Melatonin-Depleted Blood from Premenopausal Women Exposed to Light at Night Stimulates Growth of Human Breast Cancer Xenografts in Nude Rats

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Abstract

The increased breast cancer risk in female night shift workers has been postulated to result from the suppression of pineal melatonin production by exposure to light at night. Exposure of rats bearing rat hepatomas or human breast cancer xenografts to increasing intensities of white fluorescent light during each 12-hour dark phase (0-345 $\mu W/cm^2$) resulted in a dose-dependent suppression of nocturnal melatonin blood levels and a stimulation of tumor growth and linoleic acid uptake/metabolism to the mitogenic molecule 13-hydroxyoctadecadienoic acid. Venous blood samples were collected from healthy, premenopausal female volunteers during either the daytime, nighttime, or nighttime following 90 minutes of ocular bright, white fluorescent light exposure at 580 $\mu W/cm^2$ (i.e., 2,800 lx). Compared with tumors perfused with daytime-collected melatonin-deficient blood, human breast cancer xenografts and rat hepatomas perfused *in situ*, with nocturnal, physiologically melatonin-rich blood collected during the night, exhibited markedly suppressed proliferative activity and linoleic acid uptake/metabolism. Tumors perfused with melatonin-deficient blood collected following ocular exposure to light at night exhibited the daytime pattern of high tumor proliferative activity. These results are the first to show that the tumor growth response to exposure to light during darkness is intensity dependent and that the human nocturnal, circadian melatonin signal not only inhibits human breast cancer growth but that this effect is extinguished by short-term ocular exposure to bright, white light at night. These mechanistic studies are the first to provide a rational biological explanation for the increased breast cancer risk in female night shift workers. (Cancer Res 2005; 65(23): 11174-84)

Introduction

The risk of developing breast cancer is up to five times higher in industrialized nations than in underdeveloped countries. Overall, nearly 50% of breast cancers cannot be accounted for by conventional risk factors (1, 2). Westernized nations have increasingly become 24-hour societies with greater numbers of people being exposed to more artificial light during the night both at home and particularly in the workplace (3). Stevens postulated that light exposure at night may represent a unique risk factor for breast cancer in industrialized societies via its ability to suppress the nocturnal production of melatonin by the pineal gland (4, 5). This hypothesis is based on studies showing that melatonin inhibits the development and growth of experimental models of breast cancer, whereas either surgical removal of the pineal gland or exposure to constant light stimulates mammary tumorigenesis in rodents (6). This postulate was further strengthened by the demonstration that physiologic, nocturnal concentrations of melatonin directly inhibited the proliferation of MCF-7 human breast cancer cells in culture (7, 8). Recent epidemiologic studies showing that women working night shifts have a significantly elevated risk of breast cancer presumably due to their potential increased exposure to light at night (9–11) provide additional support for this hypothesis.

Cohen et al. (12) originally hypothesized that diminished pineal function may promote the development of human breast cancer. Their primary argument was that increased pineal calcification, ostensibly leading to lowered melatonin production, was most strongly associated with increased breast cancer risk. Although they were the first to refer to, in general terms, environmental lighting, which necessarily includes both sunlight and artificial light, as one of several endocrine abnormalities that may underlie the development of breast cancer, these workers did not postulate light at night as an etiologic factor. In fact, they incorrectly suggested that altered visual stimulation, as by blindness or darkness, would impair pineal melatonin production leading to unopposed estrogen secretion and increased breast cancer risk. We now know that overall melatonin production is not compromised in blind individuals (13), and breast cancer risk is actually decreased in blind women (14). Subsequent to the Stevens' hypotheses (4, 5), Kerenyi et al. (15) were the first to propose that "light pollution" may be a potentially important etiologic influence on the genesis of other human cancers. Some indirect support for this postulate was provided by a recent epidemiologic study showing a higher risk of colorectal cancer in women night shift workers (16).

The circadian synthesis and secretion of pineal melatonin during the night results from the rhythmic neuronal activity generated by the central circadian pacemaker in the suprachiasmatic

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Note: D.E. Blask, G.C. Brainard, R.T. Dauchy, and J.P. Hanifin contributed equally to this work.

We dedicate this article to the memory of our colleague and friend, the late Dr. William B. Guiney, Jr.

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nuclei of the hypothalamus, which is entrained (i.e., synchronized) by the light/dark cycle. The duration of melatonin production during the night is directly proportional to the length of the dark period (17–19). The alternating light/dark cycle entrains circadian melatonin production to a 24-hour cycle, whereas ocular exposure to light during darkness rapidly suppresses melatonin production depending on the intensity, wavelength, and duration of the light exposure (20, 21). In experimental systems, melatonin plays a fundamental role in the regulation of seasonal reproduction, circadian rhythm activity, retinal physiology, cardiovascular effects, immune function, and cancer (17–19).

It has been generally assumed that the breast cancer–promoting effects of either pinealectomy (i.e., pineal removal) or constant light exposure in rodents are due to the absence of the nocturnal melatonin signal because pharmacologic melatonin replacement either attenuates or prevents these tumorigenic effects (6). Physiologic nocturnal circulating levels of melatonin inhibit the growth of tissue-isolated rat hepatoma 7288CTC via melatonin receptor–mediated suppression of tumor linoleic acid uptake/metabolism to the mitogenic signaling molecule 13-hydroxyoctadecadienoic acid (13-HODE; refs. 22, 23). Pinealectomy or light exposure during darkness prevents these effects (22, 24, 25).

