in the virtual absence of glia. Under these conditions, neurons die within several days of culture. Even though all neurotrophic factors tested (BDNF, bFGF, NT3, NGF and EGF) failed to rescue cell viability, melatonin, dopamine plus KCl or trolox prevented cell death. The molecular mechanism implicated in melatonin's protection was not totally elucidated, but the authors surmised that the antioxidant activity of trolox or melatonin may limit oxyradical damage to cells.

It seems remarkable that most of the experimental models of apoptosis in which melatonin has been shown to be successful are related to oxidative damage. However, to date there are no established direct mechanisms to explain the anti-apoptotic properties of melatonin in the neuronal systems tested. Indirect data have confirmed that melatonin prevents most of the cellular damage induced by free radicals, and this damage sometimes triggers the apoptotic program. In some situations melatonin reduces parameters of oxidative damage without preventing cell death [156, 157], implying that the mechanism proposed to prevent neuronal cell death may not relate to its antioxidant properties.

In order to assess the neuroprotective potential of melatonin and the mechanism responsible, Harms and colleagues [158] carried out a detailed study using several models of neuronal programmed cell death. As a model of caspase-dependent apoptotic cell death, they used either the drug staurosporine or the neurotoxin ethylcholine aziridinium (AF64A) and, as an alternative caspase-independent model, the apoptotic component of oxygen-glucose deprivation unmasked by glutamate antagonists. Pretreatment of cortical neurons with melatonin (up to 2 mM) significantly reduced starurosporine-induced malondialdehyde formation, a marker of oxidative membrane damage, but did not prevent staurosporine-induced neuronal cell death. Likewise, melatonin did not influence the exaggerated cell death caused by AF64A. On the other hand, when cells were deprived of oxygen and glucose, melatonin dose-dependently preserved cell viability; however, when the apoptotic component of hypoxia was unmasked by using the NMDA receptor antagonist MK-801, melatonin was also unable to prevent cell death. The limited effect of melatonin in hypoxia without blockade of NMDA receptor was explained by the fact that melatonin's protective effect is restricted to the necrotic component to ischemic cell death. Even though the results seem clear, the research conducted by Harms and colleagues [158] contains several contradictions. First, this is the only instance in which melatonin alone was shown to increase oxidative parameters in neuronal cultures or increase cell death after a single-dose treatment. Second, even using the general caspase inhibitor Z-VAD-FMK (100 µM), a well-known suppressor of apoptosis induced by many factors, they were unable to prevent the apoptotic component of oxygen-glucose deprivation unmasked by glutamate antagonist. From our point of view, although the work seems to have been carefully performed, the findings are so different from those in so many other reports that the validity of the data must be questioned.

More numerous are the reports that support the idea that melatonin is a protective agent in neurons. The anti-apoptotic role of melatonin in neuropathology is widely accepted by researchers that study brain aging, AD, PD, stroke and others, where an increased rate of cell death occurs [159, 160]. Perhaps the major criticism regarding melatonin, and this is emphasized by some melatonin researchers more than by neurologists, comes from the dosage of melatonin and its relative physiological impor-

tance. First, it must be borne in mind that the development of a neurodegenerative disease is not a physiological situation. Thus, neither the levels of free radicals nor the rate of apoptosis in the brain areas affected in neurological disorders are in the range of normal, so it is naive to imagine that any antioxidant, including melatonin, in physiological concentrations would be significantly protective in these pathological conditions. For example, despite the presence of physiological concentrations of many antioxidants (vitamin C, vitamin E, carotenes, glutathione, melatonin, uric acid and so on) combined, they are unable to combat all free radical damage and loss of neurons (and other cells) that occur in these pathological states. Second, physiological melatonin levels are typically in nanomolar concentrations in the serum (although they can be much higher in other compartments); no other antioxidant or anti-apoptotic agent is effective in nanomolar range concentrations in reducing cellular death under such severe circumstances.

The actual levels of melatonin in specific cells and subcellular organelles of the central nervous system are unknown. Recent reports affirm that melatonin levels in both the cerebrospinal fluid of the third ventricle [161] and in bile [162] are several orders of magnitude higher than blood concentrations of the indole. Thus, certain tissues, including the brain, are bathed by fluids that contain significantly higher concentrations of melatonin than those found in the blood. These findings emphasize the importance of defining physiological concentrations of melatonin relative to a particular fluid, cell or subcellular organelle [163].

