Experimental Gerontology 46 (2011) 18–22

Contents lists available at ScienceDirect

Experimental Gerontology

journal homepage: www.elsevier.com/locate/expgero

Extended exposure to dietary melatonin reduces tumor number and size in aged male mice

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ARTICLE INFO

Article history:
Received 21 July 2010
Received in revised form 31 August 2010
Accepted 2 September 2010
Available online 16 September 2010

Section Editor: R. Effros

Keywords:
Aging
Melatonin
Cancer
Tumors
Lcn2

ABSTRACT

Several sets of male mice were given dietary melatonin over a series of experiments performed during a nine year period. Overall, melatonin-supplemented mice aged ≥26 months at sacrifice had significantly fewer tumors with lower severity than similarly aged control animals. The studies were originally designed to explore the potential of this agent for reducing the rate of onset of some genetic indices of brain aging. When these animals were sacrificed they were routinely examined for overt evidence of tumors and when these were found, a note was made of their occurrence, and of their size. Tumors are commonly found during senescence of several strains of mice. Since tumorigenesis was not the original intent of the study, these observations were recorded but not pursued in greater detail. In this report, these data have now been collated and summarized. This analysis has the disadvantage that tumor origin and morphology were not recorded. However, the study also has the advantage of being conducted over an extended period of time with many groups of animals. In consequence, many extraneous factors, which could be potential confounders, such as seasonal or dietary variations, are unlikely to have interfered with the analysis. The use of more than one mouse strain strengthens the possibility that the findings may have general relevance. Both aged and young animals were included in the original experiments but the tumor incidence in animals younger than 25 months was very low.

1. Introduction

In addition to the well-known relation between darkness and melatonin production, there are many previous reports suggesting that melatonin may be oncostatic or cancer-preventive (Srinivasan et al., 2008). Cancer incidence greatly increases in older animals at the same time that the circadian pulsatile production of melatonin in the pineal gland declines; there is increasing mechanistic evidence relating these two phenomena (Jung-Hynes et al., 2010). Human epidemiological studies are necessarily focused on specific cancer types, notably breast cancer (Davis et al., 2001; Viswanathan and Schernhammer, 2009). Studies involving blindness suggest a lower incidence of cancer in those lacking sight (Feychting et al., 1998). This correlation has been further refined and the degree of protection shown proportional to the extent of visual impairment (Verkasalo et al., 1999; Pukkala et al., 2006); this observation has been attributed to the absence of light-induced depression of circulating melatonin. Melatonin levels are often inferred rather than assayed in such studies, which may also involve other endocrine factors influenced by the absence of normal circadian cycles. Other human studies have been carried out on populations such as night workers exposed to abnormal circadian rhythms (Schernhammer and Schulmeister, 2007), and the IARC unit of the World Health Organization has classified circadian rhythm-disrupting shift-work as potentially carcinogenic (Kantermann and Roenneberg, 2009). Suggestive results reporting protective effects of melatonin against the spread of pre-existing cancer in humans (Lissoni et al., 2003; Lissoni, 2007; Mills et al., 2005) are reinforced by several animal studies in which melatonin treatment is reported to give a degree of protection against cancer implants and carcinogens (Otálora et al., 2008; Tanaka et al., 2009). Further confirmation of this concept comes from evidence that hormone-dependent cancers are less common in those who live north of the Arctic Circle and thus experience a light deficit during extended periods of winter darkness. This is unlikely to be compensated for by extended light during summer months due to protective measures employed to permit sleep (Erren and Piekarski, 1999). Several reports concerning the effect of administered melatonin on the onset and progression of tumors in experimental animals reinforce human epidemiological and clinical studies. Using carcinogens and tumorigenic cell lines, melatonin treatment has been reported to give a degree of protection against both the onset and rate of progression of tumors (Otálora et al., 2008; Tanaka et al., 2009; Vesnushkin et al., 2006).