Inasmuch as the Stevens' hypothesis (4, 5) has not been experimentally tested, we evaluated the response of rats, bearing either rat hepatomas or human breast cancer xenografts, to increasing intensities of ocular light exposure during darkness on tumor growth, linoleic acid uptake/metabolism, and signal transduction activity. More importantly, we also determined the proliferative, metabolic, and signal transduction responses of hepatomas and breast cancer xenografts to perfusion *in situ* with blood collected from premenopausal human female volunteers whose physiologic circadian melatonin signal was suppressed by ocular exposure to bright, white fluorescent light at night. Our sole rationale for including tissue-isolated rat hepatomas was based on the fact that they exhibit remarkable sensitivity to melatonin, and most of the information we have on the biochemical/molecular mechanisms of melatonin's anticancer action *in vivo* has been obtained in this model system (22–25). We wanted the ability to compare the biological/molecular responses to light at night in the new tissue-isolated human breast cancer xenograft model with those in our highly melatonin-sensitive rat liver cancer model serving as a positive control. It is important to note, however, that unlike breast cancer, human liver cancer is much more common in the developing world (i.e., less light pollution) than in industrialized nations (i.e., more light pollution), and its incidence decreases as societies become more westernized (26).

Materials and Methods

Animals, tumors, and histopathology. Adult male Buffalo [BUF(BUF/Ncr)] rats were implanted with rat hepatocarcinoma syngeneic grafts (Morris 7288CTC), and adult inbred female nude rats (HSD:RH-rnu) were implanted with either steroid receptor–negative (SR^-; i.e., no estrogen or progesterone receptor expression) or steroid receptor–positive (SR^+) MCF-7 human breast cancer xenografts in a tissue-isolated manner as previously described (27, 28). SR^- tumors evolved over several passages from a subset of SR^+ xenografts that had become estrogen unresponsive. Both SR^+ and SR^- human breast cancer xenografts were histopathologically confirmed to be poorly differentiated, grade 3, infiltrating ductal breast adenocarcinomas. Adult male rats (Hsd:Sprague-DawleySD) were used as whole-blood donors for some tumor perfusions. Immunoshistochemical staining of cell nuclei for estrogen and progesterone receptor expression in tissue sections of human breast cancer xenografts was done by IMPATH (New York, NY). SR^+ tumors were highly estrogen receptor positive (80% staining) and minimally progesterone receptor positive (10% staining); in SR^- xenografts no staining for either receptor was evident (data not shown). Animals were maintained in an Association for Assessment and Accreditation of Laboratory Animal Care International–accredited facility on a 12-hour light/12-hour dark cycle (lights on 06:00 to 18:00 hours) and provided with standard laboratory chow and water *ad libitum* in accordance with an Institutional Animal Care and Use Committee–approved protocol. A series of ceiling fixtures containing 32-W fluorescent tubes (GE Watt Miser, cool white, F40CW.RS.WM) provided 7.5 $\mu W/cm^2$ of white light at rodent eye level during the light phase.

Tissue-isolated tumor perfusions *in situ*. SR^+ MCF-7 human breast cancer xenografts were perfused *in situ* with rat donor whole blood to which either synthetic melatonin (Sigma, St. Louis, MO), and/or 13-HODE (Cayman Chemicals, Ann Arbor, MI) was added as previously described (22, 23). Sets (three tumors per perfusion) of tissue-isolated rat hepatomas and human breast cancer xenografts were perfused *in situ* for 1 hour as previously described (22, 23) with whole blood collected from either donor rats or human subjects exposed to each of the lighting conditions outlined below.

Tumor reverse transcription-PCR. Rat hepatoma 7288CTC and SR^+ human breast cancer xenograft tissues were harvested from rats under nembutal anesthesia between 08:30 and 10:30 hours (zeitgeber time), quickly frozen, and stored at −80°C until subsequent isolation of total RNA. Total cellular RNA was isolated from human breast cancer xenograft tissue and rat hepatoma using the SV Total RNA Isolation System (Promega Corp., Madison, WI) The RNA was quantitated spectrophotometrically, and its integrity was checked by agarose gel. The mRNA was reversed transcribed with Moloney murine leukemia virus reverse transcriptase (Invitrogen Corp., Carlsbad, CA). The resulting cDNA was amplified using the PCR for MT_1 or MT_2 melatonin receptor product with Taq Polymerase (Promega) at 1.5 mmol/L $MgCl_2$ and a 58°C annealing temperature for 36 cycles using a RoboCycler (Stratagene, La Jolla, CA). Forward and reverse primers used for MT_1 and MT_2 melatonin receptors in tumor tissues are the same as previously published for rat (29) and for human (30, 31).

