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Bone marrow toxicity and antitumor action of adriamycin in relation to the antioxidant effects of melatonin

Valentina Rapozzi[1], Laura Perissin[1], Sonia Zorzet[2], Marina Comelli[1], Irene Mavelli[1], Marjeta Šentjurc[3], Alja Pregelj[3], Milan Schara[3] and Tullio Giraldi[1]

[1]Department of Biomedical Sciences and Technologies, University of Udine,[2] Department of Biomedical Sciences, University of Trieste, Italy,[3] Jozef Stefan Institute, Ljubljana, Slovenia

Melatonin has been reported to possess numerous properties, including antioxidant effects. Some antitumor drugs, such as anthracyclines, display a pro-oxidant activity which is held responsible for their toxicity to normal tissues of the host. The aim of this work was, therefore, to preliminarily examine the effects of melatonin on the bone marrow toxicity caused by the treatment with adriamycin in CBA mice bearing TLX5 lymphoma. After a single treatment with adriamycin (28-40 mg/kg i.v.), the administration of a single pharmacological dose of melatonin (10 mg/kg s.c.) reduced the acute mortality of the hosts from 9/16 to 2/16. The antitumor action of adriamycin, consisting in the increase in survival time of animals which were not affected by the acute toxicity of the drug, was not reduced by melatonin. Melatonin also attenuated the reduction in the number of bone marrow GM-CFU caused by adriamycin, and significantly restored the reduced and total glutathione levels. Moreover, the use of Fenton reaction and free radical determination via spin trapping, show that melatonin acts as a direct free radical scavenger. The data reported indicate that melatonin attenuates the bone marrow toxicity of adriamycin with a mechanism consistent with its antioxidant properties.

Key words: lymphoma; doxorubicin-adverse effects; bone marrow; melatonin; mice

Introduction

The pineal gland and its indole hormone, melatonin, have been shown in numerous experimental studies to be involved in cancer progression. In the 30's, Engel suggested a link between the pineal gland and cancer.[1,2] Cancer treatment with pineal extracts has been performed later in the clinic, resulting in a reported retardation in the progression of the disease and in an improvement of the quality of life of the patients.[3]

The role of pineal gland and of melatonin for cancer growth has been investigated rather extensively in laboratory animals. Surgical pinealectomy resulted in the increased growth *in vivo* of different types of experimental tumors.[4-8] Tumor growth was correspondingly attenuated in pinealectomized animals by the administration of exogenous

Correspondence to: Prof. Tullio Giraldi, Department of Biomedical Sciences, University of Trieste, Via L. Giorgieri 7, 34100 Trieste, Italy. Tel: +39-40-6763539; Fax: +39-40-577435; E-mail: giraldi@univ. trieste.it

melatonin.[9-11] Tumor growth inhibition *in vivo* and *in vitro* following treatment with melatonin in non pinealectomized animals has been described in some instances,[12-16] although contrasting reports showing a stimulation of tumor growth are also available in the literature.[17,18]

In Lewis lung carcinoma bearing mice melatonin has been shown to increase the therapeutic index of the antitumor drugs cyclophosphamide and etoposide, since it protects the bone marrow stem cells from the apoptosis induced by these drugs while it does not reduce their antitumor action. This effect of melatonin was suggested to occur via interaction with its receptors on T-helper lymphocytes in the bone marrow,[19] leading to the stimulation of the production of a Th cell factor constituted of two cytokines named MIO (melatonin induced opioids). In turn, this factor would act on bone marrow stromal cells inducing the release of hematopoietic growth factors.[20]

On the other hand, melatonin has been shown to cause potent direct antioxidant effects, rapidly scavenging hydroxyl[21,22] and peroxyl radicals.[23] Additionally, melatonin can also upregulate endogenous antioxidant defenses, as shown for glutathione peroxidase activity.[24,25] The antioxidant action of melatonin is also supported by experiments indicating that it decreases the DNA damage caused by ionizing radiation in cultured cells,[26] the *in vivo* cataract formation induced by BSO in rats,[27,28] the DNA damage caused by chemical carcinogen safrol,[29] as well as the kainate excytotoxicity in cerebellar granular neurons.[30]

