Photoperiod and Testosterone Modulate Growth and Melanogenesis of S91 Murine Melanoma

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Abstract: *In vivo* and *in vitro* assays were performed with S91 murine melanoma cells aiming to investigate the effects of testosterone and photoperiod on tumor growth and melanogenesis (tyrosinase activity). *In vivo* assays were performed by inducing melanoma tumors in castrated mice receiving increasing concentrations of testosterone and submitted to varying photoperiod regimens. The results demonstrated that the increase of melanin content was higher in animals submitted to the longest days, thus demonstrating the importance of photoperiod length in melanin synthesis. Increase in tumor growth and protein content was observed in testosterone-treated animals submitted to 12L:12D; in testosterone-treated animals submitted to 4L:20D and 20L:4D tumor growth was significantly smaller. In S91 cultured cells, testosterone increased cell proliferation and reduced tyrosinase activity in a dose-dependent manner. Radioactive binding assays demonstrated that the hormone was acting through low affinity testosterone receptors, since the presence of aromatase inhibitor did not affect the binding assay in a statistically significant way, and all the *in vitro* experiments were performed in the presence of the inhibitor. Our *in vivo* data added to the *in vitro* results corroborate the hypothesis that S91 melanoma cells directly respond to testosterone and that this effect is modulated by light.

Key Words: Testosterone, light, S-91 melanoma cells, tumor growth, tyrosinase activity, DBA/2J mice.

INTRODUCTION

The mammalian skin and fur coloration is given by integumental pigments mainly melanins which are deposited in melanosomes within specialized dermal cells, the melanocytes. Melanin is a heteropolymer widely distributed in plants and animals. The major enzymatic pathway for melanin synthesis (melanogenesis) has also been conserved and involves a series of steps catalyzed by tyrosinase and tyrosinase-associated proteins, TRP1 and TRP2 [1].

Melatonin, the pineal hormone, known as the darkness messenger [2], has been reported to modulate melanogenesis. Amelanotic S91 melanoma cells exhibited diminished melanogenesis after melatonin treatment [3], whereas melanotic S91 cells [4,5] and RPMI 3460 hamster melanoma cells [6] responded to the indoleamine with increase of tyrosinase activity. In the same rodents, as well as in human melanoma [7] melatonin was able to decrease cell proliferation. The indoleamine was also effective to reduce melanoma growth *in vivo* in animal models [3,5,8] and in humans. [9,10] The length of the photophase has been reported to affect tumor growth [11], most probably due to its influence on melatonin secretion. *In vivo* and *in vitro* proliferation assays of various tumor types have demonstrated that short photoperiods and/or melatonin have an inhibitory effect. [7,8,12-15 among others]

It is well known that photoperiod affects cell cycle. In the epidermis of rodents maintained 12L:12D (lights on at 6AM), the peak of S phase occurs at 3h30min, and of M phase happens at 8h30min [16-19]. Interestingly, in humans, diurnal mammalians, in opposition to the rodents (nocturnal animals), the peak of the S phase is seen at 15h30min and of the M phase at 23h30min [20,21].

The stimulatory effect of androgens on tumor growth has been demonstrated in a variety of cancers, but little is known about testosterone actions on mammalian melanoma. Exogenous testosterone not only increases the incidence of tumors such as thyroid tumor and carcinoma [22], but also stimulates melanoma growth and metastasis [23-26]. Testosterone may be converted, in the target cells, to dihydrotestosterone and estradiol. Steroid receptors are hormone-dependent transcription factors which regulate a variety of genes affecting cell proliferation, differentiation and development [27].

Here we demonstrate that photoperiod and testosterone affect growth, melanin synthesis and protein content in S91 melanoma tumor inoculated in DBA/2J mice, and that these effects may be directly exerted by the hormone since it increases cell proliferation and decreases tyrosinase activity of S91 cells in culture, through specific testosterone binding sites.

MATERIAL AND METHODS

Cell Culture

S91 murine melanoma cells were maintained as monolayer cultures in Ham's F10 medium (Cultilab, Campinas, Brazil), supplemented with 5% fetal calf serum (Cultilab), in the absence of antibiotics, at 37°C. To minimize the presence of steroids in the medium, the serum was previously incubated at 4°C with activated charcoal (25g/L) for 1h, and centrifuged at 16,000g for 15min.

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1573-4064/08 $55.00+.00

After passage 12, the cells ($2x10^6$ cells/0.1ml of PBS) were reinoculated in DBA/2J mice to obtain primary tumors or for the *in vivo* assays.

