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Review

Melatonin influences pancreatic cancerogenesis

Jolanta Jaworek and Anna Leja-Szpak
Department of Medical Physiology, Jagiellonian University CM, Krakow, Poland

Summary. Pancreatic cancer has fatal prognosis because of the absence of early symptoms, late diagnosis and the resistance to radio- and chemotherapy. Melatonin, an indoleamine discovered in the pineal gland, has also been detected in the gastrointestinal system and its specific receptors have been identified in the pancreas. Some evidence indicates that melatonin could modulate the process of pancreatic oncogenesis: 1) Melatonin, as direct scavenger of radical oxygen and nitrogen species (ROS and RNS) and activator of antioxidant enzymes effectively protects the pancreatic tissue against oxidative stress and inflammatory damage. 2) In pancreatic carcinoma cell line (PANC-1) melatonin used at high doses affects the Bax/Bcl protein balance, and stimulates the expressions of caspase-9 and caspase-3, thus activating the mitochondrial pathway of apoptosis. On the contrary, low concentrations of melatonin turn on the production of anti-apoptotic heat shock proteins: HSP27, HSP70, and HSP90, which prevents the activation of caspase-3. 3) Melatonin reduces angiogenesis and decreases proliferation of endothelial cells through inhibition of vascular endothelial factor (VEGF). 4) Melatonin strengthens the immune defense of the organism *via* activation of peripheral effector T cells and suppression of T regulatory cells. 5) In animal studies melatonin has been found to increase the efficacy of oncostatic drugs, to reduce the side effects of chemotherapy and to decrease morbidity. These observations suggest that melatonin at high doses could be potentially taken into consideration as the supportive treatment in the therapy of pancreatic cancer, although the effect of melatonin on apoptosis requires further study.

Key words: Melatonin, Pancreatic cancer, Antioxidant enzymes, Apoptosis, Angiogenesis, Immunomodulation

Offprint requests to: Prof. Jolanta Jaworek MD, PhD, Department of Medical Physiology, Faculty of Health Care Jagiellonian University CM, Michalowskiego 16, 31-126 Krakow, Poland. e-mail: mpjawore@cyf-kr.edu.pl

Introduction

Pancreatic cancer represents the fifth leading cause of adult death from cancer in the world (Qiu et al., 2011). It is one of the most dangerous tumors with fatal prognosis. Because of the absence of early symptoms and serious difficulties in the examination of the pancreatic gland, pancreatic tumors are usually recognized in the advanced stage. Late diagnosis together with the resistance of pancreatic cancer to chemo- or radiotherapy results in high patient mortality (5-year survival is lower than 5%) (Jemal et al., 2009). Over the last years the incidence of pancreatic tumors has been increasing, with the peak of incidence found between 60 and 80 years of age (Krejs, 2010).

Established risk factors for pancreatic cancer include tobacco smoking, hereditary or chronic pancreatitis, obesity, diabetes mellitus type 2, high-fat diet, family history of pancreatic cancer, or perhaps bacterial infection (Krejs, 2010; Michaud, 2013). Molecular mechanisms play an important role in the pancreatic neoplasia. Cancerogenesis is associated with mutations of proto-oncogenes such as KRAS, $CTNNB_1$, or AKT_1, and with impairment of multiple tumor-suppressor genes, such as TP_{16}, TP_{53}, APC, $SMAD_4$ (Zavoral et al., 2011).

Almost 95% of pancreatic cancers develop from the

exocrine part of the pancreas, including the ductal epithelium, acinar cells, connective tissue or lymphatic tissue (Beger et al., 2003). The remaining 5% includes adenosquamous carcinomas, hepatoid carcinomas, colloid carcinomas and undifferentiated carcinomas (Han et al., 2006).

The treatment offered to the patients is a combination of complete surgical tumor removal and chemo- or radiotherapy. Unfortunately, at the moment of diagnosis, metastases have been found in 50-90% of patients with pancreatic neoplasm, which limits the effectiveness of surgical treatment (Lloyd and Chang, 2013; Niess et al., 2013).

