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Putting Cancer to Sleep at Night

The Neuroendocrine/Circadian Melatonin Signal

David E. Blask, Robert T. Dauchy, and Leonard A. Sauer

Laboratory of Chrono-Neuroendocrine Oncology, Bassett Research Institute, Cooperstown, NY, USA

Physiological and pharmacological blood concentrations of melatonin inhibit tumorigenesis in a variety of in vivo and in vitro experimental models of neoplasia. Evidence indicates that melatonin's anticancer effects are exerted via inhibition of cell proliferation and a stimulation of differentiation and apoptosis. A new mechanism by which physiological and pharmacological blood levels of melatonin inhibit cancer growth in vivo is via a melatonin-induced suppression of tumor linoleic acid (LA) uptake and its metabolism to the important mitogenic signaling molecule 13-hydroxyoctadecadienoic acid (13-HODE). Melatonin suppresses cAMP formation and inhibits tumor uptake of LA and its metabolism to 13-HODE via a melatonin receptor–mediated mechanism in both tissue-isolated rat hepatoma 7288 CTC and human breast cancer xenografts. It has been postulated that in industrialized societies, light at night, by suppressing melatonin production, poses a new risk for the development of breast cancer and, perhaps, other cancers as well. In support of this hypothesis, light during darkness suppresses nocturnal melatonin production and stimulates the LA metabolism and growth of rat hepatoma and human breast cancer xenografts. Nocturnal dietary supplementation with melatonin, at levels contained in a melatonin-rich diet, inhibits rat hepatoma growth via the mechanisms described above. The nocturnal melatonin signal organizes tumor metabolism and growth within circadian time structure that can be further reinforced by appropriately timed melatonin supplementation. Dietary melatonin supplementation working in concert with the endogenous melatonin signal has the potential to be a new preventive/therapeutic strategy to optimize the host/cancer balance in favor of host survival and quality of life.

Key Words: Melatonin; pineal gland; circadian rhythm; cancer growth; linoleic acid metabolism; host/cancer balance.

Introduction

The central, long-standing dogma of the complex and heterogeneous disease called cancer is that malignant tumors initially evolve from the sequential acquisition of genetic alterations in specific genes (i.e., mutations and amplifications) in the DNA of individual, fully differentiated somatic cells. Just as natural selection is the mechanism driving the evolution of both simple and complex organisms, cancer cells are "selected" for their ability to proliferate. New mutations become fixed as a result of a wave of clonal expansion due to the relative growth advantage that a new mutation confers on the cell. Genomic instability induced by faulty cell division or defective DNA repair mechanisms may further increase the rate of potentially tumorigenic mutations. Additionally, changes in cell cycle progression, alterations in the operation of cell survival mechanisms, and/or modifications in the signal transduction pathways that regulate these processes, appear to make cancer cells exempt from the normal constraints of cell proliferation and apoptosis. Cancer cells are selected not only for their proliferative capacity, but also for their ability to stimulate their own blood supply in support of unlimited expansion, and to invade the circulation and form metastases in distant organs that ultimately lead to the demise of the host. Furthermore, the renewal of a small subset of pluripotent stem cells within the cancer tissue may ultimately be responsible for the maintenance of the malignant phenotype *(1–6)*.

The circadian system exerts an important influence over many physiological and metabolic activities such as the sleep/wake cycle, body temperature, intermediary metabolism, and endocrine functions as well as a number of disease processes including myocardial infarction, stroke, and asthma *(7)*. That mitotic activity in normal and neoplastic mammalian tissues follows a daily rhythmic pattern was discovered over 60 yr ago *(8,9)* and while evidence that cell-cycle progression and apoptosis in normal proliferating tissues are coordinated within circadian time structure was obtained over 30 yr ago *(10)*. More recently, it has become clear that cancer cell proliferation exhibits circadian rhythmicity at all stages of tumor growth. Evidence is emerging that disruption of the activity of the central circadian pacemaker, the suprachiasmatic nuclei (SCN) of the hypothalamus, is associated with cancer in experimental models of tumorigenesis such that desynchronization of internal time-

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Author to whom all correspondence and reprint requests should be addressed: David E. Blask, Ph.D., M.D., Laboratory of Chrono-Neuroendocrine Oncology, Bassett Research Institute, One Atwell Road, Cooperstown, NY, 13326. E-mail: david.blask@bassett.org

keeping adversely tips the host/cancer balance in favor of cancer development and growth *(11–13)*. Interestingly, cancer cells express the very same clock genes found in the SCN and many common molecular elements are shared between the circadian clock and cell cycle *(14,15)*. Recent findings indicate that a mutation in the circadian clock gene *per2* results in an increase in spontaneous tumorigenesis in mice *(15)*, while an elevated risk of premenopausal breast cancer has been associated with length polymorphorism in the clock gene *per3 (16)*. In spite of this exciting progress, however, no definitive outputs linking the human circadian clock with the processes governing human malignancy have as yet been identified.

The pineal synthesis and secretion of melatonin represents an important nocturnal output component of the central circadian pacemaker mechanism *(17,18)*. As the only known chronobiotic, neurohormonal regulator of cancer development and growth in experimental models of neoplasia, melatonin, at nocturnal circulating concentrations, inhibits the proliferation of human cancer cell lines in vitro. This is achieved by delaying the progression of cells from the $G_{1/0}$ to the S phase of the cell cycle. At pharmacological levels, melatonin has also been shown to exert cytotoxic effects on cancer cells in vitro. In fact, either alone or in combination with other agents, melatonin induces apoptotic cancer cell death. In some neoplastic cells, this indoleamine acts as a differentiating agent and diminishes their invasive/metastatic potential via alterations in adhesion molecule expression and the support of mechanisms responsible for gap junctional intercellular communication. Additionally, evidence in support of a variety of biochemical and molecular mechanisms of melatonin's oncostatic action has been presented including the regulation of estrogen-receptor expression and transactivation, calcium/calmodulin activity, protein kinase C activity, cytoskeletal architecture and function, intracellular redox status, melatonin receptor–mediated signal transduction cascades, aromatase and telomerase activity, and fatty acid transport and metabolism. Several clinical trials have confirmed melatonin's efficacy as a single therapeutic agent, and its capacity to raise the therapeutic index when used in conjunction with more conventional anticancer therapies *(19–21)*.