Often, neurodegenerative diseases are not diagnosed until extensive brain damage has occurred. For example, in patients judged to have Parkinsonism, in excess of 75% of the dopaminergic neurons of the SN have already died. Since melatonin levels drop with increasing age [164], it has been suggested that the loss of melatonin may contribute to increased neuronal apoptosis that occurs in advancing age. If so, supplementing melatonin and/or other antioxidants in the diet after middle age may be helpful in reducing the rate of neuronal loss. Fortunately, even pharmacological doses of melatonin given to experimental animals throughout a lifetime or to humans for prolonged periods have shown the indole to have virtually no toxicity [165–167] and to be devoid of prooxidant actions [168], which are characteristic of some other antioxidants. Additionally, its utility in potentially delaying neurodegeneration becomes even a bigger issue given melatonin's ability to readily cross the blood-brain barrier.

Melatonin and apoptosis in peripheral tissues

There are other experimental models of apoptotic cell death in which melatonin was shown to be protective. Melatonin may also ameliorate damaging processes in unicellular organisms, i.e. *Saccharomyces cerevisiae,* where it is found in high concentrations. Aoshima and colleagues [169], in fact, showed that antioxidants, including melatonin or vitamin E, reduced cell death in yeast. Additionally, melatonin's anti-apoptotic properties were also noted in bovine cerebral endothelial cells, contributing to its protective role in ischemia models [170].

Ionizing radiation for the treatment of malignant tumors is limited by its side effects. Radioprotective substances are still sought for reducing this damage, and antioxidants are good candidates since radiation damage is mainly mediated by the hemolytic scission of H_2O generating $^{\bullet}OH$. Because of its antioxidant activity, melatonin was tested as to its ability to influence apoptosis induced by X-irradiation in skin fibroblasts. In this system, melatonin prevented cell death caused by irradiation and decreased lipid peroxidation without affecting p21 or p53 genes, which are related to X-ray-induced apoptosis [171]. In addition, melatonin was shown to diminish apoptotic cell death induced by ultraviolet-B radiation of dermal fibroblasts at very low concentrations (1 nM or 100 nM). Much higher doses of ascorbate (10 μM) are necessary to achieve the same level of protection [172].

Mercury hydrochloride ($HgCl_2$) has been widely used to study the pathophysiology of nephrotoxicity and acute renal failure. Using this agent, renal damage is particularly severe in the proximal tubules, which undergo marked structural and functional abnormalities. While the role of ROS in this condition is not definitively proven given that administration of antioxidants has failed to provide a consistent beneficial effect, an accumulation of hydroperoxides (products of free radical damage) in the kidneys has been reported. $HgCl_2$ treatment also causes a drop in renal GSH, and if animals are treated with a GSH-depleting drug, the severity of $HgCl_2$-induced acute renal failure is exaggerated [173]. Apoptosis is known to be induced by mercury in cultured proximal tubes and in other models of renal failure. Here, even though superoxide dismutase, allopurinol, tryptophan, *N*-acetyl tryptophan, vitamin E and ascorbic acid have failed to reduce mercury-related damage, melatonin prevented the pathogenic participation of apoptosis in renal failure when it was given at least 30 min in advance of mercury treatment. In this situation melatonin also decreased parameters of oxidative damage, including restoring GSH levels [174].

Additionally, hepatic cells are protected by melatonin against apoptosis induced by aflatoxin B1 (AFB1). Besides being a dietary hepatocarcinogen, AFB1 has been detected in dust generated in the processing and transportation of AFB1-contaminated products. Inhalation of grain dust contaminated with AFB1 may be a risk factor in human lung cancer. After 8 weeks of aflatoxin treatment in rats, it was found that caspase-3 enzyme activity was significantly increased, showing a positive correlation with markers of oxidative damage such as MDA, a lipid peroxidation product, and NO. In addition, a nega-

tive correlation was found when antioxidant defenses such as GSH levels, GPx or glutathione reductase (GRd) activities were measured. The rats treated with melatonin (5 mg/kg BW) exhibited a decreased apoptotic frequency, and they also showed reduced NO and MDA levels. Furthermore, melatonin recovered GSH levels and restored GPx and GRd activities [175].

As mentioned above, melatonin often has been used to prevent the damage caused by drugs; in these situations it is typically more effective than other antioxidants [89]. Many clinically useful drugs, e.g. anticarcinogens, are highly toxic. This is the case with doxorubicin (DOX), an effective antitumor anthracycline antibiotic that, unfortunately, induces a wide variety of acute cardiotoxic effects. Among others, DOX induces apoptosis in cardiomyocytes, which is significantly reduced when melatonin or its analog, 6-hydroxymelatonin, is present. Both preserved animal survival and improved cardiac function after DOX injection. Although the mechanisms were not totally deduced, these workers showed that the melatonin receptor analog, 8-methoxy-2-propionamidotetralin, which does not possess antioxidant capability, was unable to protect cardiac cells [176].