Melatonin – production in the organism and receptors

Melatonin (5-methoxy-N-acetyltryptamine) originally isolated from the pineal gland, is a simple indoleamine, produced from amino acid L-tryptophan with serotonin as an intermediate step in this process (Lerner et al., 1958). Mitochondria appear the primary site of melatonin production in animals, which was evidenced by identification of the main enzymes required for synthesis of this molecule: arylalkylamino-N-acetyl-serotonin-transferase (AA-NAT) and hydroxyindolo-O-methyl-transferase (HIOMT) (Stefulj et al., 2001; Shimozuma et al., 2011; Tan et al., 2013). Melatonin is catabolized to kynuramines: N1acetyl-N2-formyl-5-methoxy-kynuramine (AFMK) and N1acetyl-5-methoxykynuramine (AMK), and these substances share a part of melatonin's properties (Hardeland et al., 2009).

Melatonin has been detected in almost all living organisms including plants, bacteria, vertebrates and invertebrates (Bubenik, 2008; Chen et al., 2011; Tan et al., 2012). In mammals, melatonin was identified in the central nervous system, in the immune cells, in the retina and in the harderian glands, although the gut appears to be the main source of this indoleamine (Bubenik et al., 1996; Messner et al., 2001; Bubenik, 2008; Chen et al., 2011; Hardeland and Poeggeler, 2012; Tan et al., 2012). It has been calculated that total amount of melatonin produced in the gastrointestinal system exceeds 400 times that of pineal origin (Huether et al., 1998).

In the gut melatonin is produced in the enterochromaffin (EE) cells of the gastrointestinal mucosa and is secreted into the gut lumen, where content of this indoleamine gradually increased from a low amount detected in the jejunum and ileum up to a high concentrations found in the colon and rectum (Raikhlin et al., 1975; Bubenik et al., 1996; Messner et al., 2001; Kvetnoy et al., 2002). Melatonin, which is present in the gastrointestinal lumen originates also from ingested food, from microorganisms living in the gut, and from the bile secreted into the duodenum (Bubenik, et al., 1996; Messner et al., 2001; Kvetnoy et al., 2002; Tan et al., 2012).

Melatonin production and secretion from the pineal gland is directed by light-dark cycle with a peak at night, when plasma levels of this substance rise up to 150-160 pg/ml, whereas light reduces this melatonin plasma concentration almost ten times (Zawilska et al., 2009; Stebelova et al., 2010). It is worth remembering that production of melatonin decreases with age, and in addition, the circadian rhythm of its secretion disappears in the elderly (Cardinali et al., 2008). In contrast to the diurnal/nocturnal rhythm of melatonin release from the pineal gland, the secretion of this substance from the gastrointestinal tract is independent from exposure to light, but related to food intake (Bubenik, 2008).

Melatonin binds to the specific receptors (MT_1, MT_2, MT_3, and RZR/ROR) which have been detected on the cell membranes, in the cytosol, and in the nucleus. Transmembrane receptors MT_1, MT_2 have been identified in many tissues, such as central nervous system, retina, heart, blood vessels, immune cells and gastrointestinal system, with the highest density in the ileum and colon (Cardinali et al., 2008). Both receptors MT_1 and MT_2 could be blocked by luzindole, a non-selective antagonist of melatonin receptors (Slominski et al., 2012; Ahmed et al., 2013). Melatonin receptor MT3 is the enzyme quinone reductase 2 (QR2), which is responsible for part of melatonin's antioxidative properties (Adamczyk-Sowa et al., 2013). The role of orphan nuclear receptors RZR/ROR is unclear (Hardeland, 2008). Furthermore, melatonin, which is characterized by high lipid solubility, could directly cross the cell membrane to exert its biological effects on the cell compartment (DiBella and Gualano, 2006).