Tumor *in situ* hybridization. At 15:30 hours (zeitgeber time), rat hepatoma and SR^- human breast cancer xenograft tissue wedges were immediately harvested from rats under nembutal anesthesia, quickly frozen, and stored at −80°C until subsequent isolation of total RNA. Rat hepatoma and SR^- human breast cancer tissue wedges were embedded in ornithine carbamyl transferase compound, rapidly frozen in 2-methylbutane kept in dry ice, and stored frozen at −80°C until tissue sections were cut and thaw mounted onto glass slides. Tumor sections were processed for *in situ* hybridization using digoxigenin-labeled oligonucleotide antisense and sense probes for MT_1 and MT_2 melatonin receptors as previously described (32). The MT_1 oligonucleotide (GGGGTCGTACTGGAGAGTTCCGGTTTGCAGG, mer 31) corresponds to bases 108 to 138 of the partial rat MT_1 receptor sequence (Genbank accession no. U14409) and to bases 518 to 548 of the human MT_1 receptor sequence (Genbank accession no. U14108). The MT_2 oligonucleotide (CGGGTCATATTCTAGAGACCCCACAAAGAAA, mer 31) corresponds to bases 111 to 141 of the partial rat MT_2 receptor sequence (Genbank accession no. U28218) and to bases 537 to 556 of human MT_2 receptor sequence (Genbank accession no. U25341).

Nocturnal light exposure studies in rats and effects on tumor growth. Groups of tumor-bearing rats were housed in light exposure chambers that contained two separate solid-state, electromagnetic fluorescent ballasts with rapid-start, cool-white lamps connected to separate 24-hour timers and separated by a metal baffle (20). One ballast/lamp system (GE Watt-Miser, F34CW-RS-WM, 34-W bulb) in each chamber was set up to provide a direct, steady, bright light stimulus (345 $\mu W/cm^2$) at the animals' eye level during the light portion of a 12-hour light/12-hour dark cycle. The second ballast/lamp system (GE Starcoat, F32T8-SP-11, 32-W bulb) was adjustable with a combination of neutral filter density filter material (CINEGEL 3403, N-9) and electronic dimmer modules to emit steady, indirect light reflected off of the rear chamber wall

measured at the animals' eye level during the dark phase. The entire chamber was painted with Pro-Mar flat white paint (Sherwin Williams, St. Simon Island, GA), which has a high content of titanium dioxide that reflects light relatively uniformly across the visible spectrum. The light intensities during each 12-hour dark phase were different in each chamber as follows: 0 (total darkness), 0.02, 0.05, 0.06, 0.08, and 345 $\mu W/cm^2$ (constant light). These intensities were measured and regularly monitored at animals' eye level in the center of each cage using a Model IL 1400a Radiometer/Photometer (International Light, Inc., Newburyport, MA). Animals were exposed to the various light intensities beginning 2 weeks before tumor implantation and continuing thereafter until the end of each tumor growth period. Throughout each experiment, animal cage positions were rotated on a daily basis to minimize the potential effects of slight variations in the target, reflected light intensity within each chamber.

Nocturnal light exposure studies in human subjects and effects on tumor proliferative activity. At Thomas Jefferson University (Philadelphia, PA), healthy, premenopausal female volunteers (n = 12; mean age, 22.6 ± 0.6 years) were recruited without regard to either race or ethnic background and signed approved institutional review board consent forms. All subjects were free of any medication, including birth control pills, melatonin supplements, and/or β-blockers, and had regular sleep patterns. Each subject had antecubital venous blood samples drawn by a phlebotomist during the daytime (10:00 to 14:00 hours), nighttime (02:00 hours) following 2 hours of complete darkness, and nighttime (03:30 hours) following 90 minutes of ocular, bright, white light exposure at 580 $\mu W/cm^2$ (i.e., 2,800 lx) at eye level reflected from the wall. This exposure protocol is described in detail elsewhere (33). On the day following the final blood collection, whole-blood samples were shipped on ice via overnight express mail to the Bassett Research Institute (Cooperstown, NY), where they were stored refrigerated with constant gentle vortexing until tumor perfusions were done 24 hours later.

Assay of blood hormone levels in rats and human subjects. Rat serum and human plasma melatonin levels were measured via RIA as previously described (21, 23). Rat serum corticosterone and estradiol levels were measured using a commercial RIA kits (Diagnostic Products Corp., Los Angeles, CA).

Determination of tumor growth, linoleic acid uptake, and 13-hydroxyoctadecadienoic acid formation. Tumor growth rates, uptake of linoleic acid, and formation of 13-HODE were determined as previously described (22, 23). Due to variations in the absolute circulating concentrations of linoleic acid, tumor linoleic acid uptake is expressed as a percent of the actual arterial supply of linoleic acid to the tumor.

Determination of tumor cyclic AMP levels, DNA content, and [^{3}H]thymidine incorporation into DNA. Tumor levels of cyclic AMP (cAMP) were determined via ELISA (Amersham Biosciences, Piscataway, NJ) and [^{3}H]thymidine incorporation into DNA, and DNA content were determined as previously described (22, 23, 28).

Determination of tumor mitogen-activated protein/extracellular signal-regulated kinase kinase and extracellular signal-regulated kinase 1/2 activation. Western blot analysis of tumor mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK) and extracellular signal-regulated kinase 1/2 (ERK1/2) activation was done as previously described (34).