The anthracycline antitumor drug, adriamycin, is being widely used in the clinic. The most serious adverse effects limiting the applicable dose intensity are myelosuppression, gastrointestinal toxicity and acute cardiac toxicity eventually leading to cumulative late cardiomyopathy.[31] Numerous studies investigated the underlying mechanisms and oxidative damage to membrane lipids and to other cellular components, which are believed to be a major factor in the cardiac toxicity of adriamycin and other anthracyclines.[32-36]

The aim of this work was, therefore, to examine the effects of the administration of adriamycin, of exogenous melatonin, or of their combination, in terms of toxicity for the host and antitumor activity in mice implanted with TLX5 lymphoma. The acute toxicity for the host has been evaluated in terms of lethality, as well as of the effects on bone marrow stem cells. The possible relevance of oxidative damage caused by adriamycin, and its prevention by melatonin, has been determined measuring glutathione levels in bone marrow cells. The direct free radical scavenger activity of melatonin was evaluated in a model system, using Fenton reaction and free radicals determination via spin trapping and EPR at different concentrations of melatonin. The results obtained are reported hereafter.

Materials and methods

Reagents

Melatonin was a kind gift of Prof. Fraschini, University of Milano, Italy. Adriamycin was obtained from Pharmacia S.p.A. Milano, Italy, and the other reagents used were purchased from Sigma Chemical Co, Sigma Chimica Divisione della Sigma-Aldrich S.r.l., Milano, Italy.

Animals and tumor transplantation

The animals used were male CBA/LAC mice weighting 22-25 g, belonging to a conventional local breeding colony. The animals were provided food and water *ad libitum*, and were kept constantly at a 12/12 light/dark cycle (lights on from 8 a.m. to 8 p.m.). TLX5 lymphoma was originally provided by the

Chester Beatty Research Institute, London, England. Tumor implantation was performed by injecting each mouse i.p. with 0.1 ml of a suspension containing 10^5 viable tumor cells. The tumor cells, obtained from donors inoculated 8 days before, were washed by centrifugation at 500xg and resuspended in PBS after counting for trypan blue exclusion.

Drug treatment

Melatonin was dissolved in 0.9 % NaCl saline containing 4 % ethanol, and was administered s.c. in a volume of 0.05 ml/10 g of body weight. Adriamycin was dissolved in 0.9 % NaCl solution, and was administered i.v. in 0.05 ml/10 g of body weight or i.p. in 0.1 ml/10 g of body weight, as indicated. The treatment with melatonin was performed at 8 p.m. (light off), whereas the treatment with adriamycin was applied at 9 p.m..

GM-CFU assay

The number of granulocyte/macrophage-colony forming units (GM-CFU) was determined after *in vivo* treatment with the drugs tested. Following sacrifice, 10^5 viable bone marrow cells were incubated in 0.3 % semisolid agar in RPMI 1640 medium containing 10 % fetal calf serum and 10 % lung conditioned medium (LCM) as a source of stimulating factors. LCM was prepared by mincing the lungs from 2 mice into small pieces and incubating the pieces at 37°C with 5 % CO_2 for 3 days in RPMI 1640 medium containing 10 % fetal calf serum. The cultures were kept for 7 days at 37°C in humidified air and then examined by phase contrast microscopy; colonies containing more then 50 cells were counted as GM-CFU.

