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Stress, Melatonin and Tumor Progression in Mice[a]

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INTRODUCTION

The possibility that psychosocial factors might influence cancer incidence and progression in humans has repeatedly been expressed.[1] Several studies have indicated associations of stressful life events,[2,3] specific personality traits,[4,5] emotional repression[6] and modalities of psychological adaptation to cancer diagnosis[4,7] with clinical cancer progression. Fundamental tools of investigation have been provided by the rapidly growing field of neuroimmunomodulation (and/or psychoneuroimmunology). Yet a definitive demonstration of the general relevance of psychosocial factors, and in particular of styles of coping with stressors, for clinical cancer progression, still constitutes a formidable and unresolved challenge.[1]

Models of laboratory animals subjected to experimental stressors have been widely used to investigate the action of stress on tumor growth.[8] However, when the relevant reports were critically reviewed, a high heterogeneity appeared both for the animal-tumor systems used and for the characteristics of the stressors employed.[9] The occurrence and magnitude of the effects of the stressors on tumor growth were correspondingly variable. Moreover, tumor metastasis was only marginally considered, in spite of its outstanding clinical relevance.[10]

Among the numerous experimental stressors studied, the application of rotational stress to mice housed in a stress-protected environment appears to be a carefully and widely characterized mild psychological stressor.[11] The differential effects of the application of rotational stress (spatial disorientation) have been examined in syngeneic mice implanted with Lewis lung carcinoma, in order to specifically determine the action of the stressor upon tumor dissemination in addition to tumor growth. Rotational stress was shown to significantly modify metastasis, independently from its effects on the primary tumor.[12] However, a high variability in the results obtained was observed, which could not be reduced

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by the accurate control of housing conditions and experimental procedures. When a retrospective analysis of the results obtained in numerous experiments repeated in different periods of the year was performed, a seasonal dependency could be recognized, consisting of an increase in metastasis by the stressor in summer, and of a decrease in winter.

The aim of the present investigation, therefore, was to examine the seasonally dependent effects of experimental stressors on tumor growth and metastasis in mice bearing Lewis lung carcinoma. The stressor paradigms used were: rotational stress; forced immobilization; and electric foot shock. The possible participation of pineal gland function and of melatonin in the seasonally dependent effects of experimental stressors on tumor progression were also investigated in summer and winter. Indeed, melatonin is a hormone which displays circadian and seasonal rhythmic variations.[13] Moreover, melatonin plasmatic levels are stress sensitive,[14] and exogenous melatonin has been shown to possess immunomodulatory[15] and antitumor properties.[16] The further experimental variables explored thus were urinary excretion of melatonin, various cycles and intensities of illumination capable of influencing pineal gland function, and the administration of exogenous melatonin. The results obtained are reported herewith.

MATERIALS AND METHODS

Animals and Tumor Transplantation

The animals used were female C57BL/6 and C57BL/6 × DBA/2F$_1$ (hereafter called BD2F1) mice weighing 18–20 g, purchased from Charles River, Calco, Como, Italy. Lewis lung carcinoma was originally provided by the National Cancer Institute, Bethesda, MD, and was maintained in C57BL/6 mice by subcutaneous injection in the axillary region of 50 mm^3 of minced tumor tissue aseptically prepared from donors similarly inoculated two weeks before. For experimental purposes, the tumor was propagated in BD2F1 mice by subcutaneous or intramuscular injection, as indicated, of a tumor cell suspension containing 10^6 viable tumor cells.[12]

Measurement of Tumor Growth and Metastasis Formation

Primary tumor weight was determined 14 days after tumor inoculation by caliper measurements of short (a) and long (b) axes (cm), taking tumor density equal to 1:

$$\text{tumor weight} = \pi/6 \times a^2 \times b \qquad (1)$$

The number of metastases was determined at sacrifice on day 22 from tumor inoculation by examining the surface of the lungs with a low-power stereomicroscope. The weight of metastasis was determined as the sum of their individual weights calculated according to equation (1) after measurement of their dimensions by an ocular micrometer.[12]