Melatonin as an antioxidant

Melatonin remains the subject of numerous studies because of its anti-oxidative and anti-inflammatory effects, which have been proved in various tissues by many investigators (Jaworek et al., 2003; Kurcer et al., 2007; Rodriquez et al., 2007; Chojnacki et al., 2011; Ochoa et al., 2011). This substance attenuated the inflammatory change, diminished tissue damage, and modulated the immune response of the organism (Carillo-Vico and Reiter, 2006; Miller et al., 2006; Paredes et al., 2007; Rodriquez et al., 2007; Terron et al., 2009; Zhou et al., 2009; Mukherjee et al., 2010; Ganguly et al., 2010; Yang et al., 2011; Liu et al., 2011).

The above beneficial effects of melatonin are dependent on its antioxidative properties. Melatonin acts as a direct scavenger of radical oxygen (ROS) and nitrogen (RNS) species and also activates the enzymatic defense mechanism against these toxic radicals (Tan et al., 2007; Ochoa et al., 2011; Miller et al., 2011; Shagirtha et al., 2011). ROS and RNS are noxious substances, which are generated as a side products of mitochondrial metabolism. These radicals are necessary to maintain the intracellular redox homeostasis under normal conditions and they are deactivated by antioxidants: enzymatic or nonenzymatic (Reiter et al., 2008; Galecka et al., 2008). Oxidative stress and

inflammation are characterized by enlarged production of ROS and RNS, which exceed the normal ability of tissues to neutralize these toxic substances. Accumulation of ROS and RNS leads to tissue damage (Bergaminil et al., 2004; Shi et al., 2005; Reiter et al., 2007; Rosanna and Salvadore, 2012).

Melatonin acts as an nonenzymatic scavenger of free radicals, together with vitamins C and E, uric acid, selenium and other substances (Cruz et al., 2007; Reiter et al., 2008; Durante et al., 2010; Rosanna and Salvadore, 2012). Moreover, melatonin strengthens the activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), or glutathione reductase (GR), amplifying indirectly the antioxidant capacity of tissues (Tan et al., 2007; Shagirtha et al., 2011; Ochoa et al., 2011; Miller et al., 2011).

Cell membranes are highly permeable to melatonin, which easily enters the cell to protect the cell structure against oxidative damage and to prevent lipid membranes from peroxidation (Reiter et al., 2008). In addition melatonin via activation of the MT3 receptor, stimulates enzyme quinone reductase 2 (QR2), known as a potent antioxidant (Slominski et al., 2012).

In experimental studies on pancreatic cancer, treatment of animals with combination of melatonin and oncostatic drugs capecitabine (precursor of 5-fluorouracil) and celecoxib, resulted in the spectacular reduction of the side effects of capecitabine and increased survival of the animals. These protective and antitumor effects of melatonin were related, at least in part, to the reduction of lipid peroxidation products and the recovery of antioxidant enzymes CAT and GPx activities in the pancreatic tissue (Ruiz-Rabelo et al., 2007, 2011; Padillo et al., 2010).

Pro-apoptotic action of melatonin

Numerous studies on cancer cells have focused on the effect of melatonin on the intrinsic pathway of apoptosis otherwise known as mitochondrial pathway (Muilenburg et al., 2010). This pathway involves the members of the BCL-2 protein family, which consists of pro-apoptotic and anti-apoptotic subgroups of protein. The pro-apoptotic subgroups includes the BAX-like group (e.g. Bax) and BH-3 subgroup (e.g., Bad, Puma, Noxa), whereas the anti-apoptotic subgroup consists of proteins known as Bcl-2, Mcl-1 or Bcl-xL (Autret and Martin, 2009; Takahashi et al., 2013). The imbalance between pro- and anti-apoptotic proteins leads to the activation of Bax or Bak and to the release of cytochrome c from the mitochondrial outer membrane to the cytosol. Consequently, cytochrome c binds to apoptotic protease activating factor-1 (Apaf-1) and procaspase-9, thereby activating the caspase cascade (Adams and Cory, 2007; Fulda, 2009). Executioner caspase-3 initiates DNA fragmentation, degradation of cytoskeletal protein and cell death induction (Degterev et al., 2003; Chowdbury et al., 2008).