This review will be limited to a discussion of evidence primarily from the authors's laboratory relative to the neuroendocrine and signal transduction mechanisms by which the endogenous, nocturnal circadian melatonin signal as well as dietary melatonin supplementation affect the regulation of cancer growth in vivo. A major theme of this review will be the relationship of melatonin's oncostatic action to signal transduction mechanisms that have an impact on the tumor uptake of LA and its metabolism. An essential omega-6 polyunsaturated fatty acid (PUFA), LA is the most prevalent PUFA in the western diet in which levels greatly exceed those required to prevent essential FA deficiency (i.e., 1% of total calories) *(22)*. As a potent promoter of both murine and human tumorigenesis, LA exerts actions on cancer cells that are diametrically opposed to many of the oncostatic actions of melatonin listed above. Its oncogenic effects, particularly on human breast cancer cells, are related to its ability to upregulate the expression of genes involved in estrogen receptor (ERα) expression, cell-cycle progression, G protein signaling, and the mitogen-activated protein kinase (MAPK) growth cascade *(23)*.

Another major pathway by which LA provokes tumorigenesis is via the lipoxygenase system and its interactions with growth factor pathways. For example, epidermal growth factor (EGF), through activation of its cognate receptor, the EGFR, stimulates the activity of a 15-lipoxgenase-1 (15-LOX-1) *(24)*. 15-LOX-1, in turn, metabolizes 1–10% of LA taken up by the cell from dietary sources and/or endogenous fat depots to the mitogenic signaling molecule 13-HODE *(22)*. Through signaling cross-talk, 13-HODE enhances both EGF- and insulin-like growth factor-1 (IGF-1)–responsive mitogenesis and decreases apoptosis by enhancing the activation of mitogen-activated protein kinases (MEK and ERK1/2). In the case of the EGFR, 13-HODE is known to augment receptor autophosphorylation and tyrosine phosphorylation of the key downstream enzymes MEK and ERK1/2 that carry the mitogenic signal from the plasma membrane into the nucleus to stimulate cell division. In the case of the IGF-1 receptor pathway, 13-HODE activates both the ERK1/2 and phosphotidylinositol-3-kinase (PI3K)/Akt pathways *(25)*.

Model System—Tissue-Isolated Tumors

Our work has focused on the role of melatonin in the regulation of the growth of transplantable murine and human tumors in rats as well as on its modulation of relevant signal transduction and metabolic pathways. Our approach involves the use of a unique system, originally devised by Guillino *(26)* and modified by two of us, Dauchy and Sauer *(27–29)*, in which tumor tissue is implanted into a rat host in a tissue-isolated manner. This system has been extensively described elsewhere *(27–29)* and is depicted schematically in Fig. 1. Briefly, a small piece (approx 3 mm cube) of an established tumor grown in a donor rat is implanted into the inguinal region the host animal. This is accomplished by surgically creating a vascular pedicle from branches of the femoral artery and vein, the superficial epigastric vessels, which normally provide arterial supply to and venous drainage of the superficial inguinal fat pad. The tumor tissue is securely attached to the end of this vascular stalk with suture material and then wrapped within a tailor-made parafilm sac that prevents vascular in-growth from all other vascular sources except the epigastric vessels. The arterial supply and venous drainage of the tumor tissue, exclusively provided by the epigastric vessels, is quickly established and

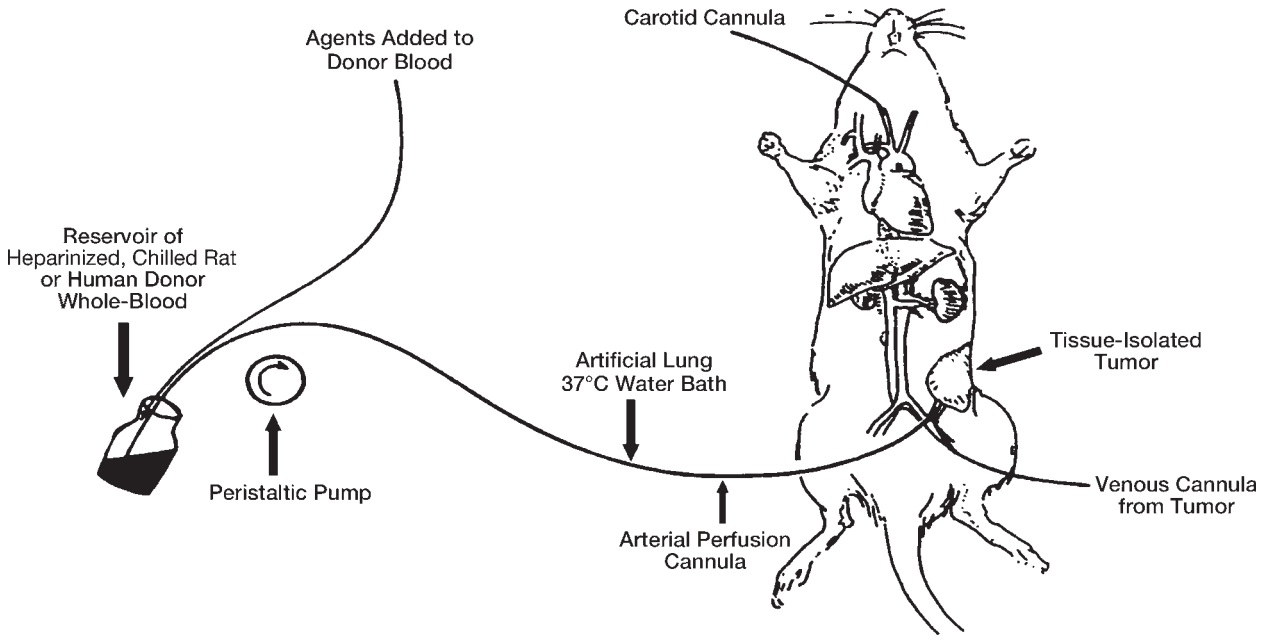

Fig. 1. A schematic diagram illustrating the tissue-isolated, *in situ* tumor perfusion system in the adult male Buffalo rat.

