in vivo *24*: 787-790 (2010)

Psychoneuroendocrine Modulation of Regulatory T Lymphocyte System: *In Vivo* and *In Vitro* Effects of the Pineal Immunomodulating Hormone Melatonin

LUIGI VIGORÈ[1], GIUSY MESSINA[2], FERNANDO BRIVIO[3], LUCA FUMAGALLI[3], FRANCO ROVELLI[4], GIUSEPPE DI FEDE[4] and PAOLO LISSONI[4]

[1]*Laboratory of Immunomicrobiology, S. Gerardo Hospital, Monza, Milan, Italy;*
[2]*Psychiatric Division, Polyclinic Hospital, Milan, Italy;*
[3]*Surgical Division, Bassini Hospital, Cinisello, Milan, Italy;*
[4]*Institute of Biological Medicine, Milan, Italy*

Abstract. *Background: At present, it is known that cancer-related immunosuppression would mainly depend on an immunosuppressive action mediated by a subtype of $CD4^+$ lymphocytes, the so-called regulatory T lymphocytes (T-reg), which are identified as $CD4^+CD25^+$ cells. Moreover, it has been shown that anticancer immunity is under psychoneuroendocrine regulation, mainly mediated by the pineal hormone melatonin (MLT). This study was performed to investigate the in vivo and in vitro effects of MLT on T-reg generation. Materials and Methods: We evaluated the in vivo effects of MLT (20 mg/daily orally in the evening) in 20 patients with untreatable metastatic solid tumor and the in vitro effects of MLT incubation (at 10 and 100 pg/ml) of pure lymphocyte cultures on T-reg cell count. Results: MLT induced a statistically significant decline in mean T-reg cell numbers in patients who achieved disease control, whereas no effect was seen in those who had progressed. In contrast, no in vitro effect of MLT incubation was apparent. Conclusion: This preliminary study would suggest that MLT may exert in vivo an inhibitory action on T-reg cell generation in cancer patients which is associated with a control of the neoplastic progression, whereas no direct effect was seen in vitro on lymphocyte differentiation. This finding would suggest that MLT may counteract T-reg cell generation in vivo by inhibiting macrophage activity which is involved in stimulating T-reg cell production.*

Correspondence to: Dr. Paolo Lissoni, Divisione di Radioterapia Oncologica, Ospedale S. Gerardo, 20052 Monza, Milan, Italy. Fax: +39 0392332284, e-mail: p.lissoni@hsgerardo.org

Key Words: Anticancer immunity, melatonin, pineal gland, regulatory T lymphocytes.

It is known that cancer progression is due at least in part to a lack of an effective anticancer immune reaction (1-3). However, recent discoveries in the immunobiology of tumors have suggested that cancer-related failure of the anticancer immune response would namely depend on the activation of an abnormally enhanced immunosuppressive response, which is mainly mediated by a subtype of $CD4^+$ lymphocytes, the so-called regulatory T lymphocytes (T-reg), wich are identified as $CD4^+CD25^+$ cells (4, 5). Several immune cell types have been proven to be potentially characterized by an immunosuppressive activity on the anticancer immunity, including T-reg cells themselves, macrophages, namely the M2 subtype (6) and myeloid-derived suppressor cells (7), consisting of myeloid precursors released from the bone marrow. However, according to recent discoveries, the end-result of the immunobiological interactions among the various immunosuppressive cells would consist of an abnormally enhanced generation of T-reg lymphocytes, which represent the most biologically active immune cells in suppressing the anticancer immune reaction through the release of immunosuppressive cytokines, namely interleukin (IL)-10 and transforming growth factor (TGF)-beta (4-7). Therefore, the inhibition of T-reg cell generation could constitute the fundamental key mechanism of the various immunotherapies of cancer in an attempt to reinduce an effective anticancer immune reaction capable of counteracting cancer progression. Several endogenous hormonal or immunological molecules capable of activating the anticancer immunity have been identified in recent years. In more detail, within these immunological substances, IL-2 (8) and IL-12 (9) would represent the most active anticancer cytokines in humans.

Within the endocrine system, the main endogenous anticancer hormones are the pineal indole hormones, the most investigated of which is melatonin (MLT), whose anticancer activity has already been well documented (10).

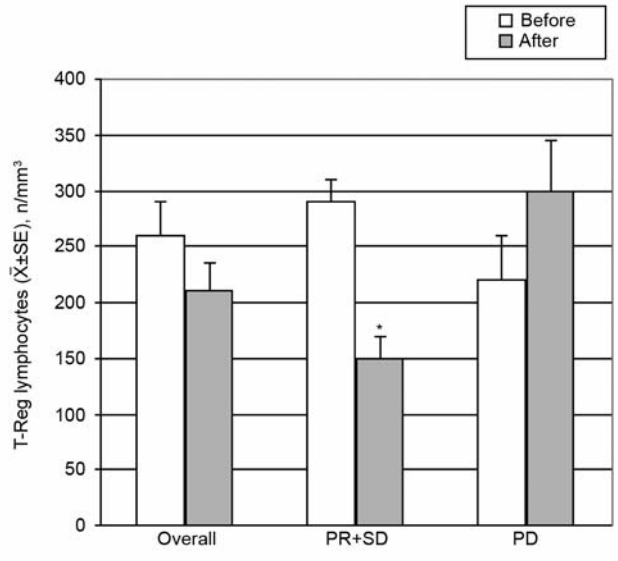

Figure 1. *Effect of 20 mg/day melatonin on the number of T-reg lymphocytes in cancer patients in relationto clinical response. PR+SD: Disease control, comprising cases with partial response, and stable disease; PD: progressive disease. *p<0.025 comparing values before and after therapy.*