Statistical analyses. Statistical differences among group means were determined by a one-way ANOVA followed by Student-Neuman-Keul's post hoc test. Differences in the slopes of regression lines (i.e., tumor growth rates) among groups were determined by regression analyses and tests for parallelism (Student's t test). Four-variable parabolic fluence (dose)-response curve fitting of the above variables was done as previously described (35). Differences were considered statistically significant at $P < 0.05$.

Results

Melatonin responsiveness of human breast cancer xenografts perfused *in situ*. We previously reported that tissue-isolated rat hepatoma 7288CTC was highly responsive to the inhibitory effects of physiologic nocturnal melatonin concentrations with respect to tumor linoleic acid uptake/metabolism and proliferative activity following perfusion *in situ* (22, 23). Furthermore, the tumor inhibitory effects of melatonin were blocked by both MT_1 and MT_2 receptor antagonists (36). We now show that SR^+ MCF-7 human breast cancer xenografts were also very sensitive to the inhibitory actions of nocturnal melatonin concentrations by perfusing tissue-isolated tumors *in situ* with exogenous synthetic melatonin (1 nmol/L; see perfusion diagram in Fig. 5*B*). Following 2 hours of perfusion with melatonin, tumor cAMP levels were reduced by 40%, whereas both tumor linoleic acid uptake and 13-HODE formation were completely blocked (Table 1). These changes were accompanied by a decrease in ERK1/2 activation (Fig. 1) and a 71% decrease in tumor [^{3}H]thymidine incorporation into DNA, which were completely prevented by the coperfusion of tumors with both melatonin and 13-HODE (Fig. 1; Table 1). These findings indicated that, similar to rat hepatoma 7288CTC (22, 23), SR^+ MCF-7 human breast cancer xenografts were highly sensitive to a nocturnal, physiologic concentration of melatonin, which is consistent with the expression of MT_1 receptors in SR^+ MCF-7 cells (37). This high degree of melatonin sensitivity was also exhibited in tissue-isolated SR^- MCF-7 breast cancer xenografts that had evolved from and retained the histopathologic phenotype of the SR^+ MCF-7 parental tumor (see Materials and Methods) and also expressed MT_1 receptors (see below). Therefore, SR^- MCF-7 xenografts were used in testing the effects of melatonin suppression, via ocular light exposure during darkness in either rats or premenopausal human subjects, on breast cancer growth progression.

Melatonin receptor mRNA expression in rat hepatoma and human breast cancer xenografts. Although human cancer cell lines, including SR^+ MCF-7 breast cancer cells (37), have been shown to express either the MT_1 or MT_2 mammalian melatonin receptors (6), information about the expression of either of these melatonin receptors in solid tumors *in vivo* is extremely limited (38). This prompted us to determine with both reverse transcription-PCR and *in situ* hybridization techniques whether one or both of these melatonin receptors are expressed in tissue-isolated rat hepatoma 7288CTC and SR^+ and SR^- MCF-7 human breast cancer xenografts. PCR products of the expected size were obtained using primers specific for MT_1 and MT_2 melatonin receptors for both rat hepatoma and human breast cancer xenograft tissue (SR^+; Fig. 2*A*). No product was observed from a reaction with RNA alone. Hybridization signal for antisense MT_1-specific oligonucleotide was found in both rat hepatoma and SR^- MCF-7 human breast cancer xenograft tissue, whereas only rat hepatoma showed specific hybridization signal with MT_2 antisense specific oligoprobe (Fig. 2*B*). These hybridization signals were localized primarily to tumor epithelial cells. From a functional standpoint, however, melatonin receptor antagonist studies reveal that whereas rat hepatoma expresses both MT_1 and MT_2 melatonin receptors (39), human breast cancer xenografts only express functional MT_1 melatonin receptors (40).

Melatonin suppressive and tumor stimulatory responses to different intensities of nocturnal light exposure in rat hepatoma. We next determined the fluence-response (i.e., dose-response) effects of light exposure during darkness on signal transduction activity, linoleic acid uptake/metabolism, and growth in tissue-isolated rat hepatoma 7288CTC in view of its exquisite melatonin sensitivity (22–24). Groups of male rats housed in specially designed light exposure chambers (Fig. 3*A*) were exposed to different intensities of light during the dark phase of an

Table 1. Response of SR^+ MCF-7 human breast cancer xenografts to perfusion *in situ* with a physiologic nocturnal concentration of melatonin

Treatment	[^{3}H]Thymidine incorporation (dpms/μg DNA)	Linoleic acid uptake (% supply)	13-HODE production (ng/min/g)	cAMP (nmol/g)
Controls	47.4 ± 3.9	16.7 ± 1.7	0.97 ± 0.17	0.55 ± 0.11
Melatonin	13.6 ± 1.6*	0	0	0.33 ± 0.12*
Melatonin + 13-HODE	74.8 ± 6.3	0	333.17 ± 19.02	0.68 ± 0.06
13-HODE	78.0 ± 6.5	17.2 ± 3.2	363.18 ± 10.62	0.78 ± 0.17