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doi:10.1016/j.exger.2010.09.004

2. Methods

The total number of animals used in these studies was 43 melatonin-treated mice and 52 controls over 20 months old for the cancer analysis; and 74 and 77, respectively, older than 16 months for the death-rate analysis. All animals were males. The corresponding values for mice aged under 7 months of age were 57 and 58 respectively. Each individual study comprised sets of between 7 and 10 mice. The period of treatment with dietary melatonin was always between 9 and 14 weeks immediately prior to sacrifice, while the melatonin content of the experimental diet was 40 ppm. The age at sacrifice of adult mice was 4.5 to 7 months while that of senescent mice was 26–27 months. Some groups included only in the death-rate analysis were sacrificed earlier, at 16.7 and 21.7 months. The strains of mouse used were B6C3F1 (C57BL/6J F×C3H M) and CB6F1 (BALB/cJ F×C57BL/6J M). All mice were from Harlan Labs (Indianapolis, IN). Mice were housed two to four per cage and maintained on a 12 h light/dark cycle in a temperature controlled (22±1 °C) room. Food and water were provided ad libitum. Young and old control animals were fed a pelleted minimal basal diet (AIN-93M, Dyets #100900, Dyets Inc., Bethlehem, PA) consisting of 10% sucrose and 14% casein (w/w) as well as a minimal salt and vitamin mix. This basal diet was supplemented with 40 ppm (w/w) melatonin (Sigma-Aldrich, St. Louis, MO) in separate groups of mice. The dosage level was confirmed by independent testing (Irvine Nutri-Chemical Laboratories, Irvine, CA). Tissue and serum levels of melatonin, but not of other hormones, were measured in one group of mice (Lahiri et al., 2004). Separately caged sentinel animals were maintained in the same room to monitor for the occurrence of infectious disease; no disease was detected during the treatment periods. All experiments were approved by the Institutional Animal Care and Use Committee at the University of California, Irvine, and conformed to the National Institute of Health guide for the care and use of laboratory animals.

Tumor severity was scored to evaluate the size and multiplicity of tumors occurring in each animal. A score of 1 was assigned to a single tumor recorded as *small*, a score of 2 to a single (unremarkable) tumor, and a score of 3 was assigned if an animal had developed multiple tumors or if a single tumor was recorded as *large*. A severity index for each treatment was computed by summing the severity scores and dividing by the number of animals in the treatment group.

3. Results

As expected, a large increase of observable tumors occurred with age. However, an effect of the melatonin dosing was apparent in aged mice where the tumor incidence was markedly reduced (Fig. 1). Furthermore, the severity of any visible tumors was lessened in melatonin-treated animals (Fig. 2). Melatonin's protective effect was apparent only in older animals (Fig. 3); in younger mice ≤25 months of age, melatonin treatment did not significantly affect cancer incidence.

While tumor size may be considered rather subjective, all observations over the period of the work were performed by a single person. This would tend to minimize variation. The mice were all originally used to study the effect of melatonin upon cortical immune function in a series of experiments ranging over six years. Non-neurological pathological changes were only noted in order to allow systematic recording of overall health. Thus, the objectivity of these examinations is enhanced by their original documentation being motivated merely by thoroughness rather than for hypothesis development.

The reduced incidence of visible tumors in melatonin-treated mice raised the issue whether normal mortality taking place during the long experimental retention periods could be affected by this treatment. After 16 months of age the overall spontaneous death rate was markedly reduced in mice receiving melatonin (Fig. 4). Over

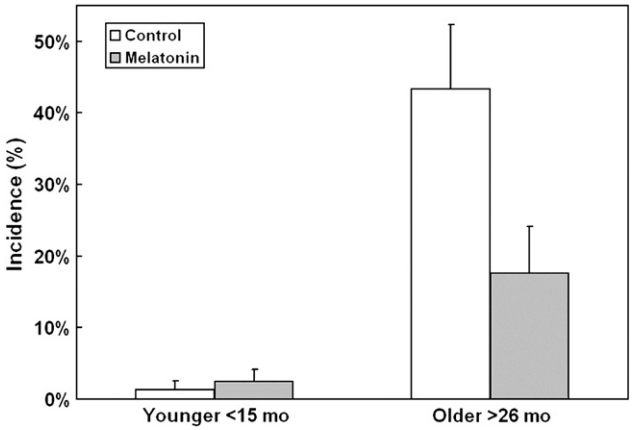

Fig. 1. Effect of melatonin supplementation on tumor incidence in mice of two different age ranges. Mice aged less than 15 months (younger) or over 26 months (older) at sacrifice were fed chemically purified diets either without (open bars) or with 40 ppm melatonin (shaded bars). Y-axis displays cancer incidence as percentage of animals in each group with observable cancers at necropsy.