In Vivo Assays

Isogenic male mice (DBA/2J) were kept in individual cages with water and food *ad libitum*, at 23°C in 12L:12D light:dark cycle (lightson at 8AM). Animals 30-40 days old were anesthetized (20mg/ml xylazine and 100mg/ml ketamine, 5:1, 60ul for each 20g of body mass in 0.1ml PBS) and the testicles and *vas deferens* surgically removed. Control sham operated animals were submitted to the same procedure with no extirpation.

Immediately after the surgery, the animals were grouped in sham operated, castrated and castrated with exogenous testosterone, and submitted to 12L:12D, 4L:20D and 20D:4L. After 7 days each animal was inoculated with $2x10^6$ cells/ 0.1ml of PBS. Testosterone (250mg/ml, Organon, Sao Paulo, Brazil) was diluted in peanut oil and subcutaneously injected every 48 hours at concentrations of 0.1, 0.3 and 1.0ng/g of body mass (between 2 and 4PM).

Tumor Growth

After further 7 days, the 3 dimensions of the tumors were measured every day, during 21 days, using the formula $(\pi/6)$xABC (A, B and C being the 3 tumor dimensions) to calculate the volume [8]. At the end, the animals were sacrificed and each tumor excised, weighed and measured.

Protein Quantification

The tumors were solubilized in 1N NaOH overnight at 4°C in order to quantify total proteins using Pierce kit, according to the manufacturer's recommendations. Briefly, triplicates of 5ul of the standard solution provided in the kit or of the solubilized tumors were transferred to an ELISA plate, received 200ul of the kit reagent, and the absorbance was read at 562nm. The experimental values obtained from the standard curve were expressed in ug protein/g of tumor or in percentage of control.

Melanin Quantification

After removal of the aliquot for protein quantification, the sample was centrifuged (1000xg, 15min) and the supernatant used to determine melanin content. The absorbance of 500ul aliquots of the solubilized tumors and of serial dilutions of synthetic melanin (Sigma) was obtained at 400nm, and the experimental values expressed in ug melanin/g of tumor, or in percentage of control.

In Vitro Assays

S91 cells (passages 3 to 10) were seeded ($2x10^5$ cells/ 25cm2 flask) in Ham's F10 medium supplemented with 5% charcoal inactivated fetal calf serum. After 24h, the medium was replaced by fresh medium containing 10^{-9} to 10^{-7}M testosterone (Sigma) in the presence of the aromatase inhibitor aminoglutethimide (10^{-6}M, Sigma). The assays lasted 48, 72 or 96h, with medium changes every 48h. Control cells were grown in the presence of 10^{-6}M aminoglutethimide.

Cell Proliferation

The cells were harvested with Tyrode/EDTA solution, centrifuged, resuspended in PBS and counted in a hemocytometer. The cell number was expressed as percentage of control.

Tyrosinase Activity

The tyrosinase activity was determined through the quantification of ^3H_2O produced along the first steps of melanogenesis and released to the medium [28 modified according to 29]. Twenty-four hours before the end of the experiment, 1uCi/ml of ^{3}H-tyrosine (Amersham, Buckinghamshire, England) was added to the medium. At the end, 200ul of medium were collected and added to 800ul of 10% activated charcoal in 10% trichloroacetic acid. The mixture was vortexed, incubated at 4°C for 30min and centrifuged at 3,000g for 10min. The samples (500ul) were added to 5ml of scintillation liquid and the scintillation counted in a Packard scintillation counter. The data were converted in cpm/10^6cells and expressed as percentage of control.

1,2,6,7-[^{3}H]-Testosterone Competition Binding Assays

S91 cells were seeded ($1.5x10^5$ cells/well) in multi-well plates, in triplicates for each treatment. Twenty-four hours after seeding, the culture medium was replaced by fresh medium containing 10^{-8}M 1,2,6,7-[^{3}H]-testosterone in the absence (total binding) or in the presence (non-specific biding) of increasing concentration of cold testosterone (10^{-12} to 10^{-4}M testosterone) for 2h at 37°C, in the presence or absence of 10^{-4}M aminoglutethimide, an aromatase inhibitor. Aromatase is the key enzyme for the conversion of testosterone into estradiol (Fig. **1**), and it was blocked in our assays to assure that any response to the hormone would be due to the androgen own action. After incubation, the solutions were discarded, and the cells rinsed with PBS (6 x 5 ml). The cells were then lysed with PBS containing 0.2% bovine serum albumin and 0.5% Triton X-100, and 200ul duplicates were added to 5ml of scintillation fluid and the scintillation counted in a Packard scintillation counter.