Determination of anti-apoptotic proteins (Bcl-2, Bcl-xL) expression is used in pancreatic cancer for prognostic purposes (Lee et al., 1996; Kapranos et al., 1997; Matsushima et al., 1999; Muilenburg et al., 2010; Okumura et al., 2008; Takahashi et al., 2013). There are strong clinical data supporting the positive correlation between Bcl-2 expression and patient survival following pancreatic cancer resection (Nio et al., 2001; Sun et al., 2002), Moreover, there is some evidence that knock-down of Bcl-xL and Mcl-1 strongly induced apoptosis in pancreatic cancer cells (Hinz et al., 2000; Takahashi et al., 2013). Bcl-2, Bcl-xL and other family members might be good targets for pancreatic cancer therapy and certainly impairment in the function of these proteins leads to an increased rate of apoptosis in pancreatic cancer cells and sensitizes these cells to oncostatic drugs, such as gemcitabine (Jiang et al., 2006). On the other hand, overexpression of Bax increases the sensitivity of pancreatic cancer cells to drug-induced apoptosis (Xu et al., 2002) although limited data regarding Bax expression and patient survival is not sufficient to draw any firm conclusions.

Extensive studies of the last years have indicated that melatonin could influence the apoptotic process in pancreatic cancer cells. The results of our previous study on human pancreatic carcinoma cell line (PANC-1) present the evidence that melatonin affects the Bax/Bcl-2 protein balance and stimulates the expression of caspase-9 in these cells (Leja-Szpak et al., 2010). Above findings were in accordance with Xu et al. (2013) previous results, showing the down-regulation of Bcl-2 and upregulation of pro-apoptotic Bax expression after treatment of pancreatic cancer cell line SW-1990 with melatonin (Xu et al., 2013). A pro-apoptotic effect of melatonin was also demonstrated in the rat pancreatic acinar cells (AR42J), where this indoleamine produced depolarization of the mitochondrial membrane, resulting in an increase of the membrane's permeability and consequently in the increased rate of apoptosis of this cell population (Uguz et al., 2012). Similar results were obtained in AR42J cells by Gonzalez et al. (2011), who confirmed that melatonin induced transient changes in cytosolic concentration of Ca^{2+}, mitochondrial membrane depolarization and calcium-dependent activation of caspase-3 (del Castillo-Vaquero et al., 2010; Gonzalez et al., 2011). In opposition to the above results, our own observations (Leja-Szpak et al., 2010) have evidenced that melatonin used at low concentrations stimulates the expression and phosphorylation of anti-apoptotic heat shock protein (HSP27), as well as the production of HSP70 and HSP90·, in PANC-1 cells. Our observation suggests that the mentioned HSPs could be responsible for the inhibition of the pro-apoptotic pathway at the level of caspase-3 activation and thus HSPs could block apoptotic cell death, as confirmed by the absence of DNA defragmentation in these experiments (Leja-Szpak et al., 2007, 2010). In addition, melatonin has been found in rat pancreatic acinar tumor cells (AR42J) to

induce an overexpression of another chaperone protein HSP60, and as suggested by investigators, this phenomena is very likely to take a part in melatonin-evoked pancreatic tissue protection against caerulein overstimulation (Bonior et al., 2005).

Experimental studies have shown a beneficial effect of melatonin used as an additive treatment with other oncostatic drugs. Melatonin-enhanced chemotherapy induced cytotoxicity and apoptosis in pancreatic AR42J cells and, therefore, co-treatment of these cells with melatonin and other oncostatic drugs (5-fluorouracil, cisplatin, and doxorubicin) increased the rate of apoptosis, as compared to the treatment with each chemotherapeutic agent alone (Uguz et al., 2012).