91% of melatonin-treated mice survived to 26.5 months age, while only 82% of untreated mice attained this age. Thus treatment with melatonin led to the overall death rate in the most aged mice being halved from 18% to 9%.

4. Discussion

The results presented here confirm that melatonin can reduce the risk of onset and delay the progression of tumors. Our extensive prior work on the effects of melatonin on the brain, suggests some of the mechanisms underlying its oncostatic effects. The levels of melatonin encountered within tissues are sufficiently low that a direct anti-oxidant effect is unlikely to play a significant role (Lahiri et al., 2004). However, melatonin is able to reverse some key genetic changes associated with senescence. Thus melatonin can specifically prevent the age-related increased expression of genes relating to inflammation (Sharman et al., 2002, 2004). This increase is not provoked by extrinsic inflammatory stimuli but reflects a change with age, in basal gene expression. In consequence, the immune response to exogenous stimuli is also altered with age (Sharman et al., 2001). Thus the protective effect of melatonin may be by way of reversal of age-related changes in gene expression (Table 1) (Perreau et al., 2007; Sharman et al., 2007). These findings are likely to be relevant to human populations since age-related changes of gene expression

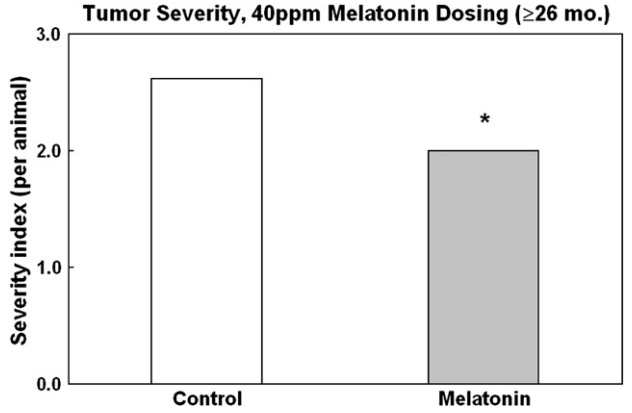

Fig. 2. Effect of melatonin supplementation on tumor severity in mice aged over 26 months at sacrifice. Mice were fed chemically purified diets either without (open bar) or with 40 ppm melatonin (shaded bar). Y-axis displays cancer disease severity index (see text). * Significantly different from Control by Mann-Whitney U-test.

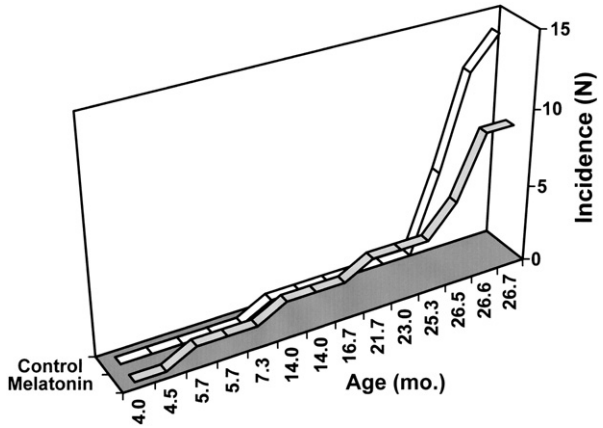

Fig. 3. Cumulative effect of melatonin supplementation on tumor incidence in mice of increasing age. Mice were fed chemically purified diets either without (open segments) or with 40 ppm melatonin (shaded segments). Y-axis displays cancer incidence as number of animals in each group with observable cancers at necropsy.

occurring in mice have close counterparts in humans (Sharman et al., 2005). Since age is a very important factor in determining cancer risk, restoration of a more youthful profile of mRNA production may reduce the likelihood of an aged organism from developing cancer.