Glutathione assays

The reduced (GSH) and oxidized (GSSG) glutathione levels were measured in bone marrow by a high-performance liquid chromatography (HPLC) technique. Bone marrow samples were processed following the method of Reed *et al.*.[37] Briefly, 1 ml of bone marrow cell suspension in 0.9 % NaCl (10^6 cells) was added to 0.05 ml of 70 % perchloric acid. After protein precipitation, 0.5 ml of the supernatant was treated immediately with 50 ml of 0.08M fresh aqueous solution of iodacetic acid and then neutralized with an excess of $NaHCO_3$. After 60 min in the dark at room temperature, 0.5 ml of an alcoholic solution of 1-fluoro-2,4-dinitrobenzene (1.5 ml/ 98.5 ml absolute ethanol) was added, and the reaction was left to proceed for 4 hours in the dark. The samples were then chromatographed using a reverse-phase ion exchange column microbondapak NH_2 3.9 x 300 mm (Waters). Glutathione levels were related to protein content in the samples, which was determined by the method of Lowry *et al.*.[38]

Spin trapping experiment

Free radical scavenging activity of melatonin was measured in phosphate buffered saline containing 0.1 mM EDTA (pH 7, 320 mosmol). The buffer was supplemented with spin trap 5,5-dimethyl-1-pyrroline N-oxide (DMPO, 1 mM), 0.02 mM $FeSO_4.7H_2O$ and 0.01 % (w/v) of H_2O_2 (final concentrations). The intensity of the EPR spectra of DMPO-OH adduct was measured in presence and absence of different concentrations of melatonin in the course of the reaction.[39]

Statistical analysis

Tabled values are group means ± SD. Data were subjected to Kruskall-Wallis analysis of variance, as well as Kaplan Meier, logrank and Cox proportional hazard analysis as appropriate. All analyses were performed using standard procedures implemented in the Systat package (SYSTAT Inc., Evanston, IL).

Results

Antitumor activity and toxicity of melatonin and adriamycin

In TLX5 lymphoma bearing mice, adriamycin displays a significant antitumor action when administered i.v. as a single dose of 28 mg/kg, the survival time of the treated mice being significantly increased in comparison with drug untreated controls, as determined by Kaplan-Meier analysis. The stratification of the data and log-rank analysis indicated a significant effect for adriamycin (chi-square = 17.2, DF = 2, $P < 0.0001$), and the absence of significant effects for melatonin 10 mg/kg s.c. (chi-square = 1.18, DF = 1, P = 0.278). Bivariate Cox proportional hazard analysis further indicated that adriamycin constituted a significant negative risk factor (HR = 0.192, 95 % CL 1.486 – 0.025), whereas the effects of melatonin were insignificant (HR = 1.253, 95 % CL 9.66 – 0.163).

On the contrary, the toxicity of adriamycin, as indicated by the number of toxic deaths occurring before day 9, was significantly reduced by melatonin. Indeed, the total number of such toxic deaths in the dose range of 28-40 mg/kg adriamycin was 9/16; when the treatment with adriamycin was combined with melatonin, the number of toxic deaths was significantly reduced to 2/16, Yates corrected chi-square = 4.987, DF = 1, P = 0.026 (Table 1).

Table 1. Toxicity-related deaths and increase in the survival of CBA mice implanted with TLX5 lymphoma and treated with adriamycin and melatonin

Adriamycin mg/kg	Melatonin	Toxicity-related deaths	Mean survival time
–	–	0/9	10.1
40	–	7/8	9
40	+	2/8	14.8
28	–	2/8	18.5
28	+	0/8	17.3

Groups of 8 CBA male mice were implanted on day 0 with 10^5 TLX5 lymphoma cells. On day 1 they were treated at 8 p.m. with melatonin (10mg/kg s.c.) and at 9 p.m. with adriamycin (i.v.) as indicated. Acute toxic deaths were those occurring before day 9. Mean survival time was determined using Kaplan Meier statistics (for the results of statistical analysis see the Results section).

Effects of melatonin and adriamycin on bone marrow granulocyte / macrophage – colony forming units

The treatment with melatonin (20 mg/kg s.c.), which was devoid of effects by itself, significantly reverted the reduction in the number of GM-CFU which was caused by the administration of a single i.v. dose of 28 mg/kg adriamycin (Kruskall-Wallis analysis of variance, chi-square=3.87, DF=1, P=0.049) (Table 2).