Anti-angiogenic action of melatonin

An adequate blood supply is the key factor in the tumor development of pancreatic cancer, hence anti-angiogenic therapy has been a challenging target for tumor therapeutics. Vascular endothelial growth factor (VEGF) is the most active, endogenous pro-angiogenic factor involved in angiogenesis in various types of tumors, including pancreatic cancer. Both VEGF and VEGF receptors are overexpressed in pancreatic cancer cells and perhaps their uncontrolled increase is responsible for cancer growth promotion, its dissemination and metastasis (Doi et al., 2012; Huang et al., 2012).

Studies of Lv et al. (2012) have shown that a high concentration of melatonin (1 mmol/L) strongly inhibited the proliferation of carcinoma PANC-1 cells by suppression of VEGF expression, thus suggesting a possible anti-angiogenic effect of this indoleamine (Lv et al., 2012). These findings confirmed the previous results, showing that pharmacological concentration of melatonin exerts a direct anti-angiogenic effect through the reduction of the expression of endogenous VEGF and HIF-1 in PANC-1 cells and through inhibition of the proliferation process in vascular endothelium (Cui et al., 2012). Endothelial cell migration and invasion is essential for the formation of new blood vessels during neo-angiogenesis. Alvarez-Garcia et al. (2013) have observed that melatonin treatment strongly inhibited the migration of endothelial cells (HUVECs) and disrupted the tubular network on basement membrane. They suggested that melatonin could be responsible for impairment of the paracrine interaction between proximal endothelial and malignant epithelial cells via down-regulation of VEGF receptor expression in these cancer cells (Alvarez-Garcia et al., 2013).

Melatonin in the treatment of pancreatic tumors

Chemotherapy is an important method of adjuvant therapy for pancreatic cancer. Gemcitabine, 2,2-difluoro-2-deoxycytidine, remains the 'gold standard' in the treatment of pancreatic tumor and its clinical benefit has been documented (Berlin and Benson, 2010). In order to increase the effectiveness of oncologic therapy in pancreatic cancer, gemcitabine is commonly used in combination with other chemotherapeutic agents such as

