Hematopoietic Rescue via T-Cell-dependent, Endogenous Granulocyte-Macrophage Colony-stimulating Factor Induced by the Pineal Neurohormone Melatonin in Tumor-bearing Mice

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ABSTRACT

We investigated whether melatonin can affect tumor growth and/or hematopoiesis in mice transplanted with Lewis lung carcinoma and treated with cyclophosphamide or etoposide. These agents were injected i.p. for 5 days at two different cumulative doses (cyclophosphamide, 40 and 160 mg/kg body weight; etoposide, 20 and 40 mg/kg body weight) from day 8 through day 12 after tumor transplantation. Melatonin was injected s.c. at a dose of 1 mg/kg body weight/day, from day 8 throughout the experiments and from days 8 through 12 or from day 13 onwards. Melatonin did not influence tumor growth but selectively counteracted bone marrow toxicity when administered together with the cancer chemotherapy compounds without interfering with their anticancer action. In vitro, melatonin proved to counteract apoptosis in bone marrow cells incubated with etoposide. Such protection was reflected by an increased frequency of granulocyte/macrophage-colony forming units but not of the pluripotent spleen-colony forming units. The effect of melatonin was neutralized by anti-granulocyte/macrophage-colony-stimulating factor monoclonal antibodies. When athymic, T-cell-deficient mice were used as bone marrow donors, melatonin did not exert any protective effect. This suggested that melatonin is able to stimulate the endogenous production of granulocyte/macrophage-colony-stimulating factor via bone marrow T-cells. Due to the well known lack of toxic and undesirable side effects of melatonin, these findings might have a straightforward clinical application.

INTRODUCTION

Melatonin is an indoleamine synthesized from serotonin in the pineal gland. Its synthesis and release follows a circadian rhythm with the highest blood concentration occurring at night in all species (1). Melatonin regulates fertility in seasonally breeding animals (2), whereas its role in other species, including human, is less clear (2). In previous work, we have shown that melatonin can augment the immune response and correct immunodeficiency states which may follow acute stress, viral diseases, aging, or drug treatment (reviewed in Ref. 3). Relevant to the present study, we observed that melatonin was able to antagonize the effect of high dose cyclophosphamide on antibody production (4). This finding has been then confirmed and extended to other immune parameters by other authors (5, 6). Such interesting effects of melatonin seem to depend on activated CD4+, T-cells which upon melatonin stimulation show an enhanced synthesis and/or release of opioid peptides, interleukin 2, and γ-interferon (3, 5–8). A large body of evidence indicates also that melatonin may inhibit carcinogenesis and tumor growth in a variety of experimental and clinical situations (9). On the basis of our animal studies (3), we have investigated the clinical effect of melatonin in association with low-dose interleukin 2 in cancer patients and found that this association represents a well-tolerated strategy capable of determining an apparent control of tumor growth in patients with advanced solid neoplasms (10–13). However, chemotherapy still remains the most used tool to fight cancer. Unfortunately, a major barrier to achieving the best possible response to cancer chemotherapy is the hematological toxicity of available agents, which limits optimal dosing (14). To circumvent this problem, hematopoietic rescue by colony-stimulating factors, interleukin 3, and/or autologous bone marrow transplantation is increasingly used (15–17). Handling of such procedures remains, however, problematic because of negative side effects or incomplete marrow regeneration, respectively (15–17). The purpose of this study is to determine whether melatonin can protect the bone marrow in mice transplanted with LLC and treated with the alkylating agent cyclophosphamide or the topoisomerase II-reactive drug, etoposide. The in vitro effect of melatonin on apoptosis of bone marrow cells and survival of hematopoietic progenitor cells upon incubation with etoposide is also studied.

MATERIALS AND METHODS

Mice. Female C57BL/6 mice (2–3 months old) were purchased from Charles River Italia, Como, Italy. Age- and sex-matched C57BL/6 athymic mice were purchased from The Jackson Laboratory, Bar Harbor, ME. The animals were kept under a 12-h light/dark cycle at 21 ± 1°C with free access to food and water.