Statistical Analysis

The *in vivo* data were analyzed with two-way ANOVA followed by Student-Newman-Keuls, and the *in vitro* were analyzed with one-way ANOVA followed by Student-Newman-Keuls. The differences were considered significant for $p<0.05$.

RESULTS

We observed a slower tumor growth in intact animals maintained in 4L:20D as compared to the other two photoperiod regimens (Table **1**). Removal of the testes did not affect the rate of tumor growth in animals submitted to 12L:12D or 20L:4D, but significantly increased the rate in animals under 4L:20D, restoring the values seen in the other two regimens (Table **1**). In the groups in which testosterone was replaced, there was a significant increase of tumor volume since the 10th day of experiment (Fig. **2**) and of the final weight (Table **1**) in the animals treated with 1.0ng hormone/g b.w. in 12L:12D photoperiod regimen.

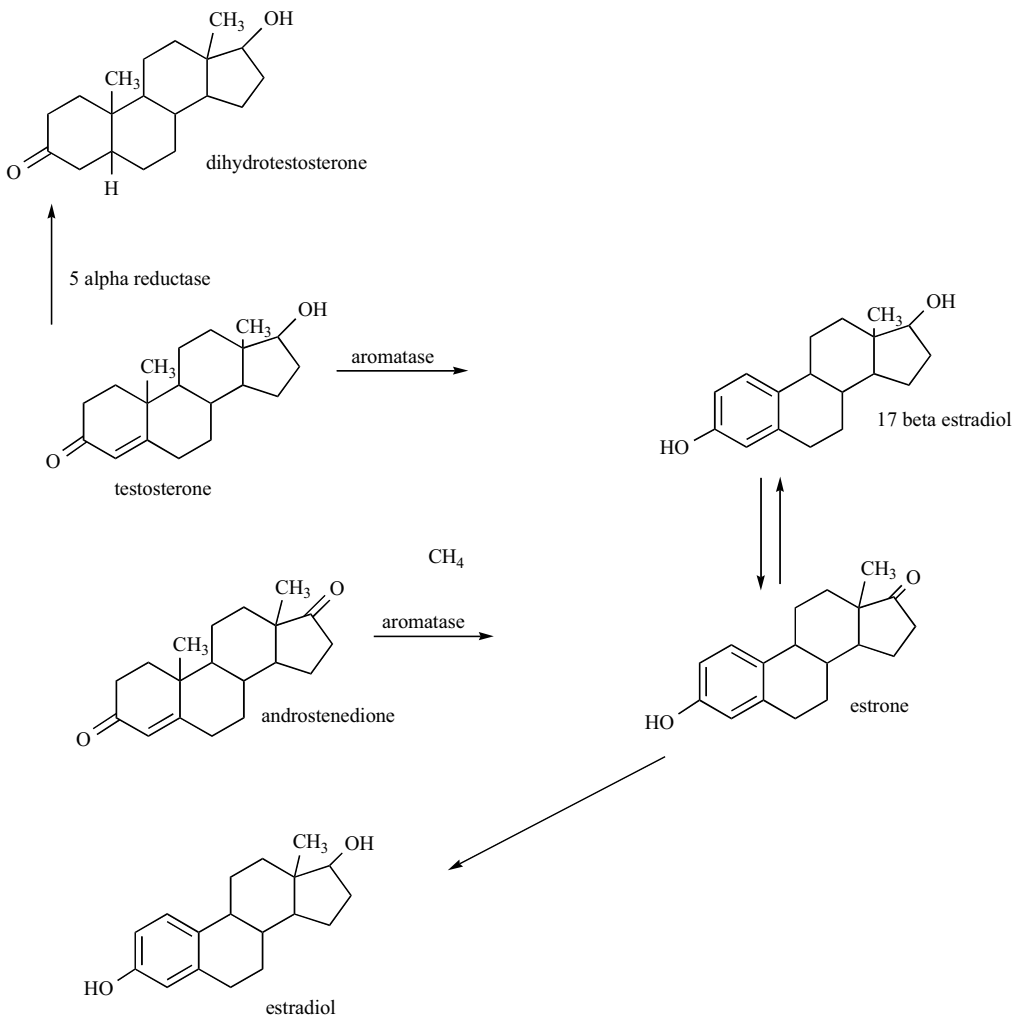

Fig. (1). Conversion of testosterone to two other possible steroid agonists, dihydrotestosterone and estradiol. These reactions may occur within target cells.