robust tumor growth soon occurs within the inguinal fossa. Once a tumor reaches a sufficient size to become palpable, it can be easily measured through the skin with calipers in three perpendicular diameters, the lineal dimensions of which are then converted to an estimated tumor weight using a special linear regression formula *(30)*. This allows frequent and remarkably accurate serial measurements of tumor size, and thus tumor weight over several weeks followed by the calculation of tumor growth rates. Growth rates can therefore be followed for individual tumors whereby the animal host serves as its own control. In addition, the mean growth rate for an entire experimental group can be calculated from individual tumor growth rates or from the mean tumor size determined at each time point of the growth curve.

The greatest advantage of this model system, however is that, upon reaching an appropriate size of at least 4–6 g, tumors can be exposed and the epigastric vessels cannulated with polyethylene tubing for perfusion *in situ* (Fig. 1). Whole blood, initially collected from donor rats, is pumped to the tumor via a peristaltic pump, through an artificial lung and a 37°C tissue bath so that by the time the blood reaches the tumor it is completely physiological in terms of its composition, pH, blood gases, and temperature *(27–29)*. It takes approx 10 min for the blood to reach the tumor from the reservoir. Frequent serial arterial and venous samples are collected over the entire course of the perfusion, which typically lasts from 1 to 2 h but can be sustained for up to 8 h without compromising tumor physiology. This permits arteriovenous (A-V) difference measurements to be made across tumors for assessing various parameters of tumor metabolism such as the rate of uptake of a biochemical substrate and/or the release of a metabolite. Agents can be added to the blood reservoir (i.e., hormones, growth factors, drugs, receptor blockers, signal transduction agents) and efficiently delivered to the tumor to assess their acute effects directly on tumor signal transduction activity, metabolism, and proliferative activity. This novel strategy permits a simultaneous and integrated assessment of the biochemical/molecular mechanisms governing tumor proliferative activity in vivo. A modification of this procedure also allows A-V difference measurements to be made at the end of a long-term tumor growth experiment in which the tumor-bearing animal host is itself treated with tumor growth modulating agents of interest.

The tissue-isolated tumor approach preserves the full physiological, circadian, and organismal integrity of the tumor. Therefore, these features make this an ideal system for studying the influence of melatonin, at both endogenously produced and exogenously supplemented levels, on the signal transduction and metabolic mechanisms regulating tumor growth in vivo. In most of our studies to date, we have relied on our "gold standard," the tissue-isolated Morris rat hepatoma 7288CTC, an epithelially derived liver adenocarcinoma. Therefore, the present discussion will be limited, for the most part, to findings obtained with this tumor type because it has provided the most complete picture thus far regarding the regulation of tumor growth by both endogenous and exogenously administered melatonin. During the past few years, however, we gradually have been phasing in the use of human breast cancer xenografts, another epithelial adenocarcinoma, derived from the human breast cancer cell line MCF-7. Thus, we will occasionally refer

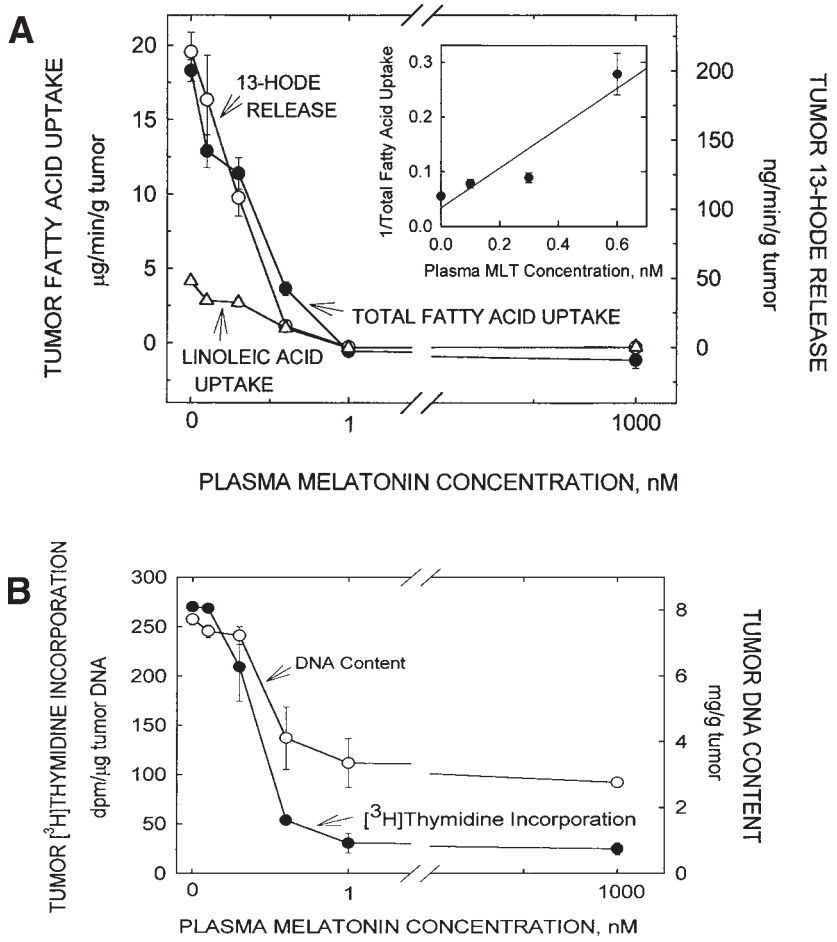

Fig. 2. Dose–response effects of increasing concentrations of melatonin on total FA and LA uptakes, 13-HODE release (**A**), DNA content and [^{3}H]thymidine incorporation into DNA (**B**) in tissue-isolated rat hepatoma 7288CTC perfused *in situ* in adult male Buffalo rats. Tumors were perfused during the early light phase of a 12L:12D light/dark cycle over a 2 h period with whole blood, from pinealectomized donor rats, to which melatonin was added to achieve final concentrations of 100, 300, and 600 p*M*, 1 n*M*, or 1 µ*M*. The inset (**A**) is Dixon plot representing reciprocal rates of total FA uptake as a function of the plasma melatonin concentration; K_i for suppression of total FA uptake = 93 p*M*. Tumors were perfused with [^{3}H]thymidine during the final 20 min of the perfusion period. $n = 3$ tumors for each melatonin dose and data points are represent the mean ± SD.