Pro-apoptotic actions of melatonin in tumor cells

In general terms, much of the research concerning the role of melatonin in apoptosis obviously has focused on immune cells and neurons. Given the importance of apoptosis in the loss of neurons with age and in age-related diseases, the role of melatonin as a regulator of the apoptotic machinery generally is important by itself. Failures at various points in the apoptotic pathway as well as mutations of some of the most important regulatory genes are sometimes related to tumor initiation and/or growth. Also, many anticancer treatments are based on increase in apoptotic rate, and several mechanisms of chemo- or radioresistance are due to an error in the proper triggering of the apoptotic pathway. Recently, the role of melatonin in influencing apoptosis in tumor cells attracted attention because it seems that it can actually increase apoptosis in some tumor cells. Given the importance of the apoptotic program in the treatment of cancer, publications on this subject regularly appear in the literature.

In contrast to the obvious inhibition of apoptotic processes in normal cells, there is evidence that in cancer cells, melatonin may actually promote apoptosis. The ability of melatonin to inhibit the development and growth of a variety of tumors has been known for decades [59]. In addition to reducing tumor growth both in vivo and in vitro, blood levels of melatonin generally seemed to be reduced in patients with cancer [177]. Perhaps the most thoroughly studied tumor types that are modulated by melatonin are endocrine cancers, including breast cancer. Melatonin reduces hormone-responsive breast cancer cell growth, an effect known for almost 20 years [178, 179]. Melatonin treatment acts directly on hormone-responsive human breast cancer cells in vitro to suppress their proliferation [60]. Hormonal inhibition of breast cancer is frequently associated with cytostatic rather than cytocidal effects, and tumors often evolve under selective pressure to become resistant to endocrine therapy in breast cancer management. A combination of drugs with anti-estrogenic molecules might be an alternative to the classical endocrine therapy.

Consistent with this, Eck and colleagues [180] demonstrated that a sequential treatment regimen with melatonin and all-*trans* retinoic acid, an inhibitor of estrogen-positive but not of estrogen-negative breast cancer cells, induced apoptosis in human breast cancer cells (MCF-7). Both melatonin and retinoic acid inhibit proliferation, but only retinoids have been shown to induce cell death [181]. The cytocidal effects produced by this sequential regimen are probably cell and treatment specific, as demonstrated by the lack of response of estrogen-negative cells. A differential regulation in the expression of 'death suppressor' or 'death inducer' genes was shown. Thus, sequential treatment reduced the levels of Bcl-2 protein and increased the levels of Bax [180]. Clearly, the development of combination therapies which would reduce the concentrations needed for clinical efficacy, yet still enhance anti-tumorigenic activity, would be of great benefit. Several authors showed that melatonin pretreatment sensitized the cells to all-*trans* retinoic acid such that 100- to 1000-fold lower levels of retinoic acid were sufficient to induce apoptosis [182]. It was also found that the upregulation of the apoptosis promoter, Bak, coupled with the downregulation of the apoptosis suppressor, Bcl-2, were primary events contributing to the induction of apoptosis in these cells. Although melatonin did not bind to the retinoic acid receptor, the authors suggested that cross-talk between melatonin and retinoic acid signaling pathways may be via phosphorylation of the receptor. Melatonin's actions through its membrane-associated, G-protein-coupled receptors, was also considered. It had been reported that the retinoic acid receptor signaling pathway is activated independent of a ligand in the presence of catalytic subunits of protein kinase A, a cAMP-dependent protein kinase [183]. Collectively, these findings suggest that melatonin, acting through its membrane receptor, might lead to modulation of cAMP levels with subsequent enhancement of retinoic acid receptor (RAR) transactivation.

In an attempt to characterize the therapeutic relevance of this research, Nowfar and colleagues [184] conducted an in vivo experiment using the *N*-nitroso-*N*-methylurea (NMU)-induced rat mammary tumor model. As expected, melatonin in combination with 9cRA more efficiently

prevented tumor development than either drug alone. In addition, the doses of 9cRA necessary to maintain antitumor efficiency were significantly reduced by the presence of melatonin.

Once melatonin's pro-apoptotic action in MCF-7 cells in combination with 9cRA was known, the question that arose is whether this effect could explain, at least in part, its inhibitory effect on tumor growth. To answer this, Cos and colleagues [185] studied the ability of melatonin by itself to induce apoptosis in MCF-7 cells. In this model, compared with vitamin D_3, a well-known antiproliferative and pro-apoptotic agent, melatonin reduced cell proliferation as demonstrated previously, but it did not increase apoptosis. Expression of p21 and p53 proteins, both related to cell cycle control, was increased after melatonin or vitamin D_3 treatment. However, vitamin D_3 significantly reduced anti-apoptotic bcl-X_L gene expression, while melatonin did not change either bcl-2, bcl-X_L or bax mRNA levels.