Tumor protein content was higher in intact and in castrated animals submitted to 12L:12D, as compared to 4L:20D and 20L:4D, regardless the hormone treatment (Table **2**).

Tumor melanin content was significantly higher in intact and in castrated animals maintained in 20L:4D, as compared to the other two regimens, regardless of the testosterone treatment (Table **3**).

We then decided to investigate wether S91 tumoral cells possessed testosterone receptors, and which would be their response to *in vitro* application of the hormone. Competition binding assays demonstrated the presence of testosterone binding sites, showing 63% displacement in the presence of the cold ligand ($Ki=10^{-5}M$). When $10^{-6}M$ aminoglutethimide, an inhibitor of aromatase, the enzyme that converts testosterone in estradiol, was added to the incubation, 93% of displacement was obtained ($Ki=5x10^{-7}M$, Fig. **3**).

Table 1. Mean Weight (g), + S.E. (n=8-9) of S91 Melanoma Tumors 21 Days After Inoculation in DBA/2J Mice

Light Regimen	Sham-Operated	Diluent Control	0.1ng Testosterone/g Body Weight	0.3ng Testosterone/g Body Weight	1.0ng Testosterone/g Body Weight
4L:20D	0.076±0.06*	0.130±0.01	0.119±0.02	0.146±0.02	0.121±0.03
12L:12D	0.157±0.02	0.122±0.02	0.141±0.03	0.168±0.02	0.196±0.03*
20L:4D	0.131±0.02	0.104±0.02	0.094±0.02	0.132±0.02	0.155±0.02

*significantly different ($p<0.05$) as compared to same treatment in other photoperiods and to other treatments in the same photoperiod.

Cultured S91 cells were treated with 10^{-9} to 10^{-7} M testosterone for 48 to 96h, in the presence of 10^{-6}M aminoglutethimide. The results demonstrated that testosterone binding sites are functional, since cells exhibited a dose-dependent increase of proliferation after 72 and 96h in the presence of 10^{-8} and 10^{-7}M testosterone (Table **4**).

Treatment with 10^{-8} and 10^{-7}M testosterone also induced a significant reduction of tyrosinase activity, after 72 and 96h (Table **4**).

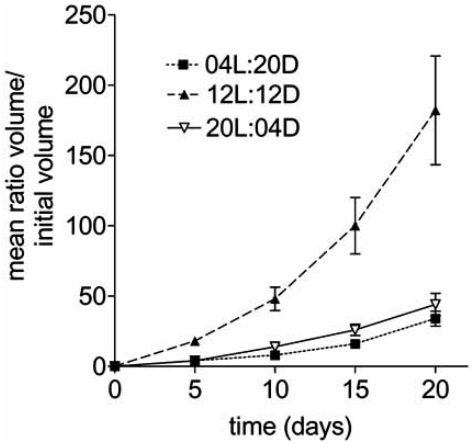

Fig. (2). Effect of exogenous testosterone (1ng/g of body weight) on the growth of S91 Cloudman melanoma in DBA/2J mice maintained in various photoperiod regimens. Each point is the mean ratio (n=9), ±S.E., of volume growth relative to the 1st day of hormone treatment.

DISCUSSION

The mouse DBA/2J is an excellent model to study melanoma *in vivo*. The facility of inoculation, success of tumorigenesis and the fast tumor growth allow the easy investigation of drug effects and therapeutics.

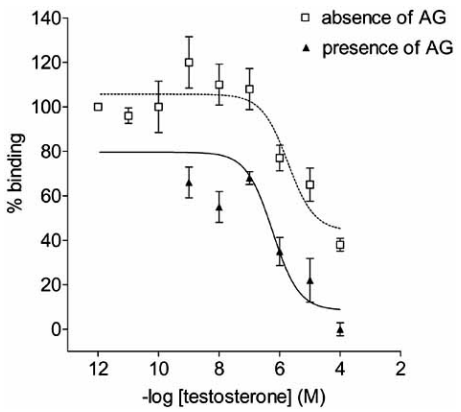

Fig. (3). Competition binding assays of 10^{-8}M [1,2,6,7-^{3}H]-testosterone in the presence and absence of 10^{-4}M aminoglutethimide (AG). Each point is the mean (n=6 wells), ±S.E., percentage of radioactive ligand binding in the presence of increasing concentrations of cold ligand (10^{-12} to 10^{-4}M), relative to the total binding in the absence of cold testosterone.