Protected Environment

The animals were kept 5 per cage in order to avoid the effects of overcrowding or isolation on tumor progression.[11,17] The cages were placed in the protected

environment for 2 weeks before tumor inoculation in order to allow the animals to recover from the stress of shipment, and to adapt them to the new housing conditions.[18,19] The protected environment consisted of cabinets containing the animal cages with laminar air flow, minimizing acoustic, olfactory and visual communication among the cages. The cabinets were placed in a room remote from other animal rooms, where staff also entered only once every 5 days to check the animals for water and food supplies, which were available ad libitum. Temperature and relative humidity were constant at 20°C and 60% respectively. The light-dark cycles used and intensity of illumination in the cages are indicated in the tables below.

Experimental Stressors

Rotational stress was applied to the animals maintained in the low stress environment, as indicated, by spinning the cages at 45 rpm for 10 min every hour from time of tumor inoculation until sacrifice.[12] Forced immobilization was imposed in the low stress environment by tying the animals' legs with strings fixed to small plastic boards, for 1 hr daily on days 1–6 from tumor inoculation. Electric foot shock experiments were performed essentially as described by Sklar and Anisman,[20] with mice individually placed in shuttle boxes measuring 30 by 14 by 15 cm. The electric shock (1 mA) was delivered through the grid floor and lasted 5 sec. Sessions, consisting of the repetition of this cycle every 10 min for 3 hrs, were repeated daily for 21 days following tumor inoculation.

Drug Treatment

Melatonin (1.25 mg/Kg body weight/night) was administered in drinking water containing 0.2% ethanol during light off hours (8 p.m.–8 a.m.) from tumor inoculation to sacrifice on day 22. Melatonin concentration (6.2 mg/l) was based on an average measured water consumption of 4.8 ml/mouse/night.

Urinary Melatonin Excretion

For urine collection, the mice were housed individually in conventional cages (21 × 27 × 14 cm), each containing a stainless steel grid. Urine was collected on chromatographic paper (Whatman 3MM) placed below this grid. The paper was subsequently removed and cut into pieces 1 cm^2, from which melatonin was extracted twice with 10 ml 0.07 M phosphate buffer, pH 5.5. The extract was purified in a single chromatographic step by absorption on a C-18 column (SEP-PAK Water Associates, Millipore) followed by elution with 1 ml chloroform. The solvent was evaporated under a gentle stream of nitrogen, and the melatonin present in the dry residue was assayed by means of a ^{125}I radioimmunoassay (Clone-System Diagnostics, Nuclear Medica, Padova, Italy). Further particulars are reported in detail elsewhere.[21]

RESULTS AND DISCUSSION

The application of stressor paradigms to laboratory animals constitutes a useful model which has been rather extensively employed for experimental investigation

on the effects and the mechanism of influence of stress on tumor progression. Numerous experimental paradigms have been used, and it is beyond the purposes of this paper to review the relevant reports (for a review, see, for instance, Ref. 9). However, the results published are in conflict, which is not surprising given that the experimental conditions used required stressors quite varied in nature and in method of administration (acute or chronic). Similarly, the choice of different tumor-host systems has apparently led to critically differing results.[9,11]

Rotational stress (spatial disorientation) is a paradigm of mild psychological stress (anxiety) originally devised, and accurately investigated, by Riley.[11] The experimental conditions require the maintenance of the mice in protected housing, since keeping the animals in a conventional animal house was shown to cause by itself high levels of stress in terms of plasmatic levels of corticosterone;[18] the potential uncontrollable variability of housing stress is also avoided. Using these experimental conditions and CH3HED lymphoma in CH3/He mice as host-tumor systems, Riley consistently reported increases in tumor growth caused by rotational stress.[19]

In the present authors' laboratory, rotational stress was applied to mice implanted with Lewis lung carcinoma, in order to determine its effects on tumor metastasis. Interestingly, this stressor was found to be effective in modifying the formation of spontaneous lung metastasis, even when the growth of the primary tumor was unaffected by the stressor.[12] Incidentally, mice kept in the protected housing showed very low levels of plasmatic corticosterone, which were increased by rotational stress; the administration of the adrenal gland suppressor, mitotane, revealed that the increase in tumor metastasis caused by rotational stress was not caused by corticosterone.[22]

Rotational stress has been repeatedly used in numerous experiments over the past several years. In spite of the most accurate control of the experimental conditions, a high variability in the results obtained was evident, and could not be reduced by a further control of the experimental setting. A similar high variability was encountered during repeated experiments with electric foot shock, physical restraint and behavioral despair.