NOTE: Effects of perfusion of tissue-isolated SR^+ MCF-7 human breast cancer xenografts *in situ* with synthetic melatonin (1 nmol/L) in the presence or absence of 13-HODE (12 μg/mL) on [^{3}H]thymidine incorporation into DNA, linoleic acid uptake, 13-HODE formation, and cAMP levels. Individual tumors (n = 3-4) were perfused over a 2-hour period (06:30 to 08:30 h) with whole blood collected from tumor-free donor rats during the early light phase when endogenous melatonin levels were low. Synthetic melatonin and 13-HODE were added to the whole-blood perfusate to achieve final concentrations of 1 nmol/L (232 pg/mL) and 12 μg/mL, respectively. Values are means ± SE.
*P < 0.05 vs controls and melatonin + 13-HODE (comparisons of relevance).

alternating 12-hour light/12-hour dark cycle beginning 2 weeks before tumor implantation and continuing thereafter until the end of the tumor growth experiment. Following 2 weeks of exposure of rats to increasing intensities of reflected light [0 (total darkness), 0.02, 0.05, 0.06, 0.08, and 345 (constant bright light) μW/cm^2] measured at rodent eye level during darkness, there was a dose-dependent increase in the percent suppression of serum melatonin levels (Fig. 4*A*). Continued exposure of rats to these light intensities following tumor implantation resulted in a corresponding dose-related increase in tumor growth rates (0.62-1.55 g/d; Fig. 3*B* and Fig. 4*E*), [^{3}H]thymidine incorporation into DNA (Fig. 4*C*), and DNA content (data not shown). In addition, the time to tumor onset decreased as the light intensity increased (Fig. 3*B*). Similarly, tumor linoleic acid uptake and 13-HODE formation (Fig. 4*B* and *D*), ERK1/2 activation (Fig. 3*B*), and cAMP levels (Table 2) increased as a function of increased light intensities during the dark phase. Four-variable parabolic fluence-response curves (Fig. 4*A-E*) showed a tight correlation (r^2 = 0.93-0.99) between percent melatonin suppression and tumor linoleic acid uptake, 13-HODE production, [^{3}H]thymidine incorporation into DNA, and growth rates. The ED_{50} values (i.e., half maximal suppression or stimulation) for melatonin suppression and tumor stimulatory responses to light during darkness ranged from 0.06 to 0.09 μW/cm^2. No significant effects of light exposure at any intensity was observed on serum corticosterone levels during the mid-dark period, albeit levels were depressed in the constant light-exposed group (Table 3). These results confirmed, in a highly melatonin-sensitive murine tumor model (22–24), that tumor signal transduction, linoleic acid uptake, and 13-HODE formation and growth could be modulated in a dose-dependent manner by changes in light intensity exposure during the dark phase of a diurnal lighting cycle.

Melatonin suppressive and tumor stimulatory responses to different intensities of nocturnal light exposure in human breast cancer xenografts. Previously, we reported that the female nude rat exhibits a robust nocturnal circadian melatonin rhythm, similar to that in humans in terms of its amplitude, phasing, and duration, that is completely suppressed by constant bright light (i.e., 300 lx or ~120 μW/cm^2 at rodent eye level; ref. 27). Furthermore, in nude rats bearing tissue-isolated SR^+ MCF-7 human breast cancer xenografts, constant bright light exposure resulted in a significant increase in tumor linoleic acid uptake/metabolism to 13-HODE and a marked stimulation of tumor growth compared with control rats on 12-hour light/12-hour dark cycle (27). We found that like tissue-isolated SR^+ MCF-7 xenografts (Fig. 1; Table 1), SR^- xenografts (Fig. 5*E* and *G*) perfused *in situ* were highly responsive to the tumor-inhibiting effects of a physiologic, nocturnal circulating concentration of melatonin (see below). We assessed the dose-response effects of light exposure during darkness on growth, linoleic acid uptake/metabolism to 13-HODE, and signal transduction activity in SR^- breast cancer xenografts under the same lighting conditions as outlined above for rats bearing rat hepatoma 7288CTC. As the light intensity increased, there was a corresponding increase in the percent suppression of serum melatonin levels in nude rats (Fig. 4*F*). There was also an accompanying marked, dose-related increase in tumor growth rates (0.22-0.74 g/d; Fig. 3*C* and Fig. 4*J*), [^{3}H]thymidine incorporation into DNA (Fig. 4*H*), and DNA content (data not shown). Latency to tumor onset decreased as the intensity of light during darkness increased (Fig. 3*C*). Tumor linoleic acid uptake and 13-HODE formation (Fig. 4*G* and *I*), ERK1/2 activation (Fig. 3*C*), and cAMP levels (Table 2) increased as a function of the light intensity exposure during the dark phase. As in the case of rat hepatoma, four-variable parabolic fluence-response curves (Fig. 4*F-J*) showed a tight correlation (r^2 = 0.97-0.99) between percent melatonin suppression and tumor linoleic acid uptake, 13-HODE formation,