Effects of melatonin and adriamycin on glutathione levels

The treatment with 10 mg/kg melatonin s.c. significantly restored the reduction in reduced, oxidized and total glutathione levels, which was caused by the administration of 3 weekly doses of 5 mg/kg adriamycin i.p (Kruskall-Wallis analysis of variance, chi-square=3.87, DF=1, P=0.049) (Table 3).

Free radical scavenging activity of melatonin

Free radical scavenging activity of melatonin was detected in spin trapping experiments using Fenton reaction as a model for production of hydroxyl radicals. DMPO-OH adducts were detected by EPR. Only a very slow decrease of this adduct with time was observed. When Fenton reaction was initiated in the presence of melatonin, the intensity of EPR spectra was decreased, indicating the scavenging activity of melatonin, which prevents binding of OH radical to DMPO. The intensity of EPR spectra decreased with increasing concentration of melatonin. A sig-

Table 2. Granulocyte/macrophage colony forming units in the bone marrow of normal CBA mice treated with adriamycin and melatonin

Adriamycin	Melatonin	GM-CFU
–	–	67.2 ± 24.1
–	+	42.3 ± 10.3[a]
+	–	15.0 ± 2.2[ab]
+	+	41.3 ± 8.1[b]

Each value is the mean ± S.D. obtained in grups of 6 tumor-free Cba male mice. The animals were treated on day 1 with melatonin (20 mg/kg s.c.) at 8 p.m. and with adrimycin (28 mg/kg i.v.) at 9 p.m. as indicated. The animals were sacrificed on day 5, and the number of granulocyte/macrophage colony forming units (GM-CFU) in 10^5 bone marrow cells was determined. The data were subjected to ANOVA analysis; the results are presented in the Results section. Means marked with the same letters are significantly different, Tukey test, $P<0.5$.

nificant decrease by about 30 % of the initial value was observed with 50 mM melatonin (Figure 1). The results are in good agreement with recently published data on the same system.[40]

Discussion

The data reported show that the administration of 10 mg/kg melatonin in the evening in TLX5 lymphoma bearing mice significantly reduces the host toxicity of adriamycin at the doses of 28 and 40 mg/kg administered 1 hour later, as indicated by the reduction in the occurrence of acute early toxic deaths. At the same time, adriamycin administered at a dose of 28 mg/kg significantly increases the survival time of the TLX5 lymphoma bearing mice which were not affected by acute treatment-related toxicity. The concurrent administration of melatonin does not reduce the magnitude of the antitumor effects of adriamycin. The reduction in the proportion of acute toxic deaths caused by adriamycin is accompanied by a significant reduction in the number of bone marrow GM-CFU in the treated animals, and this reduction is significantly attenuated by melatonin which is devoid of significant effects by itself. The doses of melatonin used are devoid of evident toxic effects on the host; they are also devoid of any antitumor action in TLX5 lymphoma bearing mice, as indicated by the lack of a significant prolongation in the life span of the treated animals (unreported results).

The presently observed attenuation of bone marrow toxicity of adriamycin by melatonin is similar to that observed by Maestroni *et al.* examining the effects of melatonin on the toxicity and antitumor action of cyclophosphamide and etoposide. In mice bearing Lewis lung carcinoma, the antitumor action of both drugs was retained after melatonin treatment, whereas their hematological

Table 3. Glutathione levels in the bone marrow cells of normal CBA mice treated with adriamycin and melatonin

Adriamycin	Melatonin	GSH	GSSG	GSH/GSSG	tGSH
–	–	5.3 ± 0.61	0.20 ± 0.04	21.5 ± 1.95	5.5 ± 0.65
+	–	3.4 ± 0.23	0.14 ± 0.01	24.6 ± 0.82	3.6 ± 0.25
+	+	6.6 ± 1.15	0.27 ± 0.07	23.9 ± 1.71	6.9 ± 1.59

Each value is the mean ± S.D. obtained in groups of 4 tumor-free CBA mice. The animals were treated once a week for 3 weeks with melatonin (10 mg/kg s.c.) at 8 p.m. and with adriamycin (5 mg/kg i.p.) at 9 p.m. as indicated. Gluathione levels were determined in the bone marrow cells of animals sacrificed 3 days after the last tretment. The values of reduced gluathione (GSH), oxidized glutathione (GSSG) and total glutathione (tGSH) are expressed as nmol/mg protein. The experiment was performed in duplicate, and the data were subjected to Kruskal–Wallis analysis of variance; the results are presented in the Results section.