A retrospective analysis of the data collected with rotational stress indicates that the results obtained could have a seasonal dependence. Indeed, data in TABLE 1 illustrate the results of representative experiments performed in winter or summer. The effects of the application of rotational stress are not significant on primary tumors. On the contrary, there is a significant increase in metastasis weight in August, whereas a significant decrease is noted in January.

Data collected in 19 different experiments with rotational stress were analyzed by the Cosinor method.[23] When these data are expressed as the variation caused by the stressor in comparison with nonstressed controls, a highly significant ($p < 0.001$) sinusoidal circannual variation results for metastasis weight and number (acrophase in summer; amplitude 45% of MESOR values). This rhythmic effect is limited to metastasis, and is not significant for primary tumor growth.[24] This finding appears of interest, since it shows that the magnitude of the variation in metastasis caused by rotational stress has a circannual seasonal pattern, consisting of an increase peaking in summer and a decrease reaching a minimum value in winter.

The number of the experiments performed with other experimental stressors was too small to permit a rhythmometric analysis. On the other hand, the application of unavoidable electric foot shock to mice implanted with Lewis lung carcinoma did display a seasonal pattern similar to that illustrated above for rotational stress. Indeed, the representative experiments reported in TABLE 2 indicate a

TABLE 1. Effects of the Application of Rotational Stress on the Weight of Primary Tumor and Lung Metastasis in Mice Bearing Lewis Lung Carcinoma, as a Function of the Month of Evaluation

Stressor	Month	Primary Tumor Weight (g)	Metastasis Weight (mg)	Metastasis Weight %[1]
–	January	2.3 ± 0.2	209 ± 28[a]	
+		2.6 ± 0.2	101 ± 17[a]	−52
–	August	2.0 ± 0.5	97 ± 14[b]	
+		2.6 ± 0.4	222 ± 41[b]	+129

[1] Variation over controls.

[a,b] Means marked with the same letter are significantly different, Mann Whitney test,[32] $p < 0.05$.

Each value in the Table is the mean ± SEM obtained using groups of 10 mice. Light cycle was 12/12 light-dark, and intensity of illumination measured in the cages is 5 lux. Tumor implantation was subcutaneous.

significant increase in metastasis by unavoidable foot shock in comparison with nonstressed mice in July, whereas a decrease is observed in December. This seasonal pattern does not appear to apply in general to other experimental stressors. The representative experiment reported in TABLE 3 indicates that forced immobilization increases metastasis formation in a way which is independent from the season of examination.

The sinusoidal pattern in the increase of metastasis caused by rotational stress could be attributed either to characteristics of the tumor cells or of the host displaying circannual rhythmicity. As far as host characteristics are concerned, several hormones capable of causing neuroimmunomodulation have been shown to display cyclic variations in their plasmatic levels.[25] Melatonin, for which a central chronobiological role has been indicated,[26] displays circadian and circannual periodicities.[27] This hormone has been shown to be involved in neuroimmunomodulation[28] and also to possess cytotoxicity *in vitro* against different tumor cell lines in culture.[16] The possible participation of pineal gland function and of melatonin in the seasonally dependent effects of rotational stress on tumor progression has consequently been examined.

TABLE 2. Effects of the Application of Electric Foot Shock on the Weight of Primary Tumor and Lung Metastasis in Mice Bearing Lewis Lung Carcinoma, as a Function of the Month of Evaluation

Stressor	Month	Primary Tumor Weight (g)	Metastasis Weight (mg)	Metastasis Weight %[1]
–	December	2.4 ± 0.4	54 ± 7	
+		2.1 ± 0.3	38 ± 8	−30
–	July	3.7 ± 0.3	17 ± 6[a]	
+		4.7 ± 1.2	82 ± 27[a]	+382

[1] Variation over controls.