Drugs and Reagents. Melatonin was graciously provided by Helsinn Chemicals SA, Breganzona, Switzerland. Cyclophosphamide and etoposide were purchased from Sigma Chemical Co., St. Louis, MO. Monoclonal rat anti-mouse GM-CSF antibody was purchased from Genzyme Co., Cambridge, MA.

Lewis Lung Carcinoma. LLC was a kind gift of Dr. Laura Perissin, Trieste, Italy. The tumor was maintained by serial passages in C57BL/6 mice as described (18). For the experiments, the primary tumor was excised from donor mice and teased by a loose-fitting teflon pestle; the cells were dissociated to obtain a single cell suspension in saline. After being washed, the cells were counted, and viable cells (2 × 10⁶) suspended in 200 μl saline were injected i.m. in the left hind leg. Primary tumor size and number of lung metastases were evaluated 20–22 days after tumor inoculation. Primary tumor size was evaluated by measuring the diameter of the leg at the tumor level with a caliper in two perpendicular directions. The tumor cross-section is considered an ellipse, and its size was calculated according to the method of Bartholomeys et al. (19) as

\[
\text{Tumor cross section (cm}^2\text{)} = \Pi/4 \left( t_1 t_2 - (c_1 c_2) \right)
\]

where \( t_1 \) and \( t_2 \) are the perpendicular axes of the tumorous leg and \( c_1 \) and \( c_2 \) are the perpendicular axes of the right control leg. Lung metastases were enumerated in the dissected lungs by a dissection microscope.

Therapeutic Protocol. Melatonin was injected s.c. once a day at 4:00 p.m. at a dose of 1 mg/kg b.w. starting on day 8 after tumor transplantation throughout the experiment. In some experiments, melatonin was injected only during treatment with cyclophosphamide (days 8–12) or afterwards (day 13 through the end of the experiment). Cyclophosphamide or etoposide was injected i.p. once a day at 12:00 p.m. from day 8 through day 12. Two

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: LLC, Lewis lung carcinoma; GM-CSF, granulocyte-macrophage colony-stimulating factor; b.w., body weight; MEM, minimal essential medium; GM-CFU, granulocyte-macrophage colony-forming units; S-CFU, spleen-colony forming units.
The cells were then washed three times and processed for GM-CFU evaluation.

Blood obtained from the tail after light ether anesthesia 16 days after tumor inoculation. Platelets was determined microscopically in a Neubauer chamber in venous blood. In certain experiments, monoclonal rat anti-mouse GM-CSF antibodies were added during incubation of bone marrow cells with etoposide + melatonin.

Cumulative doses of 40 and 160 mg/kg b.w. or 20 and 40 mg/kg b.w. were used for cyclophosphamide and etoposide, respectively.

**In Vitro Induction of Apoptosis.** Bone marrow cells were collected from the long bones, suspended in α-MEM and 5% horse serum, and incubated for 8 h in triplicate and at a concentration of 3 × 10⁶ cells/ml in presence of the reported concentrations of etoposide ± melatonin. After incubation at 37°C and 5% CO₂ in 24-well tissue culture plates (Becton Dickinson, Oxnard, CA), cells were harvested by repeated rinsing of the wells. Cell aliquots were processed for the GM-CFU and S-CFU assays and/or fixed in 4% formaldehyde in ethanol overnight, washed three times in water, stained with 10 μg/ml acridine orange (Fluka AG, Buchs, Switzerland) in saline and examined for nuclear chromatin morphology in a fluorescence microscope. Apoptotic cells were clearly identified by their condensed or fragmented chromatin. At least 200 cells were counted from each well, giving reproducibility to within 4%. In certain experiments, monoclonal rat anti-mouse GM-CSF antibodies were added during incubation of bone marrow cells with etoposide + melatonin.

The cells were then washed three times and processed for GM-CFU evaluation.

**Leukocyte and Platelet Counts.** The concentration of leukocytes and platelets was determined microscopically in a Neubauer chamber in venous blood obtained from the tail after light ether anesthesia 16 days after tumor inoculation.