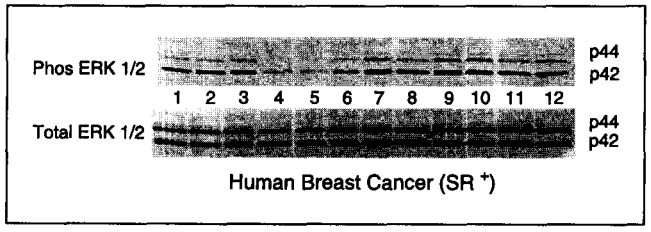

Figure 1. Tumor expression of ERK1/2. Western blot analysis of the effects of perfusion of tissue-isolated SR^+ MCF-7 human breast cancer xenografts *in situ* for 1 hour with melatonin (1 nmol/L) in the presence or absence of 13-HODE (12 μg/mL) on ERK1/2 (mitogen-activated protein kinase p44/p42) activation. *Top*, phosphorylated ERK1/2; *bottom*, total ERK1/2. Total protein (27 μg) was loaded per well. Controls (*lanes 1-3*), melatonin-treated (*lanes 4-6*), melatonin + 13-HODE (*lanes 7-9*), 13-HODE (*lanes 10-12*), and MW markers (*lane 13*). See Table 2 for further details.

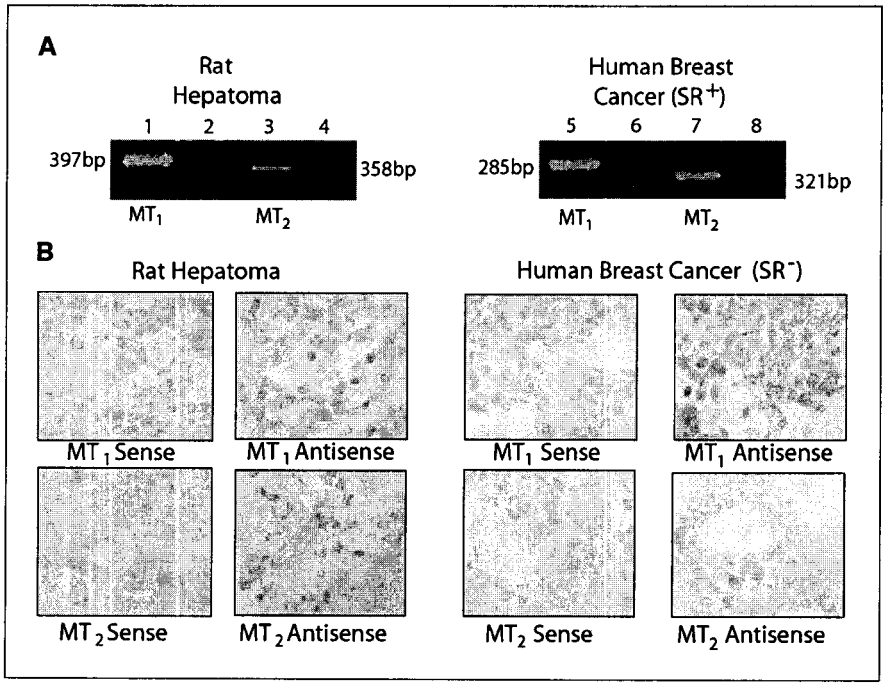

Figure 2. *A*, expression of MT_1 and MT_2 melatonin receptor mRNA transcripts in tissue-isolated rat hepatoma 7288CTC and SR^+ MCF-7 human breast cancer xenograft tissue. *Lane 1*, reverse transcription-PCR reaction with rat hepatoma cDNA using primers specific for the rat MT_1 melatonin receptor, yielding the expected 397-bp product. *Lane 2*, negative control reaction with the MT_1 primers using an equivalent amount of rat hepatoma total RNA. *Lane 3*, reaction with rat hepatoma cDNA using primers specific for the rat MT_2 melatonin receptor, yielding the expected 368-bp product. *Lane 4*, negative control reaction with the MT_2 primers using an equivalent amount of rat hepatoma total RNA. *Lane 5*, reaction with human breast cancer xenograft cDNA using primers for the human MT_1 melatonin receptor, yielding the expected 285-bp product. *Lane 6*, negative control reaction with MT_1 primers using an equivalent amount of human breast cancer xenograft total RNA. *Lane 7*, reaction with human breast cancer xenograft cDNA using primers specific for the human MT_2 melatonin receptor, yielding the expected 321-bp product. *Lane 8*, negative control reaction with MT_2 primers using an equivalent amount of human breast cancer xenograft total RNA. *B*, representative photomicrographs showing *in situ* hybridization of tumor tissue from tissue-isolated rat hepatoma 7288CTC and an SR^- MCF-7 human breast cancer xenograft tissue using digoxigenin-labeled sense and antisense oligonucleotides specific for either MT_1 or MT_2 melatonin receptors. Hybridization signal for the antisense MT_1-specific oligonucleotide was found in both rat hepatoma and human breast cancer, whereas only hepatoma showed specific hybridization signal with the MT_2 antisense-specific oligoprobe.

[^{3}H]thymidine incorporation into DNA, and growth rates. ED_{50} values for these processes ranged from 0.06 to 0.07 $\mu W/cm^2$. No significant effects of light exposure at any intensity was observed on serum either corticosterone or estradiol levels during the mid-dark period, albeit levels of these hormones tended to be decreased or increased, respectively, in the constant light-exposed group (Table 3). These results confirmed that suppression of physiologic, nocturnal melatonin levels was dependent upon the magnitude of the light intensity to which nude rats were exposed during the dark phase. This suppression of melatonin levels translated to an intensity-dependent stimulation of human breast cancer signal transduction activity, linoleic acid uptake/metabolism, and growth.