MLT may inhibit cancer cell proliferation through both antiproliferative and immunomodulating effects (11). In particular, within the immune effects of the pineal hormone, the most important anticancer immune activities of MLT would consist of the stimulation of IL-2 secretion through a direct effect on T-helper lymphocytes, by acting on specific MLT receptors (12) and the inhibition of macrophage-mediated immunosuppressive effects (13), whereas at present there are no data about the possible influence of MLT on T-reg lymphocytes. There are only some preliminary data, showing that MLT does not stimulate IL-10 secretion in response to IL-2 (11). The present preliminary study was performed in an attempt to evaluate the effect both *in vitro* and *in vivo* of MLT on the T-reg cell system.

Materials and Methods

In a preliminary study, we evaluated the *in vivo* effects of MLT as a palliative therapy of cancer in 20 patients with metastatic solid tumor (male/female:12/8; median age 61, range 48-71 years), who failed to respond to conventional anticancer therapy and were not schedulable for other standard anticancer treatment. Tumor histotypes were as follows: non-small cell lung cancer: 8; colorectal cancer: 6; breast cancer: 4; prostate cancer: 1; pancreatic cancer: 1. According to previous clinical studies (14), MLT was given orally at a pharmacological dose of 20 mg/day every day in the evening, in accordance with its physiological light/dark circadian rhythm (10), without interruption. The treatment was continued for at least 3 consecutive months. The clinical response was evaluated according to WHO criteria. To evaluate T-reg cell number, venous blood samples were collected in the morning before and after 3 months of MLT therapy. No patient was under treatment with corticosteroids or with other immunosuppressive agents during the study. The clinical experimental protocol was explained to each patient and informed consensus was obtained.

Table I. *Mean number of regulatory T lymphocytes (T-reg) and mean percentage of total lymphocytes under incubation with melatonin (MLT) or control medium alone.*

	T-regs (Mean±SE)	
	n/mm^3	%
Control medium	219±24	9±2
MLT 10 pg/ml	216±26	9±3
MLT 100 pg/ml	208±19	8±2

In a second study, we investigated the *in vitro* effects of MLT incubation on T reg cell number and percentage. Peripheral blood mononuclear cells were isolated from venous blood samples collected from healthy volunteers by centrifugation on Ficoll-Ipaque (Pharmacia, Uppsala, Sweden). After separation of monocytes by two-step Percoll density gradient centrifugation, cultures of pure lymphocytes were prepared in RPMI-1640 supplemented with 10% fetal calf serum. Cells were cultured in 24-well plates and each 1 million of cells was maintained in a final volume of 1 ml of medium. Cells were incubated in a humidified atmosphere of 5% carbon-dioxide in air at 37˚C for 6 days with MLT at 10 pg/ml and at 100 pg/ml, corresponding to its physiological blood concentrations during the light and the dark period of the day, respectively (10). MLT was added daily until the end of the incubation or with medium alone, as control. In both studies, T-reg cell number was measured by a flow cytometric assay and monoclonal antibodies supplied by Becton-Dickinson (Milan, Italy), by identifying T-reg lymphocytes as $CD4^+CD25^+$ cells. Data are reported as mean±SE and statistically analyzed by chi-square test, Student's *t*-test and analysis of variance, as appropriate. Normal values of T-reg lymphocytes determined in our laboratory (95% confidence limits) were below 240/mm^2, comprising less than 11% of the total lymphocytes.

Results

As far as the results of the first study are concerned, the clinical response to MLT consisted of a partial response (PR) in 1/20 patients and stable disease (SD) in 11/20 patients, whereas the remaining 8/20 patients had progressive disease (PD). Therefore, disease control (DC) (PR+SD) was achieved in 12/20 patients. Before MLT therapy, an abnormally high T-reg cell count was observed in 9/20 patients. MLT therapy induced a normalization of T-reg cell numbers in 4/9 patients who showed abnormally high values prior to therapy. Moreover, the percentage of MLT-induced normalization of T-reg cell count was significantly higher in patients with DC than in those who had a PD (4/6 *vs*. 0/38,

$p<0.001$). Mean T-reg cell values before and after therapy in the overall patient group and in relation to their clinical response are illustrated in Figure 1. By taking into consideration the overall patient group, the mean T-reg cell number decreased on MLT therapy, without, however, statistically significant differences. On the contrary, by evaluating T-reg cell variations with respect to the clinical response, a significant decline in mean T-reg cell count was found in patients with DC ($p<0.025$), whereas the T-reg count increased in patients with PD, even though in a non-significant manner.

