ABSTRACT

We have reported that melatonin may rescue bone marrow cells from apoptosis induced either in vivo or in vitro by cancer chemotherapy compounds via bone marrow T-cells and endogenous release of granulocyte-macrophage colony-stimulating factor. Here we show that the number of granulocyte/macrophage colony-forming units cultured with suboptimal concentrations of colony-stimulating factor was higher in the presence of melatonin both at physiological and pharmacological concentrations. CD4+Thy-1.2+ cell depletion or addition of anti-mouse interleukin 4 monoclonal antibodies prevented both effects of melatonin. Upon incubation with etoposide, the concentration of myeloid precursors was 43 ± 8 per 105 cells. The melatonin + etoposide value was 68 ± 7, whereas that of melatonin + etoposide + anti-interleukin 4 was 38 ± 6. Melatonin was also ineffective when bone marrow cells were separated in adherent and nonadherent populations. Supernatants from nonadherent cells incubated with melatonin proved to contain interleukin 4 activity which, however, showed its influence on unseparated bone marrow and adherent cells but not on nonadherent cells. It is proposed that melatonin represents a neuroendocrine regulator of interleukin 4 production in bone marrow T-helper cells. Interleukin 4 may then stimulate adherent stromal cells to produce granulocyte/macrophage colony-stimulating factor. Such a neuroendocrine-cytokine mechanism may explain the hematopoietic rescue of melatonin as well as its antitumoral and immunoenhancing properties.

INTRODUCTION

In the past several years, it has been recognized that the pineal neurotransmitter melatonin can augment the immune response, correct immunodeficiency states (reviewed in Ref. 1), and exert a potent antineoplastic influence (reviewed in Ref. 2). In humans, the association of melatonin with low-dose interleukin 2 has been found to represent a valuable tool to control tumor growth in patients with advanced solid neoplasms (3-6). Most recently, we have shown that melatonin can protect hematopoietic functions in mice from the toxic effect of cancer chemotherapy compounds without interfering with their anti-cancer action (7). In line with previous findings (1, 8-12), this effect appeared to involve bone marrow T-cells and the endogenous production of a colony-stimulating factor, which has been immunologically and functionally identified as GM-CSF (7). Our finding is consonant with previous reports indicating that GM-CSF promotes survival of hematopoietic progenitors by suppressing apoptosis (13, 14). However, the melatonin-induced GM-CSF proved to rescue lineage-committed GM-CFU. In contrast, more primitive, multipotent progenitor cells, such as those defined as spleen colony-forming units, were not protected from the toxic effect of anticancer compounds but rather were decreased upon incubation with melatonin alone (7). A T-cell cytokine which may exert a selective inhibitory effect on primitive progenitor cells is IL4 (15). In addition, IL4 has been also reported to stimulate macrophages and fibroblasts to produce colony-stimulating activity (15, 16). Our clinical studies with melatonin and interleukin 2 have shown that melatonin may enhance the interleukin 2-induced eosinophilia by mechanisms which presumably involve cytokines such as interleukin 5 and/or IL4 (11, 12). The purpose of this study is to determine whether the melatonin-induced GM-CSF is released by bone marrow T cells or by other cell types via IL4. Here we show that melatonin acts on Thy-1.2+CD4+ bone marrow cells and induces the release of a factor which is immunologically and functionally indistinguishable from IL4. In turn, IL4 acts on adherent bone marrow cells, inducing the production of colony-stimulating activity.

MATERIALS AND METHODS

Mice. Female C57BL/6 mice, ages 2-3 months, were purchased from Charles River Italia, Como, Italy. 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). Anti-mouse IL4 mAb was purchased from Genzyme Co. (Cambridge, MA). Anti Thy-1.2 and anti-L3T4 (CD4) mAbs coupled to paramagnetic microbeads were purchased from Miltenyi Biotec GmbH (Bergish Gladbach, Germany). Etoposide was purchased from Sigma Chemical Co. (St. Louis, MO).