Our results indicating a reduction in spontaneous tumor incidence by melatonin are supported by studies of chemically-induced or implanted cancers. Melatonin (5 mg/kg bw, *i.p.*) prevented N-nitrosodiethylamine/CCl_4-induced hepatocarcinoma in rats (Subramanian et al., 2007). Growth of hepatoma tumors implanted in mice is inhibited by 200 μg/day of melatonin administered either *s.c.* or in the diet over a 32 day period (Blask et al., 1999). In a model of pancreatic cancer induced in Syrian hamsters by N-nitrosobis(2-oxopropyl)amine, melatonin (20 μg/ml supplied in tap water for 12 weeks) reduced the number of tumors and presence of differentiated adenomacarcinoma tissue (Ruiz-Rabelo et al., 2007).

There is evidence that melatonin treatment reduces tumor incidence in female mice as well as males. In particular, reductions in mammary cancers by melatonin treatment are consonant with the generally inhibitory seasonality input it provides to the female reproductive system of many mammalian species, and with its antiestrogenic and aromatase-inhibiting actions (Sanchez-Barcelo et al., 2005). Melatonin has been shown to reduce tumor incidence and/

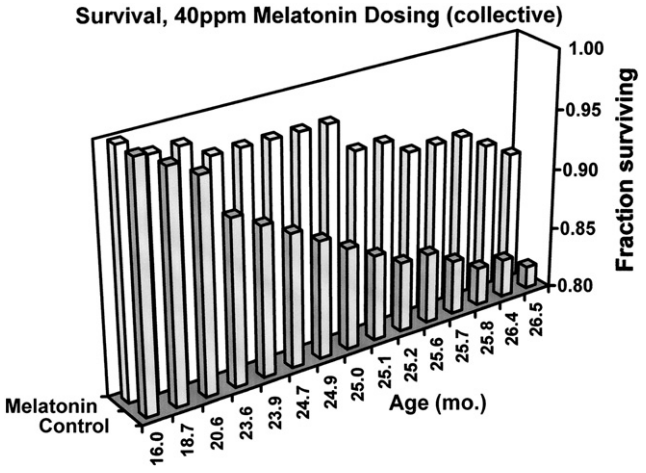

Fig. 4. Survival fraction with age, of mice receiving dietary melatonin after 16 months of age. Mice were fed chemically purified diets either without (open segments, N = 77) or with 40 ppm melatonin (shaded segments, N = 74). Y-axis displays proportion of surviving mice in either group, calculated from records of spontaneous deaths occurring prior to planned sacrifice dates.

Table 1
Comparison between cancer-associated, age-related and melatonin-induced changes in gene expression. Age and melatonin data from Sharman et al. (2007). GEO = Gene Expression Omnibus website http://www.ncbi.nlm.nih.gov/geo/.

Gene	Age-induced change	Melatonin-induced change	Cancer-associated change (trend)	Reference to cancer-associated change
LCN2	↑ 4x	↓	↑ multiple human cancers	Zhang et al. (2007)
EST AV057155	↑ 24x	↓	↑ Liver cancer in Mdr2-ko mice	GEO link, from Katzenellenbogen et al. (2006)
Igk-V1	↑ 8x	↓	↑ Liver cancer in Mdr2-ko mice	GEO link, from Katzenellenbogen et al. (2006)
Igk-V1	↑ 5x	↓	↑ Immune-resistant cancer cell line	GEO link, from Lin et al. (2007)
Igh-6	↑ 2x	↓	↑ Immune-resistant cancer cell line	GEO link, from Lin et al. (2007)
Lrg1	↑ 2x	↓	↑ mouse pancreatic cancer (protein)	Kakisaka et al. (2007)
Igj	↑ 2x	↓	↑ High-relapse lymphoblastic leukemia	Hoffmann et al. (2008)
Gvin1	↑ 1.3x	↓	— Immune-resistant cancer cell line	GEO link, from Lin et al. (2007)
TiPARP	↓ 0.7x	↑	↑ Immune-resistant cancer cell line	GEO link, from Lin et al. (2007)
			↑ Head/neck squamous cell carcinoma	Redon et al. (2002), Katoh and Katoh (2003)
Agxt2l1	↓ 0.7x	↑	↓ Human squamous cell carcinoma	GEO link, from Nindl et al. (2006)

or latency in a number of mammary cancer-prone mouse models, including virus-associated (Subramanian and Kothari, 1991), and the SHR strain (Anisimov et al., 2003).