Our results indicated the possibility that the length of the light phase affects the growth of melanoma, since intact animals maintained in short photoperiod (4L:20D) exhibited a slower tumor growth than those kept in the other two photoperiods. Although the literature report the influence of light on proliferating activity of rodent epidermis [16-19], one has also to consider possible inhibition of protein synthesis and angiogenesis, leading to reduced tumor growth.

In short photoperiods, the animals present a higher melatonin plasma level [30]. This would inhibit tumor growth as demonstrated by numerous reports of melatonin inhibitory effect on tumor progression [3,5,7-10,12-15]. Castration in the absence or presence of exogenous testosterone, however,

Table 2. Mean Protein Content (mg/g Tumor), ± S.E. (n=8-9) of S91 Melanoma Tumors 21 Days After Inoculation in DBA/2J Mice

Light Regimen	Sham-Operated	Diluent Control	0.1ng Testosterone/g Body Weight	0.3ng Testosterone/g body Weight	1.0ng Testosterone/g body Weight Testosterone
4L:20D	12.38±1.42	10.45±1.33	8.87±0.54	9.71±0.93	22.58±13.21
12L:12D	36.67±3.62*	38.16±5.59*	91.95±25.4*	55.74±8.39*	45.62±8.65*
20L:4D	5.24±0.80	5.89±0.90	6.92±0.47	6.47±0.54	8.81±2.38

*significantly different ($p<0.05$) as compared to same treatment in other photoperiods.

Table 3. Mean Melanin Content (ug/g Tumor), ± S.E. (n=8-9) of S91 Melanoma Tumors 21 Days After Inoculation in DBA/2J Mice

Light Regimen	Sham-Operated	Diluent Control	0.1ng Testosterone/g Body Weight	0.3ng Testosterone/g Body Weight	1.0ng Testosterone/g Body Weight
4L:20D	173.4±18.2	208.5±34.0	155.5±32.0	123.6±25.2	184.5±45.5
12L:12D	212.7±37.1	254.5±43.0	330.5±80.6	153.7±14.1	199.5±23.1
20L:4D	356.2±32.9*	716.1±407*	413.2±82.8*	659.7±317.7*	1087.9±540*

*significantly different ($p<0.05$) as compared to same treatment in other photoperiods.

Table 4. Proliferation of Cultured S91 Melanoma Cells (n=6-7 flasks), in the Presence of the Aromatase Inhibitor, Aminoglutethimide (10^{-6}M), in the Presence or Absence of Testosterone (10^{-9} to 10^{-7}M)

Treatment	48 Hours		72 Hours		96 Hours	
	Cell Number (x 10^6 Cells)	Percentage of Control	Cell Number (x 10^6 Cells)	Percentage of Control	Cell Number (x 10^6 Cells)	Percentage of Control
control	0.41±0.02	100.0±5.5	0.90±0.04	100.0±4.4	1.24±0.02	100.0±1.3
10^{-9}M testosterone	0.46±0.02	111.9±5.3	0.90±0.04	98.8±5.0	1.27±0.03	101.9±2.8
10^{-8}M testosterone	0.41±0.02	100.2±4.7	1.00±0.03*	117.0±2.9*	1.63±0.02*	130.8±1.9*
10^{-7}M testosterone	0.43±0.03	103.9±6.3	1.26±0.03*	140.0±3.2*	1.69±0.04*	135.5±3.6*

*significantly different ($p<0.05$) as compared to the respective time point control

restored the growth rate to a similar range observed in all three photoperiod regimens.

The robust increase of volume, weight and protein content in tumors from testosterone-treated animals in 12L:12D photoperiod was probably due to the anabolic effect of the hormone directly on cell metabolism, what led to an increase in tumor growth. As these effects were not seen in the other photoperiod regimens, it is certain that there was an interaction between light and hormone treatments.

Wardlaw [31] has demonstrated that castration induces a rise in hypothalamic content of the melanogenic hormone α-MSH. As keratinocytes, melanocytes and Langerhans cells are cutaneous sources for α-MSH, one could expect higher MSH content, thus leading to an increase of melanin content in the castrated animals. However, tumor melanin content was unaffected by testosterone treatment, indicating that the hormone did not regulate melanogenesis *in vivo*, or if it did, the result was masked by the photoperiod. In all animal groups, long photoperiod significantly enhanced tumor melanin content, probably due to an increase in cell membrane availability of MSH receptors by UV components of light [32].