**GM-CFU and S-CFU.** To evaluate the concentration of GM-CFU either after in vivo treatment or after in vitro incubation, 10⁵ viable bone marrow cells were incubated in 0.3% semisolid agar in α-MEM containing 20% horse serum and 20% lung conditioned medium as source of GM-CSF. Lung conditioned medium was prepared by mincing the lungs from 2-month-old mice into small pieces and by incubating the pieces at 37°C in 5% CO₂ in α-MEM and 20% horse serum for 3 days. Cultures were maintained for 7 days at 37°C in 5% CO₂ in humidified air and then examined by phase microscopy. Colonies containing more than 50 cells were counted as GM-CFU. S-CFU were evaluated by injecting i.v. 10⁵ viable bone marrow cells after lethal irradiation (900 cGy; X-ray exposure performed by a linear accelerator; 15 MV energy equivalent) of recipient mice. Spleen colonies were counted 12 days after bone marrow inoculation and 24 h after fixation in Bouin’s fluid.

**Statistics.** Differences were evaluated for significance by analysis of variance.

**RESULTS**

**Effect in LLC-bearing Mice.** Table 1 reports the effect of the association of melatonin with cyclophosphamide or etoposide on tumor growth, blood counts, and number of bone marrow GM-CFU in mice transplanted with LLC. In accordance with previous reports (20), the presence of LLC per se induced an increase of myelopoiesis which, in our hands, appeared to be significant only for leukocyte counts (phosphate-buffered saline versus control; Table 1). Such an increase appeared to be counteracted by melatonin treatment. In the opposite direction, melatonin exerted a significant protection against bone marrow toxicity induced either by cyclophosphamide or etoposide. Such an effect was apparent and highly significant on leukocytes, platelets, and marrow GM-CFU. On the contrary, melatonin per se or in association with the antitumor compounds did not influence either the size of the primary tumor or the number of lung metastases; also, it did not interfere with the action of the cytotoxic drugs. To investigate whether melatonin acted by preventing bone marrow toxicity or by enhancing the posttreatment recovery of hematopoiesis, experiments were devised in which melatonin was injected only during cyclophosphamide treatment or only thereafter. Table 2 demonstrates that melatonin was able to antagonize the hematopoietic toxicity of cyclophosphamide when injected together with the drug. Bone marrow protection appeared to be less effective if melatonin was injected after cyclophosphamide treatment. These results indicated that melatonin is able to protect the bone marrow in the course of cytotoxic anticancer treatments. However, a similar although less apparent effect was also observed when melatonin treatment followed cyclophosphamide.

**In Vitro Effect of Melatonin on Drug-induced Apoptosis and Survival of Progenitor Cells.** We reasoned that the protection of hematopoiesis exerted by melatonin in vivo might reflect a direct action on bone marrow cells. To challenge this hypothesis, experiments were set up in vitro by incubating normal bone marrow cells with etoposide in the presence of various concentrations of melatonin. The first question we asked was whether melatonin is able to rescue marrow cells from apoptosis induced by the anticancer drug. Fig. 1 shows that from 10 nm to 1 μM, melatonin is able to prevent apoptosis in bone marrow cells incubated with 10 μM etoposide. Cyclophosphamide was not used because it is well known to be inactive in vitro. However, similar results were obtained with carboplatin, which has a
mechanism of action close to that of cyclophosphamide metabolites (Ref. 21 and data not shown). When bone marrow cells, preincubated with 1 μM melatonin and 10 μM etoposide were assayed for GM-CFU and S-CFU content, it appeared that melatonin protected GM-CFU precursors but not the less differentiated, pluripotent S-CFU progenitor cells (Table 3). Thus, the cell types which were rescued from drug-induced apoptosis seems to include lineage-committed, myeloid progenitor cells but not pluripotent S-CFU precursors. When anti-mouse GM-CSF antibodies were added during the preincubation of bone marrow cells with etoposide and melatonin, no protection was evident (Fig. 2). This suggested that the effect of melatonin was mediated by a factor which is released by bone marrow cells and that is biologically and immunologically indistinguishable from GM-CSF.