In Vitro Induction of Apoptosis. Bone marrow cells were collected from the long bones, suspended in α-MEM-5% horse serum, and incubated for 24 h in triplicate and at a concentration of 3 × 105 cells/ml in the presence of 10 mM etoposide ± 1 µM melatonin. After incubation at 37°C and 5% CO2 in 24-well tissue culture plates (Becton Dickinson, Oxnard, CA), cells were harvested by repeated well-rinsing. Cell aliquots were processed for the GM-CFU assay 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%. GM-CFU. To evaluate the effect of melatonin on GM-CFU, 105 viable bone marrow cells were incubated in 0.3% semisolid agar in α-MEM containing 20% of horse serum and 0.2 or 10% LCM as a source of GM-CSF. LCM was prepared by mincing lungs in small pieces from 2-month-old mice and by incubating the pieces at 37°C and 5% CO2 in α-MEM-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.

Separation of Bone Marrow Cells by Adherence. Bone marrow cells were collected, suspended in culture medium constituted by α-MEM with 5% horse serum, and incubated for 2 h at a concentration of 3 × 106 cells/ml in tissue culture flasks (Costar, Cambridge, MA). After incubation, nonadherent cells were separated by collecting the medium and by rinsing the flasks gently with 10 ml of culture medium. Adherent cells were harvested after addition of 5 mM EDTA for 10 min at 4°C.

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The abbreviations used are: GM-CSF, granulocyte/macrophage colony-stimulating factor; GM-CFU, granulocyte/macrophage colony-forming units; IL4, interleukin 4; mAb, monoclonal antibody; MEM, minimal essential medium; LCM, lung conditioned medium; NK, natural killer cells.

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Preparation of Supernatant from Nonadherent Bone Marrow Cells. Melatonin (1 nm) was added to nonadherent bone marrow cells suspended in culture medium at a concentration $3 \times 10^6$ cells/ml and incubated overnight at 37°C and 5% CO$_2$. After incubation, cells were pelleted by centrifugation, and the supernatant was collected and dialyzed (molecular weight cutoff, 8,000) against phosphate-buffered saline to remove melatonin.

Magnetic Cell Sorting. Magnetic cell sorting was accomplished with a MACS magnetic cell separator, purchased from Miltenyi Biotec GmbH. Un-separated bone marrow cells or nonadherent bone marrow cells were incubated in phosphate-buffered saline/2% horse serum for 30 min at 4°C and at a concentration of $10^7$ cells/ml with 30 μl/0.1 ml of anti-L3T4 or anti-Thy.2 mAbs coupled to paramagnetic microbeads. Labeled and unlabeled cells were separated in a high-gradient magnetic field generated in a steelwool matrix inserted into a permanent magnet. Depletion was obtained by eluting unlabeled cells within the magnetic field and using an A2 column with a G25 needle for flow regulation. The recovery of the eluted, nonmagnetic cells was between 72–79% of the total number of cells. A cytofluorimetric analysis using fluorescein-labeled antibodies revealed that the percentage of labeled cells in the eluted fraction was always less than 2%.

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

RESULTS

Effect of Anti-Mouse IL4 mAb on Hematopoietic Rescue Induced by Melatonin. To evaluate whether IL4 is involved in the ability of melatonin to rescue hematopoietic cells from the toxic effect of cancer chemotherapy compounds (7), we added rat anti-mouse IL4 mAb to bone marrow cells that were incubated with etoposide in the presence of melatonin. Table 1 shows that anti-IL4 mAb neutralized the effect of melatonin evaluated both on apoptosis and number of GM-CFU.