Proliferative activity in rat hepatoma and human breast cancer xenografts perfused *in situ* with blood collected from human subjects following ocular exposure to bright while light at night. We next evaluated the acute effect of the human circadian melatonin signal and its suppression by light at night on signal transduction activity, linoleic acid uptake/metabolism, and proliferative activity in rat hepatomas and human breast cancer xenografts. This involved the unique approach of perfusing these tumors, *in situ*, with whole blood collected from healthy, premenopausal human volunteers either during the daytime, nighttime, or following exposure to 580 $\mu W/cm^2$ (i.e., 2,800 lx) of white fluorescent light at night (ref. 33; Fig. 5*A* and *B*). As expected, plasma melatonin titers in human volunteers were 5-fold higher during the night compared with daytime levels of melatonin (Fig. 5C). The perfusion of tissue-isolated rat hepatomas and human breast cancer xenografts *in situ* with melatonin-rich blood collected during the night resulted in tumor cAMP levels that were reduced by 86% compared with control tumors perfused with melatonin-deficient blood collected during the daytime (Fig. 5*D* and *E*). Similarly, tumor linoleic acid uptake and 13-HODE formation were reduced by as much as 96% following perfusion with melatonin-rich blood collected from human subjects during darkness compared with control tumors perfused with daytime-collected, melatonin-deficient blood (Fig. 5*D-G*). Furthermore, tumor incorporation of [^{3}H]thymidine into DNA following perfusion with melatonin-rich blood collected during the night was decreased by 63% to 73% compared with control tumors perfused with daytime-collected blood (Fig. 5*F* and *G*). Tumor DNA content was also significantly ($P < 0.05$) reduced following perfusion with nighttime-collected versus daytime-collected human blood (data not shown). Following perfusion with nighttime-collected, melatonin-rich blood, tumor MEK and ERK1/2 activation was markedly reduced compared with tumors perfused with daytime-collected, melatonin-deficient blood (Fig. 5*D-G*). In contrast, tumors perfused with melatonin-deficient blood obtained from subjects during the night following 90 minutes of light exposure evinced signal transduction activity,

linoleic acid uptake/metabolism, and proliferative activity that remained at the same levels as control tumors perfused with daytime-collected, melatonin-deficient blood (Fig. 5*D-G*). Similar results were obtained in a small subset of SR^+ human breast cancer xenografts perfused *in situ* with blood collected from human subjects during the night and following exposure to light at night (data not shown).

We then determined whether the tumor-suppressive effects of nighttime-collected, melatonin-rich blood from human subjects were, in fact, due to melatonin. Tissue-isolated tumors were perfused with melatonin-deficient blood, collected at night following 90 minutes of bright light exposure, to which a nocturnal concentration of synthetic melatonin was added (i.e., 500 pmol/L or 116 pg/mL). The tumor-stimulating effects of melatonin-deficient blood collected at night following light exposure were prevented with this melatonin replacement strategy (Fig. 5*D-G*). Moreover, the addition of the nonselective MT_1/MT_2 melatonin receptor antagonist S20928 (36) to the blood perfusate collected during the night completely blocked the tumor-suppressive effects of this nocturnal, physiologically melatonin-rich blood (Fig. 5*D-G*).

Discussion

Our results show that tissue-isolated rat hepatoma 7288CTC expressed mRNA transcripts for both MT_1 and MT_2 melatonin receptors both of which are functionally expressed and mediate appropriate tumor responses to nocturnal, physiologic melatonin concentrations and to melatonin receptor antagonists (22, 23, 39). Tissue-isolated SR^+ MCF-7 human breast cancer xenografts also expressed mRNA transcripts for both MT_1 and MT_2 melatonin receptors, whereas SR^- xenografts expressed only MT_1 melatonin receptors that are functionally expressed and mediate appropriate tumor responses to melatonin and melatonin receptor antagonists (40). In the present investigation, we confirmed that rat hepatoma 7288CTC, and both SR^+ and SR^- human breast cancer xenografts were highly sensitive to the ability of nocturnal, physiologic circulating levels of melatonin to suppress tumor proliferative activity *in vivo*. Additionally, ocular exposure of tumor-bearing rats to increasing intensities of light during darkness resulted in a dose-dependent suppression of melatonin blood levels that translated to a light intensity–dependent stimulation of signal transduction activity and linoleic acid–dependent rat liver and human breast tumor growth rates. Rat hepatomas and human breast cancer xenografts perfused *in situ* with nocturnal, physiologically melatonin-rich blood collected from human, premenopausal female volunteers during the night exhibited suppressed signal transduction and linoleic acid–dependent proliferative activity. Suppressed tumor growth signaling activity was absent in tumors perfused with melatonin-deficient blood collected from human subjects during the daytime or following ocular exposure to white light at night.