**In Vitro Effect of Melatonin on Bone Marrow Cells from Athymic, T-Cell-deficient Mice.** In bone marrow cell suspensions, the principal sources of GM-CSF are macrophages and T-cells. Athymic nude mice are T-cell-deficient and have a poor capacity to mount immune responses, which is in part balanced by enhanced macrophage functions (22). Therefore, we chose T-cell-deficient, nude mice as donors of bone marrow cells to investigate which cell type could release GM-CSF upon melatonin stimulation. Table 4 shows that when bone marrow cells were obtained from T-cell-deficient mice, melatonin did not exert any protection against etoposide toxicity. This indicated that T-lymphocytes are involved in the hematopoietic effect of melatonin and that bone marrow T-cells may be the melatonin target.

**DISCUSSION**

In this study, we show that melatonin can protect bone marrow functions from the toxic effect of cancer chemotherapy compounds without interfering with their anticancer action in vivo. This effect is exerted directly on bone marrow T-cells, which may directly release or participate in the induction of a GM-CSF-like factor upon melatonin stimulation.

Melatonin per se, did not influence LLC growth and metastases formation. This is in line with previous results about the effect of melatonin on LLC growth (23), but it is in contrast with the widely reported inhibiting action of melatonin on most tumor histotypes (24). However, melatonin was able to counteract, in part, the tumor-induced increase of leukocyte counts. It has been reported that the LLC-induced myelopoiesis is associated with decreased prostaglandin production by tumor-macrophages and immune suppression (25). The effect of melatonin on leukocyte counts might thus be related to its immunoenhancing properties (3, 5), possibly up-regulating macrophage activation and prostaglandin production. The cancer chemotherapy compounds decreased the tumor burden and this, in turn, might have resulted in diminished colony-stimulating activity, activation of macrophages, and prostaglandin production.

The most interesting effect of melatonin was, however, exerted in the opposite direction, i.e., melatonin protected bone marrow cells from toxicity induced either in vivo or in vitro by the anticancer compounds. Melatonin can rescue myeloid progenitor cells from drug-induced apoptosis via a mechanism involving the endogenous production of GM-CSF. Consistently, melatonin protected the lineage-committed GM-CFU progenitors but not the less differentiated CFU-S precursors which are not affected by GM-CSF (26). In line with previous reports concerning the mechanism of the immunopotentiating action of melatonin (3, 6–8, 27), the experiments performed with bone marrow cells from T-cell-deficient mice suggest that T-cells are involved in the melatonin action. Whether the melatonin-induced colony-stimulating factor is directly released by T-cells or...
induced by another T-cell cytokine is presently unknown. Recent clinical studies about the mechanism of action of melatonin in cancer patients are also consonant with a T-cell-mediated effect (28).

Programmed cell death or apoptosis is a normal process by which cells are eliminated during embryonic development and in adult life. Programmed cell death can be induced in leukemic cells by removal of colony-stimulating factors, by cytotoxic therapeutic agents, or by the tumor suppressor gene wild-type p53. All these forms of induction of apoptosis in leukemic cells can be suppressed by the same colony-stimulating factors that suppress apoptosis in normal cells (29, 30). In our case, the rescued GM-CFU precursors are contained in a larger cell population which apparently is also protected from the melatonin-induced GM-CSF. This might depend on the presence of GM-CSF receptors on cell types other than GM-CFU precursors (26).

The evidence of a neuroendocrine influence on endogenous colony-stimulating factor production has important basic and practical implications. A major problem remains, in fact, the hematological toxicity of cancer chemotherapy compounds (14). The systemic administration of GM-CSF has significant dose-related toxicity (31). In contrast, melatonin is well known to have no toxic effects (10–13, 28, 32). A modulation of the production of endogenous CSFs is likely to present several and substantial advantages over systemic administration. Our finding may thus have straightforward and wide clinical applications.

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