Effect of Melatonin on GM-CFU. As GM-CSF seems also involved in the hematopoietic rescue exerted by melatonin (7) and IL4 has been reported to synergize with or stimulate GM-CSF production (15–17), we investigated whether melatonin could substitute for or augment the effect of suboptimal concentrations of LCM (GM-CSF) on GM-CFU. The effect of our LCM reached a plateau over 10% of concentration (data not shown). On this basis, we added various concentrations of melatonin in GM-CFU cultures in the presence of 0 or 2% LCM. Fig. 1 shows that, starting from 0.1 nm, a concentration which approaches the lower physiological plasma level (18), melatonin significantly increased the number of GM-CFU. However, melatonin was effective only in the presence of 2% LCM (Fig. 1). As in the rescue experiment (Table 1), anti-IL4 mAb proved to neutralize the effect of melatonin (Fig. 2). On the other hand, the addition of recombinant mouse IL4 increased the number of GM-CFU as reported previously (15, 16) and mimicked the effect of melatonin (data not shown). Our previous work has indicated that T-cells mediate both the immunoenhancing and hematopoietic effects of melatonin (1, 7). Consistently, melatonin failed to show any action when added to GM-CFU cultures performed with bone marrow cells that were depleted from Thy$^+$,CD4$^+$ cells (Fig. 3).

Effect of Melatonin on Adherent versus Nonadherent Bone Marrow Cells. Although IL4 gene expression has also been found in bone marrow stromal cell clones (19), CD4$^+$ T-cells are considered the major source of IL4 (15). Conversely, GM-CSF may be equally synthesized in both T-cells and stromal cells (20). A simple method to separate bone marrow stromal cells is by adherence (21). Therefore, we investigated the effect of melatonin in adherent versus nonadherent bone marrow cells. Fig. 4 shows that melatonin looses its activity when added in GM-CSF cultures performed either with adherent or nonadherent bone marrow cells. The activity reappeared when the two cell populations were mixed again. Because T-lymphocytes are non-adherent cells, this result suggested that the colony-stimulating activity was released by adherent cells upon stimulation of the melatonin-induced, T-cell-derived IL4. To prove this hypothesis, we incubated nonadherent cells with melatonin in order to produce the IL4 activity in the supernatant. The supernatant was dialyzed to get rid of melatonin and then tested for its activity on GM-CFU in unseparated nonadherent or adherent bone marrow cells. The results shown in Fig. 5 demonstrate that the supernatant was active on unseparated and

<p>| Table 1: Effect of anti-IL4 mAb on melatonin-induced hematopoietic rescue |
|-----------------------------|--------------------------|
| Total bone marrow cells were obtained by carefully flushing out the marrow from the femurs of C57BL/6 mice. The cell suspension obtained, which obviously also contained adherent stromal cells, were then aliquoted and incubated in culture medium alone (Medium) or in presence of anti-IL4 mAb (aIL4), 10 μg/ml, etoposide (ETO, 10 μg/ml) and/or melatonin (MLT, 1 μM). The values represent the mean of three experiments ± SD. |</p>
<table>
<thead>
<tr>
<th>Apoptosis %</th>
<th>GM-CFU</th>
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<tbody>
<tr>
<td>Medium</td>
<td>16 ± 1.9</td>
</tr>
<tr>
<td>aIL4</td>
<td>18 ± 2.7</td>
</tr>
<tr>
<td>ETO</td>
<td>54 ± 3.1</td>
</tr>
<tr>
<td>ETO + MLT</td>
<td>38 ± 2.0</td>
</tr>
<tr>
<td>ETO + MLT + aIL4</td>
<td>52 ± 2.8</td>
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* P < 0.01 (ETO + MLT versus ETO, ETO + MLT + aIL4)
Thy1.2+ and CD4+ bone marrow cells was obtained by magnetic cell sorting. Melatonin (MLT) was added at a concentration of 1 nM. The values represent the means of three experiments; bars, SD. UC, unseparated cells; a, P < 0.004 (UC+MLT versus UC, Thy1.2+ MLT and CD4+ MLT).

Fig. 3. Prevention of the melatonin action in GM-CFU cultures depleted of Thy1.2+ cells. Depletion of Thy1.2+ and CD4+ bone marrow cells was obtained by magnetic cell sorting. Melatonin (MLT) was added at a concentration of 1 nM. The values represent the means of three experiments; bars, SD. a, P < 0.004 (UC+MLT versus UC, Thy1.2+ MLT and CD4+ MLT).