[a] Means marked with the same letter are significantly different, Mann Whitney test,[32] $p < 0.05$.

Each value in the Table is the mean ± SEM obtained using groups of 10 mice. Light cycle was 12/12 light-dark, and intensity of illumination measured in the cages is 5 lux. Tumor implantation was subcutaneous.

TABLE 3. Effects of the Application of Forced Immobilization on the Weight of Primary Tumor and Lung Metastasis in Mice Bearing Lewis Lung Carcinoma, as a Function of the Month of Evaluation

Stressor	Month	Primary Tumor Weight (g)	Metastasis Weight (mg)	Metastasis Weight %[1]
−	January	1.5 ± 0.2	59 ± 7[a]	
+		1.2 ± 0.1	121 ± 11[a]	+105
−	August	3.7 ± 0.3	202 ± 29[b]	
+		3.6 ± 0.1	328 ± 30[b]	+62

[1] Variation over controls.

[a,b] Means marked with the same letter are significantly different, Mann Whitney test,[32] $p < 0.05$.

Each value in the Table is the mean ± SEM obtained using groups of 10 mice. Light cycle was 12/12 light-dark, and intensity of illumination measured in the cages is 5 lux. Tumor implantation was intramuscular.

An initial series of experiments was performed in order to relate the effects of rotational stress on metastasis with the urinary excretion of melatonin. Intensities of illumination of 5 or 2000 lux, with cycles of 12/12 hours or continuous lighting were the experimental variables used, which have been shown to be capable of influencing pineal gland function. The seasonal effects of rotational stress on metastasis are substantially retained when the intensity of illumination is increased from 5 to 2000 lux, and lighting has a 12/12-hr light-dark cycle (TABLES 4 and 5). With continuous illumination and 2000 lux, a statistically significant tendency to reduction in December is observed (TABLE 5). A noninvasive and

TABLE 4. Effects of Rotational Stress on Tumor Metastasis and Melatonin Urinary Excretion, as a Function of Cycles and Intensity of Illumination: Results Obtained in April

Illumination		Rotational Stress	Metastasis Weight		Melatonin[1]	
Cycle[2]	Intensity[3]		(mg)	%[4]	Day[5]	Night[6]
12/12	5	−	183 ± 41[a,b]		58 ± 9	81 ± 17[a]
12/12	5	+	332 ± 62[a]	+81	18 ± 16	750 ± 104[a]
12/12	2000	−	309 ± 37[b,c]		59 ± 20	82 ± 25[b]
12/12	2000	+	438 ± 70[c]	+42	12 ± 5	646 ± 137[b]
24/0	2000	−	125 ± 26		6 ± 3	46 ± 15[c]
24/0	2000	+	90 ± 22	−28	6 ± 2	152 ± 20[c]

[1] Urinary excretion.

[2] 12/12 lights on from 8 a.m. to 8 p.m.; 24/0 light continuously on.

[3] Lux measured in the cages.

[4] Variation over controls.

[5] 8 a.m.–8 p.m.

[6] 8 p.m.–8 a.m.

[a,b,c] Means marked with the same letter are significantly different, Mann Whitney test,[32] $p < 0.05$.

Each value in the Table is the mean ± SEM obtained using groups of 10 mice implanted subcutaneously with the tumor. Melatonin assay was performed in the 24-hour period before the day of sacrifice. Primary tumor growth was not significantly different in any of the experimental groups and for this reason is unreported.