Testosterone effects on S91 cells become evident with the *in vitro* experiments, demonstrating that the binding sites seen in the competition assay are probably functional. However, the affinity was low, the Ki being in the micromolar range, what might be due to variant receptors resulting from the malignancy transformation. The hormone stimulated cell proliferation, in a dose-dependent manner, after 72 and 96h treatment with the concentrations of 10^{-8} and 10^{-7}M. Although the majority of studies in human melanoma reports growth increase by testosterone *in vivo* and *in vitro* [23-25]. Kanda and Watanabe (2001) showed in an elegant work that dihydrotestosterone, progesterone and beta-estradiol inhibit proliferation of human metastatic melanoma cells, by inhibitin interleukin-8 production [33]. This difference might be due to variations among the several melanoma cell lines and/or to differences between metastatic and non-metastatic cell lines.

We have also demonstrated a robust inhibition of melanogenesis in S91 cells in culture after testosterone treatment. Abdel-Malek and collaborators [34] have demonstrated that when MSH stimulates tyrosinase activity in S91 cells in culture, cell cycle is arrested in phase G2, thus inhibiting proliferation. If S91 cell differentiation (increase of melanin production) is associated with cell cycle blockade, then testosterone-induced stimulation of proliferation as seen here would probably lead to an inhibition of melanogenesis. This inhibitory effect of testosterone was not, however, evident in the *in vivo* assays, what could have been due to the more efficient stimulatory effect of light. In fact, the longest the exposure of the animals to light, higher was the tumor melanin content.

One has to bear in mind the possibility that testosterone may be converted to dihydrotestosterone (DHT) or to estra-

Table 5. Tyrosinase Activity of Cultured S91 Melanoma Cells (n=6-7 flasks), in the Presence of the Aromatase Inhibitor, Aminoglutethimide (10^{-6}M), in the Presence or Absence of Testosterone (10^{-9} to 10^{-7}M)

Treatment	48 Hours		72 Hours		96 Hours	
	cpm/10^6 Cells	Percentage of Control	cpm/10^6 Cells	Percentage of Control	cpm/10^6 Cells	Percentage of Control
control	6954±379	100.0±5.5	2977±132	100.0±4.4	2114±41	100.0±1.9
10^{-9}M testosterone	6527±304	93.9±4.4	2899±157	97.4±5.3	2034±64	96.2±3.0
10^{-8}M testosterone	7849±301	112.9±4.3	2360±70*	79.3±2.3*	1663±28*	78.7±1.3*
10^{-7}M testosterone	8218±494	118.2±7.1	2039±44*	68.5±1.5*	1594±43*	75.4±2.0*

*significantly different ($p<0.05$) as compared to the respective time point control.

diol (Fig. **1**), and therefore, the effects here described could be due to testosterone, DHT or estradiol. Although the enzymes 5-α-reductase and aromatase are usually expressed in typical testosterone targets, aromatase [35], and testosterone and DHT receptors, although abnormal [24], have been characterized in human melanoma cell lines. The conversion of testosterone to estradiol may be discarded, since all the *in vitro* assays were performed in the presence of the aromatase inhibitor, aminoglutethimide.

In summary, our results indicate that light exerts a pronounced regulatory effect on tumor growth, and that a possible interaction between light and androgen actions may occur. Our results may also explain gender differences found for melanoma prognostics [23]. In cultured cells, testosterone increased cell proliferation and inhibited melanin synthesis, even in the presence of the aromatase inhibitor aminoglutethimide, thus demonstrating a direct effect of testosterone and the functionality of testosterone binding sites.

In fact, melanoma cells have the extraordinary ability to synthesize hormones, growth factors and their receptors, which support and accelerate tumor development and progression [36]. On the other hand, these same mechanisms may be used as targets in therapeutic strategies, for example providing the patient with hormone antagonists. However, one should bear in mind the genetic heterogeneity of melanomas, as well as the variability of the pathology, particularly regarding tumor growth (vertical or horizontal), ulceration degree of the primary tumor and metastasis [36].

If we take in account that a great majority of receptors and signaling factors are under the control of hormones and circadian rhythms, the present work contributes with important clues for the use of light, testosterone antagonists and/or melatonin for melanoma therapeutics.

ACKNOWLEDGEMENT

Supported by FAPESP and CNPq. We are thankful to Telma Pazini and Márcio de Patto Lima for the excellent technical assistance.

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Received: 05 May, 2007 **Revised: 30 May, 2007** **Accepted: 30 May, 2007**