Fig. 4. Effect of melatonin in GM-CFU cultures of adherent versus nonadherent cells. Unseparated bone marrow cells (UC), adherent cell (AC) or nonadherent cell populations (nAC) were plated in the presence or absence of melatonin (MLT). In the mixing plates (nAC+AC), the ratio between AC and nAC was 1:10. The values are from four experiments; bars, SD. a, P < 0.005; b, P < 0.01 (MLT versus MEDIUM).

adherent bone marrow cells but not on nonadherent cells. As expected, anti-IL4 mAb neutralized the supernatant activity.

DISCUSSION

Our previous work (7) and the data shown here suggest that melatonin may rescue bone marrow progenitor cells from the toxic effect of cancer chemotherapy compounds via a two-step cytokine cascade. The first step involves the release of a factor from bone marrow T-cells. The selective ability of melatonin to decrease the number of pluripotent hematopoietic progenitors (7), together with the present results and the considerations hereunder reported, allow such factor to be defined as immunologically and functionally indistinguishable from IL4. In turn, IL4 seems then to induce adherent bone marrow cells to produce a colony-stimulating factor which has been previously identified as GM-CSF (7). When incubated with cancer chemotherapy compounds or injected in mice that are treated with the same drugs, melatonin can exert a significant hematopoietic rescue (Ref. 7; Table 1). Nevertheless, if injected in normal untreated mice (7) or added in a GM-CFU assay in the absence of LCM (GM-CSF), melatonin does not show any activity, in spite of the apparent ability of melatonin to induce IL4 production in normal bone marrow T-cells (Fig. 5). Adherent bone marrow stromal cells include macrophage and fibroblast-like cells (21) which have been reported to release GM-CSF upon IL4 stimulation (15, 16). In our case, the adherent bone marrow cells which release GM-CSF seem to need an additional signal besides that of IL4. Such a signal might be given by GM-CSF (LCM) or by cancer chemotherapy drugs, both of which have been reported to induce macrophage activation (22, 23). IL4 has also been reported to synergize with GM-CSF (17); however, this mechanism does not appear to be involved in our case because melatonin did not increase the number of GM-CFU in nonadherent bone marrow cells (Fig. 4) and supernatants which contained IL4 were not active on nonadherent cells (Fig. 5). The melatonin-cytokine hematopoietic network here described is outlined in Fig. 6.

The very low concentration (0.1 nM) at which melatonin is active may well reflect a physiological function of endogenous melatonin. The pineal gland has been, in fact, reported to signal to the blood-forming system (24). How melatonin can induce IL4 in resting bone marrow T-cells is an interesting question that needs further studies. However, the IL4 involvement seems relevant to our understanding of many melatonin effects. One of the most prominent effect of IL4, formerly B-cell growth-factor-1, is stimulation of the antibody response (15). Furthermore, IL4 has been reported to inhibit production of cytokines such as tumor necrosis factor and to exert an antitumor effect via an enhanced generation of cytototoxic T-cells and NK cells (15) as well as of eosinophils (25). Consistently, melatonin can augment the antibody response (1), T-helper cell function (8, 9) and NK activity (26). Most interestingly, the association of melatonin with interleukin 2 in cancer patients not only has been shown to improve the antitumor activity of low-dose interleukin 2 in cancer histotypes that are generally nonresponsive to interleukin 2 alone, it also...
constantly induced an amelioration of performance status (4–6). Both of these effects might be due to IL4 via T or NK cell cytotoxicity enhancement and inhibition of tumor necrosis factor, respectively. In conclusion, our findings which help in elucidating the mechanism of the hematopoietic and antitumor properties of melatonin may constitute a new rationale for clinical interventions.

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Colony-Stimulating Activity and Hematopoietic Rescue from Cancer Chemotherapy Compounds Are Induced by Melatonin via Endogenous Interleukin 4

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