A potential gene of especial relevance for carcinogenesis is that for the glycoprotein lipocalin 2 (lcn2, ngal). Substantially increased levels of lipocalin 2 are associated with a wide number of human cancers, including esophageal (Zhang et al., 2009), rectal, pancreatic, ovarian (Zhang et al., 2007), thyroid and breast cancers (Yang et al., 2009). High levels of the *lcn2* gene are also expressed in human hepatocellular carcinoma tissue (Patil et al., 2005). *Lcn2* gene expression is substantially increased in old mouse brains (Perreau et al., 2007) and in liver (unpublished observation). In brain, dietary melatonin supplementation reduces this expression level to that found in young animals (Perreau et al., 2007). In a breast cancer cell line, increased *lcn2* expression is associated with a more aggressive and invasive morphology, and repression of *lcn2* expression with siRNA reduced cell migration and engendered a more clustered morphology (Yang and Moses, 2009). There is clearly a mechanistic involvement of lcn2 in at least one type of cancer and high levels of the hepatic *lcn2* gene are associated with hepatocellular carcinomas. Therefore any reduction of gene expression of this gene in liver paralleling that found in brain following administration of melatonin, may account for the reduced numbers of liver cancers observed in the melatonin-treated animals. It should be kept in mind, however, that even though elevated lcn2 levels are associated with all the organ cancers listed above—particularly at early stages of progression—heightened levels are not always associated with more advanced disease, and in some cases may subdue disease aggressiveness (Yang and Moses, 2009). Although our studies were carried out with male animals, the link between elevated Lcn2 and breast cancer in females is particularly strong (Yang et al., 2009). This link suggests that inclusion of female animals in future studies should be both important and fruitful.

Some genes become over-represented as a consequence of the aneuploidy typical of many cancers. The expression of one such gene, TiPARP, is increased about 10-fold in human head and neck cancer (Redon et al., 2002; Katoh and Katoh, 2003), and is also increased in a cancer cell line (Lin et al., 2007). TiPARP (Parp7) is a member of the poly-ADP-ribose polymerase (PARP) protein family, other members of which are intimately involved with DNA damage sensing and repair, cell death, and immunity—all functions that become dysregulated in cancers. In contrast to the overexpression of *TiPARP* in human head and neck cancer, its transcription is decreased by age in non-cancerous tissue, but restored by melatonin (Table 1).

Very few tumors were observed in animals sacrificed prior to 15 months of age (1 in 78 control animals and 2 in 82 melatonin-fed). At such low levels of tumor incidence it was not possible to measure the effect of melatonin feeding. Assuming a binomial distribution about the observed probabilities of incidence, the difference between these tumor incidence levels was not significant.

Since melatonin administration decreased the size of detectable tumors, it is possible that, in addition to reducing their overall incidence, melatonin was able to slow down the rate of growth of pre-existing tumors. It is also noteworthy that the overall mortality rate of mice irrespective of the cause of death was reduced in the melatonin-treated group.

Melatonin increased the proportion of mice not dying before reaching the fully aged phase. Since malignancies, especially hepatocellular tumors and lymphoma, are the major cause of death in mature male B6C3F1 mice (Haseman et al., 1998), this finding may be related to the reduced number of tumors found in treated mice. However, the results do not directly address the issue of whether melatonin affects maximum life span. It is significant that the strains of mouse used here were relatively long-lived. Unlike many strains used for cancer research the mice were not bred for susceptibility to cancer but rather for robustness. This increases the relevance of our findings to the real world situation.

These results serve to emphasize the importance of continuing to investigate the beneficial qualities of melatonin especially in relation to the interface between cancer and aging. Melatonin is very non-toxic, inexpensive and readily available and this further adds to its attractiveness as a potential carcinostatic agent.

Acknowledgements

This work was supported in part by a grant from the National Institutes of Health (AG 16794) and in part by a UC Davis Center for Human and Nutrition Research Pilot Award (CHNR08-318) to E.S.

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