to parallel results, mostly unpublished, obtained in these human breast cancer xenografts where appropriate.

Melatonin Responsiveness of Tissue-Isolated Tumors

Several years ago, two of us, Sauer and Dauchy *(31)*, were the first to demonstrate that LA was taken-up by tumors via a saturable process that was dependent on the concentration of LA in the arterial blood. We subsequently demonstrated that approx 1–10% of the LA taken-up by tumors was converted to 13-HODE and that this metabolite was responsible for the mitogenic action of LA *(30)*. Recently, we reported for the first time that perfusion of tissue-isolated rat hepatoma 7288CTC *in situ* with melatonin for 2.5 h, at a concentration considered to be at the upper limit of the nocturnal, physiological circulating range (1 n*M*), caused a 70% decrease in the tumor uptake of total FAs, particularly LA. This was accompanied by a near complete suppression of 13-HODE formed by and released from the tumor as well as a marked decrease in tumor proliferative activity reflected by decreased DNA content and incorporation of [^{3}H]thymidine into DNA *(32)*. Dose-dependent suppression of tumor LA and total FA uptake, 13-HODE formation, DNA content, and [^{3}H]thymidine incorporation occurred entirely within the nocturnal, physiological range of circulating melatonin concentrations and reached saturation at 1 n*M* (Fig. 2). Interestingly, during tumor perfusion approx 30% of the melatonin supplied to the tumor was taken-up and retained by the tumor across all concentrations tested (see additional discussion below). The ability of melatonin to inhibit the tumor uptake of LA and its metabolism to 13-HODE is rapid (approx 5–10 min), reversible, and specific, because melatonin metabolites and precursors had no effect *(33)*. Additionally, the melatonin inhibitory effects on tumor LA uptake and 13-HODE formation are not restricted to rat hepatoma because similar results were obtained in tissue-isolated human breast cancer xenografts *(34)*, chemically

induced rat mammary tumors, and human head/neck squamous cell carcinoma xenografts (unpublished data). Our discovery that nocturnal, physiological circulating concentrations of melatonin inhibit the tumor uptake of LA and its metabolism to 13-HODE respresents a novel mechanism by which endogenous melatonin levels exert their oncostatic effects on cancer growth in vivo. Moreover, this unique mechanism of melatonin action is not restricted to tumors because melatonin also inhibits FA uptake in normal tissues such as inguinal white adipose tissue *(35)* as well as skeletal muscle *(36)*. The signal transduction mechanisms involved in the inhibition of LA uptake and 13-HODE formation will be discussed in more detail below.

Melatonin-Driven, Circadian Rhythm of Inhibition of LA Uptake/Metabolism and Growth in Tissue-Isolated Tumors

The exquisite sensitivity of rat hepatoma 7288CTC to the inhibitory effects of melatonin at nocturnal, physiological levels in our perfusion studies *(32,33)* begged the question of whether there would be a circadian rhythm of tumor LA uptake and metabolism that was driven by the endogenous melatonin signal. Because rats are nocturnally active, approx 90% of their food consumption occurs during the dark phase of an alternating light/dark cycle. In fact, the arterial concentration of LA and, thus, the arterial supply of LA to tumors is twofold higher during the middle of the dark phase as compared with FA levels during the light phase. When measured around the entire 24-h light/dark cycle, tissue-isolated rat hepatomas exhibit a nocturnal rhythm of maximal suppression of total FA uptake, LA uptake, and 13-HODE formation that is coincident with peak plasma levels of nocturnal melatonin during the middle of the dark phase (Fig. 3). The fact that surgical pinealectomy extinguishes this rhythm, causing a twofold stimulation of tumor growth, proves that the rhythm of tumor LA uptake and metabolism to 13-HODE is a slave-oscillation driven by the circadian rhythm of melatonin production and release by the pineal gland. In fact, as much as a 10–16-fold increase in tumor LA uptake and a 200-fold higher rate of 13-HODE formation occur in tumors in pinealectomized animals. These high rates of tumor LA uptake/metabolism and growth are due to the fact that melatonin is no longer present to prevent the uptake of twofold higher arterial concentrations of LA that are normally available to tumors during the dark phase *(32)*.