TABLE 5. Effects of Rotational Stress on Tumor Metastasis and Melatonin Urinary Excretion, as a Function of Cycles and Intensity of Illumination: Results Obtained in December

Illumination		Rotational	Metastasis Weight		Melatonin[1]	
Cycle[2]	Intensity[3]	Stress	(mg)	%[4]	Day[5]	Night[6]
12/12	5	−	209 ± 28[a]		12 ± 4	177 ± 38[a]
12/12	5	+	101 ± 17[a]	−52	10 ± 3	8 ± 1[a]
12/12	2000	−	155 ± 26		6 ± 1	41 ± 5
12/12	2000	+	107 ± 10	−31	15 ± 3	39 ± 11
24/0	2000	−	219 ± 38[b]		13 ± 7	146 ± 22[b]
24/0	2000	+	109 ± 24[b]	−50	11 ± 6	325 ± 18[b]

[1] Urinary excretion.
[2] 12/12 lights on from 8 a.m. to 8 p.m.; 24/0 light continuously on.
[3] Lux measured in the cages.
[4] Variation over controls.
[5] 8 a.m.–8 p.m.
[6] 8 p.m.–8 a.m.
[a,b] Means marked with the same letter are significantly different, Mann Whitney test,[32] $p < 0.05$.

Each value in the Table is the mean ± SEM obtained using groups of 10 mice implanted subcutaneously with the tumor. Melatonin assay was performed in the 24-hour period before the day of sacrifice. Primary tumor growth was not significantly different in any of the experimental groups and for this reason is unreported.

nonstressing procedure for urine collection and endogenous melatonin excretion measurement was used.[21] Nocturnal urinary excretion of melatonin is in general higher than that measured during day hours, with the exception of nonstressed mice with a 12/12-hr light-dark cycle in April (TABLE 4). Melatonin urinary excretion during light-on hours does not show any significant variation in relation to application of rotational stress or to lighting intensity (TABLES 4 and 5). On the contrary, nocturnal melatonin urinary excretion is significantly increased in April upon application of the stressor (TABLE 4). In December, with a 12/12-hr light-dark cycle, nocturnal melatonin urinary excretion is significantly decreased by rotational stress with 5 lux, is not modified with 2000 lux, and is significantly increased with 2000 lux and continuous lighting (TABLE 5). It thus appears that with a 12/12-hr light-dark cycle, a significant increase in metastasis formation is accompanied by a significant increase in nocturnal urinary excretion of melatonin in a way independent from intensity of illumination. On the other hand, an inverse correlation between metastasis and nocturnal melatonin excretion appears for continuous illumination with 2000 lux and application of the stressor.

The latter findings were intended to be obtained with functional pinealectomy by lighting. An abrogation of the nocturnal melatonin peak, and of its increase by rotational stress, is indeed obtained by continuous illumination and 2000 lux. This abrogation is limited to the initial phase of continuous lighting; after two weeks, high nocturnal melatonin levels, further increased significantly by the stressor, are observed (TABLE 6). The data obtained with 21 days of continuous illumination, which are reported in TABLES 4 and 5, thus involve an initial phase of functional pinealectomy followed by a recovery of pineal gland function, and are difficult to interpret because of this biphasic trend.

The possible rôle of melatonin in tumor metastasis was also examined by the oral administration of exogenous melatonin to tumor-bearing mice during night hours. This examination has so far been made only once, in April. Exogenous melatonin was administered in drinking water, since handling and injecting the animals was found to be a stress capable of influencing tumor metastasis.[12] The administration of 1.25 mg/Kg/night of melatonin to mice kept with continuous lighting was shown to be devoid of any significant effect either on primary tumor or on metastasis. On the contrary, with a 12/12-hr light-dark cycle, the administration of melatonin to nonstressed mice induced the statistically significant absence of primary tumors and metastasis in 5/10 mice on necroscopy at sacrifice; metastasis weight in the remaining 5/10 mice was also significantly reduced. This remarkable antitumor effect of melatonin was abolished by the application of rotational stress (TABLE 7). These findings rule out the possibility that exogenous melatonin at the dose and with the treatment schedule used exert a direct cytotoxic action on tumor cells, and rather seem to indicate a control in tumor growth exerted by means of the immunomodulatory properties of the hormone.