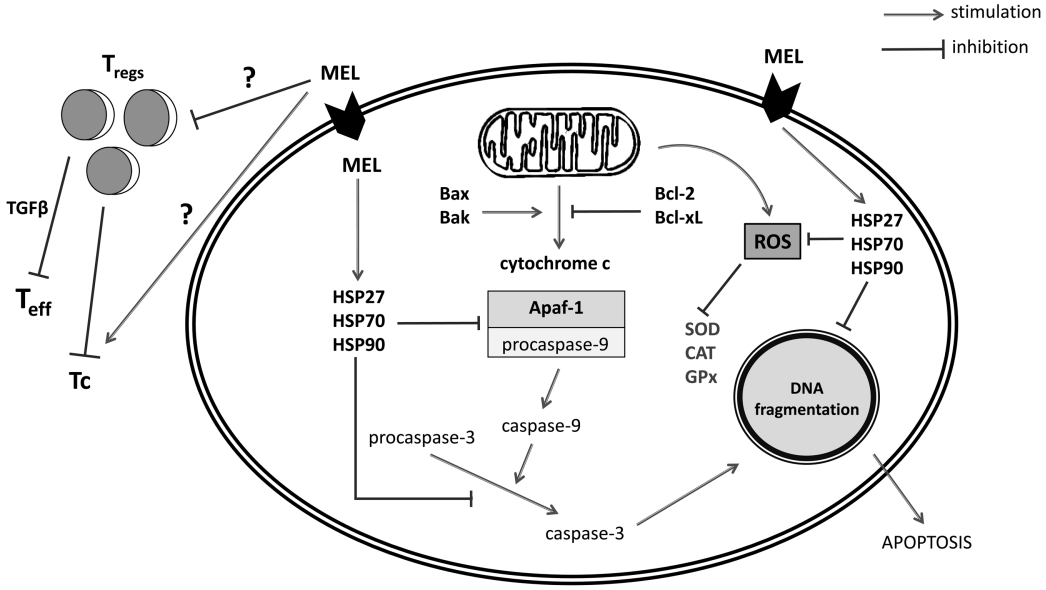

Fig. 1. Hypothetical mechanism of melatonin effects on pancreatic carcinoma cells. Melatonin triggers the intrinsic apoptotic pathway through stimulation of pro-apoptotic protein Bax or/and Bak production and depletion of the anti-apoptotic protein (Bcl-X_L or Bcl-2). This leads to the destabilization of the mitochondrial membrane, to the release of the cytochrome c, and to the formation of apoptosis protease activating factor-1 (Apaf-1). Complex of Apaf-1 and cytochrome c (apoptosome), activates a procaspase-9 and subsequently, make active executioner caspase-3. Active caspase-3 is responsible for DNA fragmentation, apoptotic body formation and cell demise. On the contrary, stimulation of HSP's by low dose of melatonin could inhibit key elements in this cascade leading to the down-regulation of apoptosome formation and preventing the activation of caspase-3. In addition, melatonin directly neutralizes ROS and RNS and increases the activity of the antioxidative enzymes (SOD, CAT, GPx), protecting the intracellular compartment. Modulation of the immune defense by melatonin could involve the suppression of T regulatory cells (T_{regs}) and activation of T effector cells (T_{eff}) and T cytotoxic cells (T_c).

5-fluorouracil or its prodrug capecitabine, which has already been introduced to the clinical trials (Hess et al., 2003; Saif et al., 2007). Moreover, many studies focused on molecular targets such a: metalloproteinase inhibitors, cycloooxygenase-2 (COX-2) inhibitors and tyrosine kinase inhibitors. Such treatment could be more effective as compared to classic therapy (Pino et al., 2004). The data published by Uguz et al. (2012) have indicated that co-treatment of pancreatic AR42J cells with chemotherapeutic agents such as 5-fluorouracil, cisplatin or doxorubicin in the presence of melatonin elevated mitochondrial membrane depolarization and increased the population of apoptotic cells (Uguz et al., 2012). The experimental studies of Ruiz-Rabelo have shown that a combination of melatonin and capecitabine increased the survival of the animals and drastically reduced the number of animals with pancreatic tumors induced by N-nitrosobis (2-oxopropyl) amine (BOP) (Ruiz-Rabelo et al., 2011). The anti-tumor effect of melatonin is probably related to its direct and indirect scavenging effects against radical oxygen and nitrogen species (Ruiz-Rabelo et al., 2007, 2011). Furthermore, the same group of researchers tried to determine the synergistic action of melatonin and celecoxib an inhibitor of cyclooxygenase-2 (COX-2) during either induction or progression phases of tumor process in BOP-induced pancreatic carcinoma (Padillo et al., 2010). This combined treatment exerted a beneficial effect evidenced as the reduction of tumor nodules, as well as the decrease of oxidative stress and diminished morbidity in BOP-treated hamsters. Moreover, melatonin by itself significantly improved the survival rate of these animals. Some authors have suggested that the application of melatonin to patients with pancreatic cancer could produce favorable effects and such therapy should be tested in clinical trials (Padillo et al., 2010).

The first clinical observation concerning the use of melatonin in the treatment of pancreatic cancer was published by Lissoni et al. (1995, 1997). They showed that melatonin combined with tamoxifen or with IL-2 exerts some beneficial effects on patients with untreatable metastatic solid tumours and amplifies the immunomodulatory and anticancer activity of the oncostatic drugs mentioned above (Lissoni et al., 1995, 1997, 2008). Moreover, despite its ability to enhance the efficacy of chemotherapy, the activity of melatonin is dependent on the psychospiritual status of cancer patients (Messina et al., 2010). A previous study exposed the circadian changes of melatonin with a few hours secretory peak delay in a patient suffering from advanced gastrointestinal neoplasms, including pancreatic cancer. These changes were negatively correlated to TNFα receptors levels detected in the tumors (Muc-Wierzgon et al., 2003).