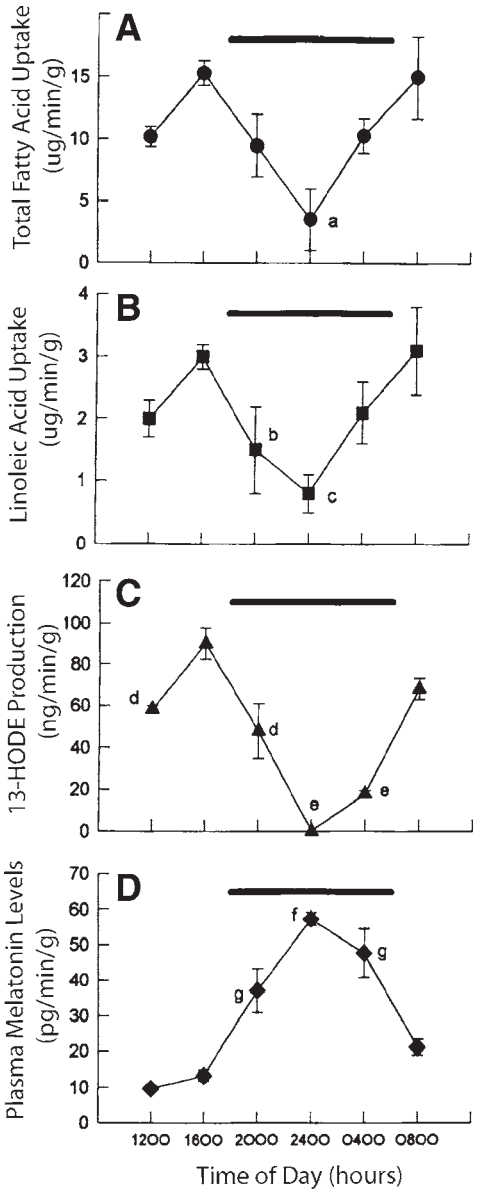

Fig. 3. Mean tumor uptake of total FAs (**A**) and LA (**B**), 13-HODE formation (**C**) by tissue-isolated rat hepatoma 7288CT in vivo, and plasma melatonin levels (**D**) over a 24-h period in adult male Buffalo rats. Animals were maintained on a 12L:12D light/dark cycle and provided with a semipurified diet containing 5% corn oil *ad libitum*. Tumor A-V difference measurements were made at 4-h intervals during a 24-h period. Tumor-bearing rats ($n = 4$ per time point) were randomized, such that the mean tumor weight at each time point was 5.4 ± 0.1 g. Data points represent the mean ± SE. The dark bar at the top of each graph represents the duration of the dark phase.

Light-Induced Suppression of Nocturnal Melatonin Production: Effects on Tumor LA Uptake/Metabolism and Growth

Like pinealectomy, constant light exposure also exerts a marked stimulatory effect on tumor development and growth of tumors induced with chemical carcinogens *(37)*. In general, following pinealectomy or exposure to constant bright light not only do tumors appear earlier, but a greater percentage of animals develop tumors and more tumors develop per animal as compared to control animals maintained on an alternating 12 h light:12 h dark cycle *(37)*. Although constant light exposure seems to stimulate tumorigenesis in the majority of investigations, clearly one-third of the studies

conducted report either inhibitory, mixed, or no effects on the development of experimental cancer. Interestingly, more consistent stimulatory actions of continuous illumination appear to occur with respect to the growth of established tumors. For example, in rats bearing either tissue-isolated rat hepatoma or rat mammary cancer and exposed to constant bright light (i.e., 300 lux at rodent eye level) for 1 wk prior to tumor implantation and continuing thereafter, there is a complete absence of the nocturnal, circadian rise in circulating melatonin levels as compared with light:dark (L:D) controls. Moreover, not only do tumors appear much earlier as a result of constant light exposure but their average daily growth rate accelerates by a factor of 2.5–6 times over the average growth rate of tumors in the L:D control group. The marked increase in the rate of tumor growth results from a substantial augmentation in the rate of tumor uptake of LA and its conversion to 13-HODE as a consequence of the suppression of the circadian melatonin signal (see above) *(38–40)*.

We recently tested the light at night hypothesis with regard to human breast carcinogenesis in female nude rats implanted with ERα+ MCF-7 cell–derived human breast cancer xenografts. During a 2-wk period following their transfer from a 12L:12D light:dark cycle (i.e., intact circadian melatonin signal) to constant bright light (i.e., 300 lux; no nocturnal melatonin signal), the average daily rate of tumor growth in constant light–exposed rats increased by sevenfold in comparison with the tumor growth rate in animals remaining on an L:D cycle. This accelerated rate of human breast cancer growth was initiated and sustained as a result of increases in the rate of tumor uptake of LA and its metabolism to 13-HODE. This augmented rate of tumor LA uptake and metabolism resulted from constant light–induced suppression of the circadian melatonin signal, which normally drives the inhibition of these processes during the dark phase *(41)*. This is the first experimental evidence to date showing a link between inappropriate exposure to continuous bright light and increased growth and FA metabolism in human breast cancer.

The ability of ocular light exposure to suppress pineal melatonin production depends on the intensity, wavelength, duration, and timing of light. As important as the constant bright light studies are, they address only one aspect of the light intensity issue. Furthermore, circadian disruption resulting from exposure to constant bright light is not limited solely to the suppression of nocturnal pineal melatonin production because general circadian activity eventually becomes desynchronized. Studies from our laboratory demonstrate that the exposure of rats to low intensity fluorescent light (i.e., 0.2 lux at rodent eye level) during the dark phase for 1 wk prior to the implantation of tissue-isolated rat hepatomas and continuing thereafter, results in a nearly complete suppression of circulating melatonin levels. The advantage of this strategy as compared with constant bright light exposure is that dim light during darkness affects only nocturnal melatonin production rather than causing an additional, general disruption of circadian clock activity. Interestingly, the tumor growth rate, LA uptake, and metabolism to 13-HODE are nearly as rapid as in constant light–exposed animals indicating that low intensity light-induced melatonin suppression during the dark phase is as effective as constant light exposure in tumor growth and LA metabolism *(38,39)*.

More recently, we examined the effects of different light intensities during darkness on nocturnal circulating melatonin levels and the growth and LA metabolism of rat hepatoma 7288CTC. Exposure of tumor-bearing rats to white fluorescent light intensities (at rodent eye level) ranging from complete darkness to constant bright light (345 μW/cm^2 or approx 840 lux) results in a dose-dependent suppression of melatonin levels with a concomitant dose-related stimulation of tumor growth, LA uptake, and 13-HODE production *(42)*. Although preliminary, these findings represent the first evidence that stimulation of tumor growth and metabolism is dependent on the degree of the suppression of melatonin production that is, in turn, dependent on the intensity of light present during darkness. We are currently in the process of examining light intensity dose–response issues relative to melatonin suppression and the growth and LA metabolism of human breast cancer xenografts in nude rats. Nevertheless, the studies outlined above represent the most definitive support, thus far, for the hypothesis that light-induced suppression of nocturnal melatonin production may be a new risk factor for human breast cancer particularly in night shift workers. Recent epidemiological studies suggest that women who work night shifts have up to a 60% increased risk of developing breast cancer *(43,44)*.