The pro-apoptotic action of melatonin in cancer cells is not restricted to breast tissue. Melatonin also suppresses the proliferation of a Colon 38 cell line both in vitro and in vivo [186]. In addition, the proliferating/apoptotic cell ratio in this cell line, originally developed from a 1,2-dimethylhydrazine-induced colonic tumor in mouse, was significantly lower when the animals were treated with melatonin, indicating that it also induces an increase in apoptosis [187]. Furthermore, CGP52608, a selective ligand for the nuclear orphan receptor RZR/ROR, which reportedly binds melatonin with high affinity [73], also increased the apoptotic index as measured by TUNEL assay [188]. On the other hand, Winczyk and colleagues [189] also found that *N*-[(4-methoxy-1H-indol-2yl)methyl] propanamide (UMC386), a membrane MT_1 melatonin receptor analog, exerted the same anti-proliferative and even pro-apoptotic role as did melatonin in these cells. While it is known that both CGP52608 and UMC386 are ligands of the melatonin receptors described to date, these molecules may have actions independent of the receptors that were not considered.

Similar results have been found in Ehrlich ascites carcinoma cells (EAC) implanted intraperitoneally into female mice [190]. Oral suplementation of melatonin reduced the viability and volume of the tumor, delayed the progression of the cell cycle and reduced the DNA content of the cells. The depressed cell viability again suggests that melatonin might be inducing apoptosis in EAC cells. The multiplicity of tumors colon tumors in rats induced by the carcinogen 1,2-dimethylhydrazine as well as the mitotic index of each was also reduced by melatonin. On the contrary, the TUNEL-positive cells were increased significantly in the tumors of the melatonin-treated rats [191].

Finally, the promotion of Fas-induced cell death by melatonin in human leukemic Jurkat cells was recently reported [192]. Monitoring the formation of free radicals by using the oxidation-sensitive dye dihydrorhodamine 123, the authors showed that melatonin (micro- to millimolar concentrations) enhanced the appearance of free radicals in a dose- and time-dependent manner; in contrast, trolox reduced intracellular rhodamine fluorescence in Jurkat cells. To evaluate the functional relevance of these findings, the effect of melatonin on Fas-induced cell death in Jurkat cells was determined. In this study, melatonin dose-dependently enhanced the percentage of cancer cells undergoing Fas-induced apoptosis and increased Fas-induced intracellular free radical formation. Differences in the redox state and susceptibility to oxidant-induced changes in cell functions among different cell types most likely explain the results. Thus, it is known that Fas-induced apoptosis was preceded by intracellular ROS formation [193], and increased intracellular ROS also modulated the expression of Fas receptor on the surface of lymphocytes [194].

The role of melatonin as a pro-apoptotic agent in cancer cells is a new field of investigation, and information is still limited. The results obtained to date appear promising, and if in fact melatonin uniformly induces apoptosis in cancer cells, the findings could have important clinical utility. Many tumors show resistance to drug treatment mainly due to their resistance to undergo apoptosis. Identifying agents which potentiate apoptosis in cancer cells is clearly of great interest. It may seem unusual that a substance can induce apoptosis in cancer cells while preventing it in normal cells. Other antioxidants, however, can initiate apoptosis in cancer cells; this effect has been described for vitamin E, vitamin C, resveratrol and a number of carotenoids [195–198]. The idea that antioxidants may increase cell death in tumors comes from clinical studies which demonstrated that a high intake of vegetables and fruits which contain abundant antioxidants sometimes reduces cancer risk. Currently, it is generally accepted that foodstuffs containing a variety of antioxidants are beneficial, and a scientific basis for the benefits of antioxidants as a hedge against cancer is slowly becoming apparent. The reader is reminded that edible plants also contain melatonin [50], and it is ingested in the diet when these foodstuffs are eaten [199].

Concluding remarks

The apoptotic program presents a fascinating series of events which have major implications in tissue homeostasis. The participation of apoptosis in a number of diseases is established, and a better definition of these processes would permit a clearer understanding of these diseases. In addition, defining the mechanisms of this process would be of great help in defining new therapies and treatment strategies. Currently, free radicals and intracellular oxidative status seem to be significantly linked

to the apoptotic program. The beneficial effects of melatonin in reducing apoptosis in the central nervous system could potentially be useful in the treatment of neurodegenerative diseases, and additionally, the recent discovery of melatonin's participation in sustaining the apoptotic program in cancer cells makes this molecule of even greater importance. While benefits of melatonin emerge in a considerably high number of studies, the mechanism(s) underlying the effects are still unclear. Both its radical-scavenging properties and other melatonin-mediated intracellular pathways may be involved. Importantly, numerous studies to date have shown melatonin to be safe over a very wide range of doses [165–167]; furthermore, hundreds of reports in both animals and humans indicate its utility in certain disease states. Finally, melatonin is inexpensive to produce and has a long shelf life. Considering its many benefits, it should be more thoroughly investigated at the clinical level in terms of specific disease processes which involve apoptosis.

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