As far as the results of the second study are concerned, as reported in Table I, MLT incubation did not induce significant variations in mean T-reg cell counts with respect to cells incubated in the control medium alone, neither at 10 pg/ml, nor at 100 pg/ml. In the same way, the percentage of T-reg cells with respect to the total lymphocytes was also not significantly influenced by MLT incubation.

Discussion

The results of these preliminary studies, carried out to better clarify the neuroendocrine physiopathological regulation of the T-reg cell system, seem to show that the pineal hormone MLT, whose immunostimulatory effects on anticancer immunity have been well demonstrated (10-13), may actually counteract T-reg cell generation *in vivo* and this effect would simply be an epiphenomenon only, since it was associated with a control of the neoplastic disease. On the contrary, MLT does not seem to exert a direct action on T-reg cells themselves since no relevant effect was observed *in vitro* for cells under MLT incubation with respect to cells incubated in control medium alone. Since the *in vivo* generation of T-reg lymphocytes is stimulated by several mechanisms, namely by the monocyte-macrophage system (6, 7), it is possible to suggest that MLT may exert its inhibitory effect on the T-reg cell system through indirect mechanisms by counteracting the macrophage-induced stimulation of endogenous T-reg cell production. In fact, previous studies have already shown that MLT may inhibit macrophage-mediated immunosuppressive effects on anticancer immunity (13). At present, however, it is not possible to establish whether the effects of MLT on the macrophage system mainly consist of an influence on the type of cytokine secretion or whether it inhibits the release of myeloid-derived suppressor cells from the bone marrow (7). Moreover, previous investigations demonstrated that MLT may enhance the therapeutic efficacy of cancer chemotherapy by counteracting the negative influence of chemotherapy on anticancer immunity (10, 11). Since chemotherapy efficacy may depend at least in part on a suppression of T-reg cell generation (14), MLT could enhance chemotherapy efficacy by further suppressing T-reg cell generation, by acting on the macrophagic system. Future clinical protocols with the various anticancer medical treatments would have taken into consideration not only their effects on tumor size, but also their actions on the immune system, namely on T-reg lymphocytes, which would represent the main cells responsible for the lack of an effective anticancer immune reaction.

References

1 Chouaib S and Fradelizi D:The mechanism of inhibition of human IL-2 production. J Immunol *129*: 2463-2468, 1982.

2 Grimm EA, Mazumder A, Zhang HZ and Rosenberg SA: Lymphokine-activated killer cell phenomenon. J Exp Med *155*: 1823-1841, 1982.

3 Atzpodien J and Kirchner H: Cancer, cytokines and cytotoxic cells: interleukin-2 in the immunotherapy of human neoplasms. Klin Wochenschr *14*: 1-10, 1990.

4 Shevach EM: $CD4^+CD25^+$ suppressor T-cells: more questions than answers. Nat Rev Immunol 2: 389-400, 2002.

5 Zou W: Regulatory T-cells, tumour immunity and immunotherapy. Nat Rev Immunol *6*: 295-307, 2006.

6 Thompton AM and Shevach EM: Suppressor effector function of $CD4^+CD25^+$ immunoregulatory T-cells is antigen non-specific. J Immunol *164*: 183-190, 2000.

7 Baecher-Allan C, Brown JA, Freeman GJ and Hafler DA: Human $CD4^+CD25^+$ regulatory T-cells. Semin Immunol *16*: 89-98, 2004.

8 Whittington R and Faulds D: Interleukin-2. Drugs *46*: 446-514, 1993.

9 Banks RE, Patel PM and Selby PJ: Interleukin-12: a novel clinical player in cytokine therapy. Br J Cancer *71*: 655-659, 1995.

10 Maestroni GJM: The immunoneuroendocrine role of melatonin. J Pineal Res *14*: 1-10, 1993.

11 Bartsch H and Bartsch C: Effect of melatonin on experimental tumors under different photoperiods and times of administration. J Neural Transm *52*: 269-279, 1981.

12 Guerrero JM and Reiter RJ: Melatonin immune system relationships. Curr Topics Med Chem *2*: 167-180, 2002.

13 Lissoni P, Barni S, Tancini G, Ardizzoia A, Rovelli F, Cazzaniga M, Brivio F, Piperno R, Aldeghi R and Fossati D: Immunotherapy with subcutaneous low-dose interleukin-2 and the pineal indole melatonin as a new effective therapy in advanced cancers of the digestive tract. Br J Cancer *67*: 1404-1407, 1993.

14 Ghiringhelli F, Larmonier N, Schmitt E, Parcellier A, Cathelin D, Garrido X, Chauffert B, Solary E, Bonnotte B and Martin F: $CD4^+CD25^+$ regulatory T-cells suppress tumor immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumors to be curative. Eur J Immunol *34*: 336-344.

Received October 15, 2010
Revised June 9, 2010
Accepted June 15, 2010