Effects of Exogenous Dietary Melatonin Supplementation on Tumor LA Uptake/ Metabolism and Growth

Not only is melatonin produced by the pineal gland in vertebrate species but it is found in significant quantities in all major non-metazoan taxa including angiosperms *(45)*. Among angiosperms, melatonin is found in fruits, vegetables, and medicinal herbs consumed by animals and humans and the levels present in edible plants are several orders of magnitude higher than peak nocturnal blood concentrations produced by the pineal *(46,47)*. In animal studies, the consumption of melatonin-rich food acutely elevates both immunoreactive and bioactive circulating blood melatonin levels *(48)*. In fact, it has been proposed that melatonin may have to be reconsidered as not only a pineal-derived neurohormone, but also as an antioxidant nutrient and/or vitamin *(49)*.

In this regard, a more abundant source of melatonin in the human diet comes from commercially available nutritional supplements regularly consumed by millions of people throughout the world most often for sleep problems and/or jet lag *(50)*. Melatonin supplements contain anywhere from

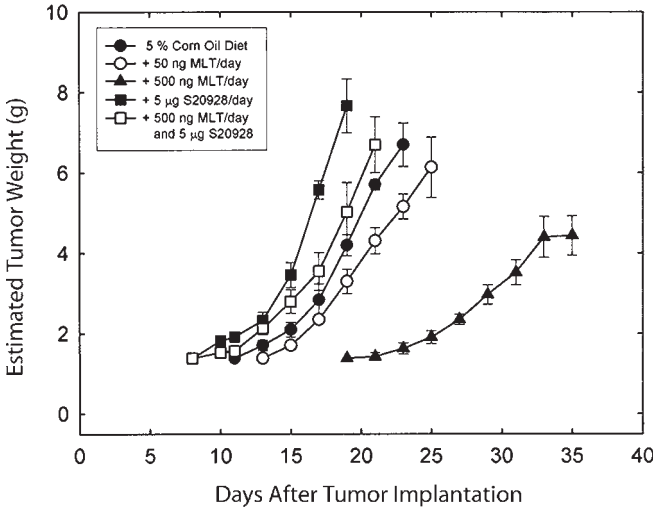

Fig. 4. Effects of dietary melatonin, in the presence or absence of non-selective MT_1/MT_2 melatonin receptor antagonist S20928, on the growth rate of tissue-isolaed rat hepatoma 7288CTC in adult male Buffalo rats. Rats were placed on a semipurified 5% corn oil diet such that they consumed either 0, 50, or 500 ng/d of melatonin or 500 ng/d of melatonin in combination with 5 μg/d of S20928. Animals began receiving diets 2 wk prior to tumor implantation and the diets were continued until the end of each growth period. Data points represent the mean ± SD; $n = 6$ rats (tumors)/group.

0.25 to 5 mg of melatonin and the oral bioavailability of 2–4 mg of this indoleamine is approx 15%, which translates to peak plasma levels of 2–4 ng/mL 1 h following ingestion *(51)*. The oral bioavailability of microgram doses varies widely from 10 to 56% ostensibly due to variations in first-pass hepatic metabolism *(52)*. Normal peak concentrations of melatonin released from the pineal gland into the circulation at night in humans are around 60–80 pg/mL. The ingestion of 300 μg of melatonin during the day can produce melatonin concentrations that mimick these nocturnal levels *(53)*.

We recently addressed the issue of whether dietary supplementation of rats, with quantities of melatonin that would hypothetically be consumed on a daily basis (0.05–5 μg/d) by humans ingesting a diet of melatonin-rich foods, would inhibit the growth of tissue-isolated rat hepatomas by suppressing tumor LA uptake and metabolism. Such a diet was implemented 2 wk prior to tumor implantation and continued for several weeks thereafter. Melatonin was added to a semipurified 5% corn oil diet, which the animals consumed *ad libitum*. This cancer prevention strategy markedly delayed tumor onset and diminshed the high rates of hepatoma growth, LA uptake, and 13-HODE formation in a dose-dependent manner (Fig. 4). Because dietary melatonin was ingested almost exclusively during the dark phase of a 12L:12D light/dark cycle, it is likely that exogenously supplemented melatonin acted in concert with and, in effect, reinforced the endogenous melatonin signal, to produce an even more potent anticancer growth effect *(33)*. That these inhibitions require a melatonin receptor–mediated process is indicated by the ability of the non-selective MT_1/MT_2 melatonin receptor antagonist S20928 *(54)* to completely block melatonin's tumor inhibitory effects when it was co-ingested with melatonin in the diet (Fig. 4). Interestingly, the ingestion of S20928 alone duplicated the same tumor-stimulatory effects produced by either pinealectomy or light exposure during darkness described above. These results are the first to indicate that interference with the action of the endogenous circadian melatonin signal, at the receptor level, is tantamount to eliminating the melatonin signal itself at its source in the pineal gland.

Tumor Uptake and Retention of Melatonin Ingested in the Diet

In addition to demonstrating the tumor growth inhibitory effect of dietary melatonin supplementation, we discovered a dose-related accumulation of melatonin in tumor tissue following several weeks of dietary melatonin intake that was consistent with the acute tumor uptake of melatonin observed in our perfusion studies described above *(33)*. Over the course of several weeks of melatonin ingestion, the daily occurrence of tumor melatonin uptake during every 12-h dark period could reasonably account for the substantial accumulation of melatonin within tumor tissue. In fact, it has been reported that human breast cancers contain melatonin levels that are three orders of magnitude higher than nocturnal blood levels. Similarly, high concentrations of melatonin have also been documented in non-neoplastic breast tissue, including adipose tissue, from both healthy subjects and breast cancer patients *(55)*. The remarkable tumor uptake and retention of melatonin indicates that, like the gut and other tissues that avidly sequester melatonin *(56)*, tissue-isolated rat hepatoma is an important repository for melatonin supplied in the diet.