Immunomodulatory effect of melatonin

Pancreatic cancer is accompanied by local inflammation and by the induction of immune suppression, through the accumulation of T regulatory cells (T_{reg}) and myeloid-derived suppressor cells (MDSC) (Hiraoka et al., 2006; Zhao et al., 2009). T_{regs}, currently defined as CD4+, CD25+ and Foxp+ T cells, have been identified in mice and in humans (Linehan and Goedegebuure, 2005; Hinz et al., 2007). T_{regs} inhibit anti-tumour immunity by suppression of peripheral effector T cells and by increasing production of anti-inflammatory cytokines interleukin 10 (IL-10) and transforming growth factor beta (TGFß) (Curiel, 2008). Moreover, TGF, exerts immunosuppressive effects such as inhibition of cytotoxic T cells ($CD8^+$) and natural killer (NK) cells, as well as by induction of $FoxP3^+$ regulatory T cells (Ellermeier et al., 2013). Increased levels of T_{regs} have been reported in patients with many malignancies, including pancreatic cancer (Shigematsu et al., 2012; Yamamoto et al., 2012; Izawa et al., 2013; Wu et al., 2013). High concentration of T_{regs} was closely related to tumor growth and have been shown in both the tumor microenvironment and in the peripheral blood (Ikemoto et al., 2006; Ino et al., 2013; Homma et al., 2013). Therefore, the inhibition of T_{reg} cell generation could be the fundamental key mechanism of the various cancer immunotherapies in an attempt to induce an effective anticancer immune reaction and to diminish cancer progression.

Another group of immune cells which are implicated in pancreatic carcinogenesis are cytotoxic T cells (CD8+ T cells or killer T cells) (Koido et al., 2011). Cytokine-induced killer cells (CIKs) have been derived from this cell population. They have been obtained from human peripheral blood mononuclear cells stimulated with IFNγ, IL-2, and CD3 antibody. It is worth to remember that CIKs are recognized as immune cells having the fastest proliferation, the strongest tumor cytotoxicity, and the most extensive range of tumor killing, including pancreatic carcinoma (Tan et al., 2011; Kim et al., 2012).

There has been an increased interest in the implication of melatonin on the immune response in cancers. The published data presents the evidence that endogenous melatonin inhibits the immunosuppressive effects of T_{reg} cells and stimulates proliferation of cytotoxic T lymphocytes. These changes were observed in patients with lung, stomach, and colon carcinomas (Kossoy et al., 2000; Liu et al., 2011; Mazzoccoli et al., 2012). The oncostatic effect of melatonin in colon cancer was probably dependent on the MT_2 receptors and perhaps on the binding of melatonin to nuclear RZR/ROR· receptors (Pandi-Perumal et al., 2008). Recent data from experimental and clinical studies have shown that melatonin could modulate the expression of anti-cancer cytokines IL-2 and IL-12 in human neoplasms (Pandi-Perumal et al., 2006). The above effects might be responsible for the oncostatic action of melatonin. Other studies have shown that addition of melatonin to MCF-7 cell culture inhibits proliferation of these tumor cells, increases the expression of pro-apoptotic proteins such as p53 and p21, and in addition reduces the metastatic potential of cancer cells due to the

increased expression of E-cadherin and beta 1-integrin proteins (Blask and Hill, 1986; Cos et al., 1998). To date, however, regular studies on the effects of melatonin use in patients with pancreatic cancer have not been carried out (Vigore et al., 2010).

Conclusion

Melatonin has been shown in experimental studies to increase the effectiveness of oncostatic drugs in animals with pancreatic cancer and to decrease their morbidity. The favorable, anti-cancer effects of melatonin could be related to the pro-apoptotic, anti-angiogenic, anti-oxidative, and immunomodulatory properties of this substance (Fig. 1). Nevertheless, the effect of melatonin on apoptosis is not completely clear and requires further study. The results of experimental studies and clinical observations indicated that melatonin at high doses could be potentially taken into consideration as a supportive treatment in the therapy of pancreatic cancer.

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