CONCLUSIONS

The data presented so far indicate that rotational stress specifically influences the metastatic spread of a solid malignant tumor—Lewis lung carcinoma—in syngeneic mice in a way which is independent from the effect on the primary tumor. The magnitude and direction of the effects of rotational stress have a significant seasonal and circannual pattern, varying from an increase to a decrease in summer or winter respectively. The seasonal nature of the effects of experimental stressors on metastasis appears to apply also to electric foot shock, but not to forced immobilization. Pineal gland function and melatonin have both been examined in relation to the circannual effects of rotational stress. No unequivocal general pattern of relationships between melatonin and metastasis appears. How-

TABLE 6. Melatonin Urinary Excretion in Mice Maintained with Constant Illumination and Subjected to Rotational Stress

Day[2]	Rotational Stress	Melatonin[1] Day[3]	Melatonin[1] Night[4]
1	−	18 ± 2	27 ± 6
	+	26 ± 8	12 ± 3
15	−	19 ± 3	66 ± 17[a]
	+	11 ± 4	172 ± 20[a]

[1] Urinary excretion.
[2] Day of evaluation from beginning of application of rotational stress.
[3] 8 a.m.–8 p.m.
[4] 8 p.m.–8 a.m.
[a] Means marked with the same letter are significantly different, Mann Whitney test,[32] $p < 0.05$.

Each value in the Table is the mean ± SEM obtained using groups of 10 normal mice. Intensity of illumination measured in the cages was 2000 lux; the experiment was performed in April.

TABLE 7. Effects of the Administration of Melatonin on Primary Tumor and Metastasis Weight, as a Function of Application of Rotational Stress and Cycle of Illumination

Cycle of Illumination[1]	Rotational Stress	Melatonin Administration	Primary Tumor Weight (g)	Metastasis Weight (mg)	Tumor-Free Animals
12/12	−	−	0.7 ± 0.1^{a}	$36 \pm 7^{a,b}$	0/10
12/12	+	−	1.4 ± 0.3^{a}	72 ± 11^{a}	0/10
12/12	−	+	0.5 ± 0.2^{b}	$14 \pm 5^{b,c}$	5/10#
12/12	+	+	1.4 ± 0.2^{b}	55 ± 13^{c}	0/10
24/0	−	−	0.8 ± 0.1	58 ± 28	0/10
24/0	+	−	1.1 ± 0.2	28 ± 6	0/10
24/0	−	+	0.9 ± 0.5	34 ± 11	0/10
24/0	+	+	1.3 ± 0.2	42 ± 12	0/10

[1] 12/12 lights on from 8 a.m. to 8 p.m.; 24/0 light continuously on.

Significantly different from 0/10, Fisher exact test,[33] $p < 0.05$.

^{a,b,c} Means marked with the same letter are significantly different, Mann Whitney test,[32] $p < 0.05$.

Each value in the Table is the mean ± SEM obtained using groups of 10 mice implanted subcutaneously with the tumor. Light intensity measured in the cages was 2000 lux; the experiment was performed in April.

ever, endogenous melatonin levels appear to directly correlate with metastasis in mice kept with a 12/12-hr light-dark cycle. Moreover, with the same light cycle the administration of exogenous melatonin exerts a remarkable antitumor effect in nonstressed mice only.

These observations are important for their experimental implications, since change of season and lighting conditions, in addition to housing stress, are influential when the effects of experimental stressors are investigated in laboratory animals. The data presented support the view that both endogenous and exogenous melatonin might be related to tumor progression. However, an unequivocal inhibitory action of melatonin on tumor progression cannot be shown by the data presented. This assumption is in agreement with the results found in similar reports, which also give conflicting evidence on the antitumor action of melatonin in experimental tumor systems.[16] The clinical implications of the results presented are even more difficult to determine. The presently reported antitumor action of melatonin, observed only in limited and defined experimental conditions, however, appears to correlate with the scant utility observed for the administration of melatonin to cancer patients so far reported;[29] more encouraging preliminary results have been obtained with the use of melatonin in combination with interleukin-2.[30,31]

In conclusion, the rôle of the pineal gland and of its indoleamine hormone, melatonin, in relation to cancer progression appears significant, but at the same time as elusive as this gland still continues to be in the more general fields of neuroendocrinology and neuroimmunomodulation. However, the stress sensitivity and the chronobiological aspects illustrated in the present report, together with the data already available, encourage the continuation of research on the basic aspects and on the resulting possible therapeutic applications of melatonin in the field of oncology.

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