Although the mechanism mediating melatonin's uptake and retention by hepatoma 7288CTC is unclear, it appears to be consistent with a receptor-mediated process inasmuch as the melatonin receptor antagonist S20928 blocks tumor melatonin uptake *(33)*. A likely explanation for the majority of tumor uptake and retention of melatonin is desensitization and internalization of ligand-occupied melatonin receptors at the cellular level *(57)*. Once within the cancer cell, melatonin may then become uncoupled from its internalized membrane receptors thus allowing it to bind to intracellular binding proteins resulting in increased intratumoral concentrations of melatonin *(56)*. Melatonin receptor desensitization is indirectly supported by the fact that despite the continued presence of melatonin, the tumor growth rate accelerates and shows signs of developing refractoriness to melatonin during the later stages of tumor growth and continued exposure to dietary melatonin *(33)* (Fig. 4). It is not exactly clear what, if any, function the tumor uptake and sequestration of melatonin serves during tumorigenesis. We

suspect, however, that this may have important clinical implications and possibly represent a mechanism by which tumors develop melatonin resistance during long-term cancer therapy thus tipping the host/cancer balance in favor of malignant growth.

A New Mechanism of Melatonin's Anticancer Action In Vivo

The tissue-isolated tumor model system allows the integration of circadian melatonin regulation with the study of the mechanisms of action of endogenous or exogenous melatonin, via biochemical and molecular signal transduction pathways at the level of the tumor *in situ*. As alluded to above, all the systemic, integrative influences of nutritional and photoperiodic interactions with the circadian system, in general, and the melatonin rhythm–generating system, in particular, are preserved. Retaining this systemic context is crucial for achieving a more thorough understanding of melatonin's anticancer mechanisms that optimize the host/cancer balance in favor of the host and to the detriment of the tumor.

We referred above to a major component of a newly discovered mechanism by which melatonin inhibits tumor growth in vivo, namely, melatonin-induced suppression of the tumor uptake of LA and its metabolism to the mitogenic signaling molecule 13-HODE *(32)*. Also, in the melatonin dietary supplementation work described above, we cited evidence that this was a melatonin receptor–mediated process *(33)*. Perfusion studies in tissue-isolated rat hepatoma *(32, 33)* with non-selective MT_1/MT_2 melatonin receptor antagonist S20928 *(54)* and selective MT_2 melatonin receptor antagonist 4-phenyl-2-proprionamidotetraline (4P-PDOT) *(57)* reveal that both MT_1 and MT_2 melatonin receptors are present in this tumor type *(58)*. Both receptors appear to be involved in mediating the melatonin-induced suppression of tumor LA uptake and its metabolism to 13-HODE culminating in the inhibition of tumor proliferative activity *(58)*. These are inhibitory G protein–coupled receptors linked to inhibition of cAMP production *(57)*, and, as would be expected, PTX, forskolin, and 8-bromo-cAMP are all effective in reversing melatonin-induced inhibition of LA uptake/metabolism and tumor proliferative activity. An interesting question that remains to be addressed is whether melatonin exerts an additional inhibitory effect on the expression and/or activity of 15-LOX-1 that is responsible for 13-HODE production from LA.

In both tissue-isolated ERα (+ and –), MCF-7 human breast cancer xenografts, which only express functional MT_1 receptors (unpublished data), melatonin acts via this receptor to suppress tumor cAMP formation, LA uptake, and 13-HODE production, and to reduce the activation of the MEK/ERK1/2 pathway leading to tumor growth inhibition *(34)*. As mentioned in the introduction, LA upregulates the expression of ERα in human breast cancer cells *(23)*, whereas melatonin inhibits the transcriptional regulation of ERα via an MT_1 melatonin receptor–mediated inhibition of cAMP in these cells *(59)*. Thus, in ERα+ human breast cancers there potentially would be ample opportunity for cross-talk among these pathways. It is conceivable that melatonin could down-regulate ERα expression via a pathway involving MT_1 melatonin receptor–mediated suppression of cAMP production leading to a suppression of tumor LA uptake and metabolism to 13-HODE. This, in turn, would result in a downregulation of the EGFR/MEK/ERK1/2 cascade culminating in a decreased tumor growth rate. This could explain why melatonin is effective in inhibiting the growth of both tissue-isolated ERα (+ and –) human breast cancer xenografts (Fig. 5).

Concluding Remarks

In this review, we have briefly summarized some of the most recent data, primarily from our laboratory, supporting the view that melatonin, produced and secreted by the pineal gland, represents an important neuroendocrine/circadian anticancer signal of the night. Although exciting new evidence continues to emerge that the central circadian pacemaker in the SCN plays an important role in regulating the mechanisms that ensure the proliferation and survival of cancer cells, the identification of the actual outputs linking the central circadian clock mechanism with processes governing oncogenesis remains elusive.