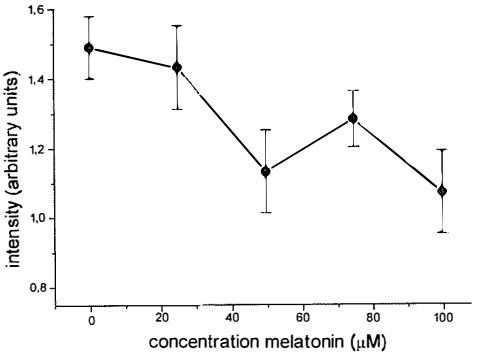

Figure 1. EPR spectra intensity decrease of spin adduct DMPO-OH with increasing concentration of melatonin. Final concentrations: 1mM DMPO, 0.02 mM Fe^{2+}. 0.01% H_2O_2 in PBS (pH 7) with 0.1 mM EDTA. Each point is a mean value of 5 measurements, the bars indicate standard deviations.

toxicity was significantly reduced.[41,42] The mechanism by which melatonin attenuates the hematological toxicity of cyclophosphamide and etoposide has been studied in detail by the same authors.[19,20,43] Their results indicate that melatonin binds to helper T-lymphocytes, inducing the release of a Th cell factor constituted of two cytokines named MIO (melatonin induced opioids). In turn, this factor acts on bone marrow stromal cells inducing the release of hematopoietic growth factors, such as GM-CSF.[44]

The reduction of bone marrow toxicity of adriamycin by melatonin might be caused by a mechanism alternative to that involving MIO, consisting of the antioxidant action of melatonin exerted against the pro-oxidant effects of adriamycin. In fact, the results obtained by Fenton reaction and EPR show that melatonin possesses a direct and concentration-dependent free radical scavenger activity within the concentration range 20-100 mM. Moreover, melatonin significantly restores to control values the levels of reduced, oxidized and total glutathione which have been lowered by adriamycin in bone marrow cells. These results indicate that melatonin may attenuate the oxidative damage caused by adriamycin in bone marrow stem cells. Assuming a distribution of administered melatonin occurring in total body water, its peak concentration should fall in a concentration range between 10 and 100 mM, which is consistent with the possibility of a direct interaction with adriamycin oxygen reactive species. This direct free radical scavenging action is accompanied by an indirect mechanism consisting in the restoration of the levels of reduced glutathione which have been lowered by adriamycin. The data presented here do not allow to assess the relative importance of the mechanisms presently proposed, and do not permit evaluation of their relevance in relation to that involving MIO forwarded by Maestroni and co-workers.[44]

In conclusion, the results presented show that the administration of a pharmacological dose of melatonin to TLX5 lymphoma bearing CBA mice does not decrease the antitumor action of adriamycin, while the acute host toxicity of this drug is significantly reduced, thus suggesting that an enhancement in the dose intensity of adriamycin can be achieved by its combination with exogenously administered melatonin. Bone marrow toxicity of adriamycin is attenuated by melatonin through a mechanism consistent with a direct and indirect antioxidant action of melatonin which is effective against the pro-oxidant action of adriamycin. These data appear to encourage the study of the effect of endogenous melatonin and of the administration of melatonin at pharmacological doses on other organ directed toxicity of anthracyclines. Early and late cardiac toxicity of anthracyclines deserve particular attention, since they are of crucial importance as factors limiting the dose intensity tolerated clinically, and since they have been attributed to an oxidative mechanism.[45-48] Moreover, the data reported also appear to encourage the study of endogenous melatonin concentra-

tion and its rhythmic variations in relation to the chronotoxicological data obtained for anthracyclines in laboratory animals [49] and in clinical antitumor chemotherapy.[50]

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