The endogenous, nocturnal melatonin signal from the pineal gland appears to be the strongest inhibitory link yet described between the central circadian system and the growth of solid cancers. A fundamental new mechanism by which this nocturnal, circadian oncostatic neurohormone slows the rate of tumor growth in vivo involves a melatonin receptor–mediated suppression of cAMP production that results in a suppression of the tumor uptake of LA and its conversion, via 15-LOX-1, to the mitogenic metabolite 13-HODE. A deficiency in 13-HODE levels leads to a downregulation in activity of the EGFR/Ras/MEK/ERK1/2 growth signaling cascade and diminished tumor growth. Therefore, as a nocturnal output signal of the central circadian pacemaker, pineal melatonin literally drives a circadian, nocturnal rhythm of tumor LA uptake/metabolism and tumor growth activity. Therefore, during the dark phase of every 24-h day, tumor growth proceeds at a slow rate due to the ability of high circulating levels of melatonin to inhibit tumor LA uptake/metabolism; in a sense, tumors "fall asleep" at night (Fig. 5). Conversely, during the light phase when melatonin levels are very low, tumors "wake-up" and growth accelerates owing to unobstructed LA uptake/metabolism. Either pineal removal or exposure of tumor-bearing animals to light during darkness extinguishes these melatonin-driven tumor rhythms resulting in unabated tumor growth and metabolism "24/7." Thus, the nocturnal melatonin signal, via its ability to suppress tumor signal trans-

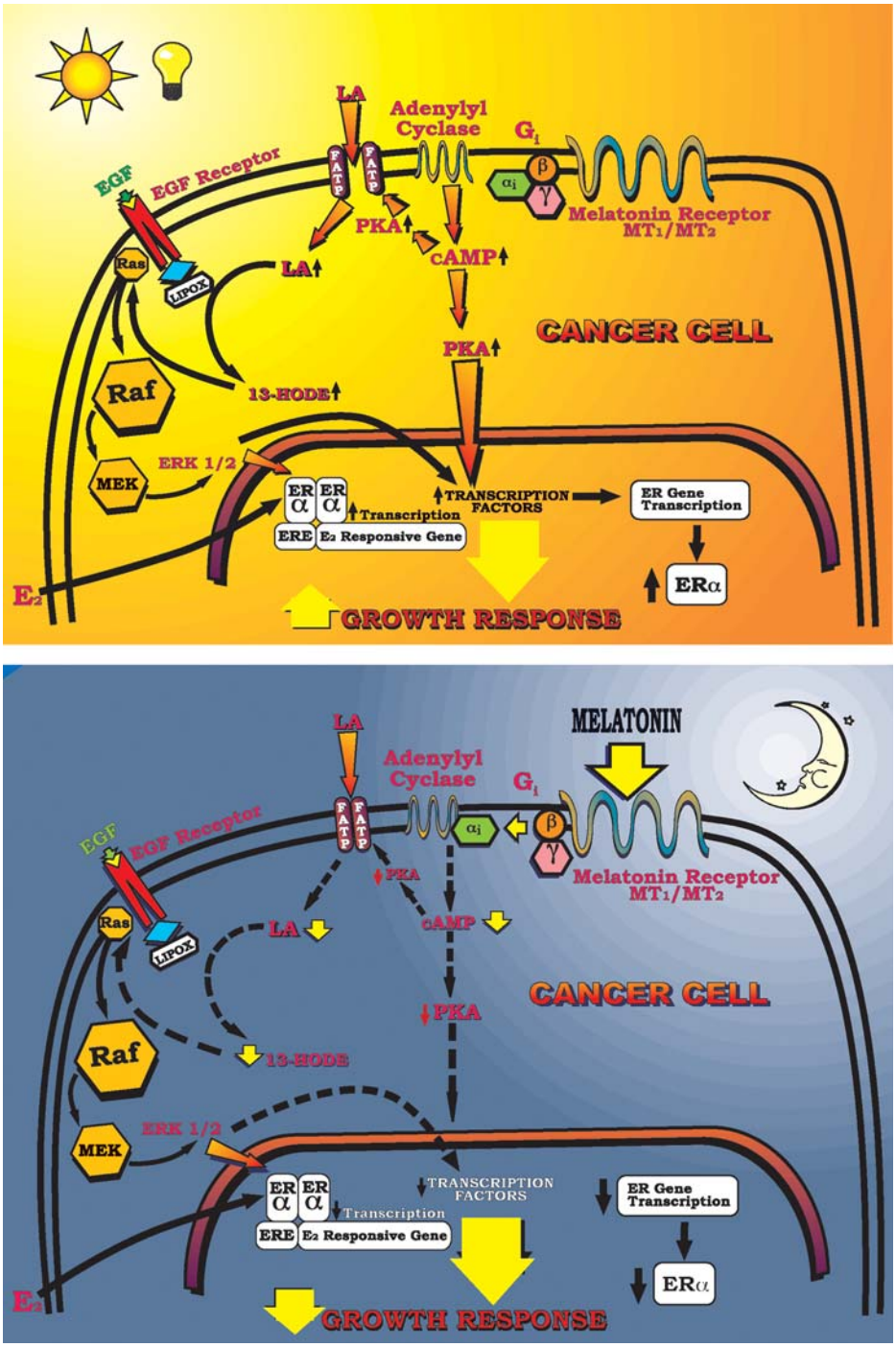

Fig. 5. Schematic diagram of the signal transduction and metabolic pathways mediating LA uptake, 13-HODE formation, and growth in tissue-isolated rat hepatoma or human breast cancer xenografts. Daytime growth stimulation is represented in the upper panel and nocturnal, melatonin-induced growth inhibition is depicted in the lower panel. Abbreviations: LA, linoleic acid; 13-HODE, 13-hydroxyoctadecadienoic acid; PKA, protein kinase A; ERα, estrogen receptor α; ERE, estrogen responsive element; EGF, epidermal growth factor; MEK, mitogen-activated protein kinase kinase; Raf, mitogen-activated protein kinase kinase kinase; ERK1/2, mitogen-activated protein kinase or extracellular signal-related kinase; LIPOX, 15-lipoxygenase-1; E_2, estradiol.

duction, metabolic, and growth activity, organizes tumor growth within circadian time structure. Dietary melatonin supplementation at the appropriate circadian stage (i.e., near the onset or during the dark phase) further reinforces the organization of this circadian time structure imposed on tumors by the endogenous, nocturnal melatonin signal leading to an even greater inhibition of cancer growth. Optimally timed exogenous melatonin administration, in the form of either dietary phytomelatonin or nutritional supplements, working in concert with endogenously, circadian-produced melatonin, may represent a new and potentially rewarding preventive and therapeutic strategy to tip the host/

cancer balance in favor of an increased quality of life and survival of the host.

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