The circadian system exerts an important influence over many physiologic and metabolic activities, such as the sleep/wake cycle, body temperature, intermediary metabolism, and endocrine functions, and a number of disease processes, including myocardial infarction, stroke, and asthma (19, 41). Indirect evidence is emerging that disruption of circadian clock activity is associated with cancer in humans, and that desynchronization of internal timekeeping adversely tips the host/cancer balance in favor of tumor development and growth in experimental models of tumorigenesis (42, 43). Cancer cells themselves express the same clock genes found in the suprachiasmatic nuclei (44), and many common molecular elements are shared between the circadian clock and cell cycle (45, 46). A mutation in the circadian clock gene *per2* results in an increase in spontaneous tumorigenesis in mice (46), whereas an elevated risk of premenopausal breast

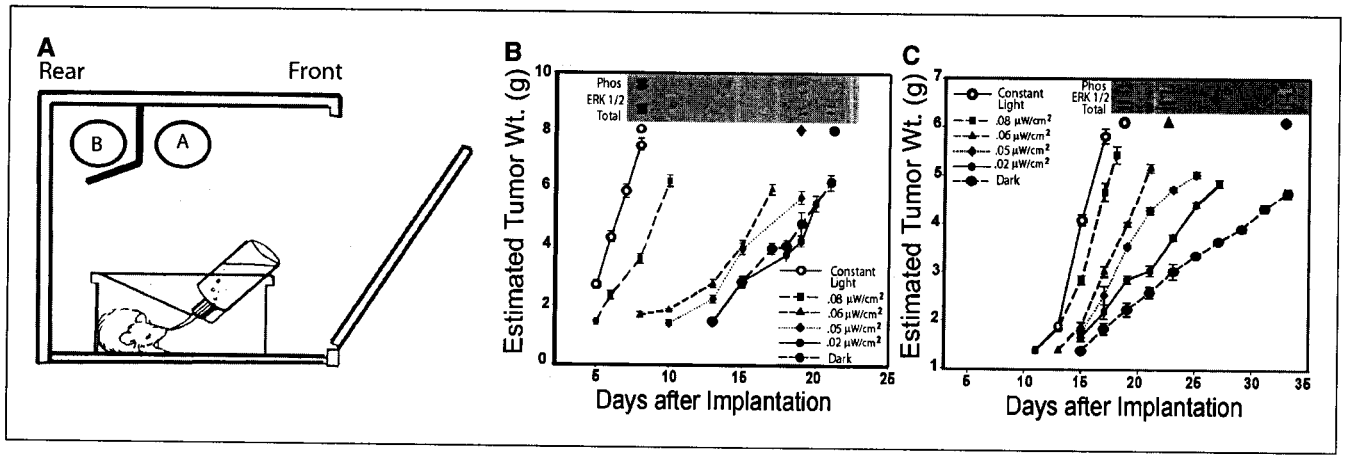

Figure 3. Ocular exposure of tumor-bearing rats to light during darkness. *A*, schematic representation of a side, cut-a-way view of a light exposure chamber showing the relationship of the two separate solid-state, electromagnetic fluorescent ballasts with rapid-start, cool-white fluorescent lamps separated by a metal baffle. The ballast/lamp system adjacent to the hinged front wall of the chamber provides a direct, steady, bright light stimulus (345 $\mu W/cm^2$) at the animals' eye level during the light portion of a 12-hour light/12-hour dark cycle. The second ballast/lamp system adjacent to the rear wall was adjustable with neutral density filters and electronic dimmer modules to emit steady, indirect light reflected off of the rear chamber wall measured at the animals' eye level during the dark phase only. Families of tumor growth curves reflecting the effects of exposure of rats bearing either (*B*) hepatoma 7288CTC (male rats) or (*C*) SR^- MCF-7 human breast cancer xenografts (nude female rats) to increasing intensities of light during the entire dark phase (12 hours) of a 12-hour light/12-hour dark cycle on tumor growth and ERK1/2 activation (phosphorylation). Light exposures began 2 weeks before tumor implantation and continued throughout each tumor growth period in the light chambers. At the end of each growth period for light treatment groups (dark, 0.05 $\mu W/cm^2$ and 345 $\mu W/cm^2$), tumors were freeze-clamped under liquid N_2 and ERK1/2 activation was determined by Western blotting (one representative tumor from each of these groups is depicted). *Points*, mean ($n = 6$ rats, tumors per group); *bars*, SE. Slopes of regression lines and tests for parallelism among growth curves show that rats exposed to 345, 0.08, 0.06, and 0.05 $\mu W/cm^2$ had significantly ($P < 0.05$) faster tumor growth rates compared with those exposed to 0.02 and 0.00 $\mu W/cm^2$ (dark control). All intensities of light versus dark control and each other ($P < 0.05$).

cancer has been associated with length polymorphism in the clock gene *per3* (47). In spite of this progress, however, no definitive outputs linking the human circadian clock with the processes governing human malignancy, particularly breast cancer, have been identified. Moreover, the way in which circadian disruption by light at night may lead to an increased risk of breast cancer, as suggested by the increased breast cancer risk in night shift workers (9–11), is unknown.

The pineal synthesis and secretion of melatonin represents an important nocturnal output component of the central circadian

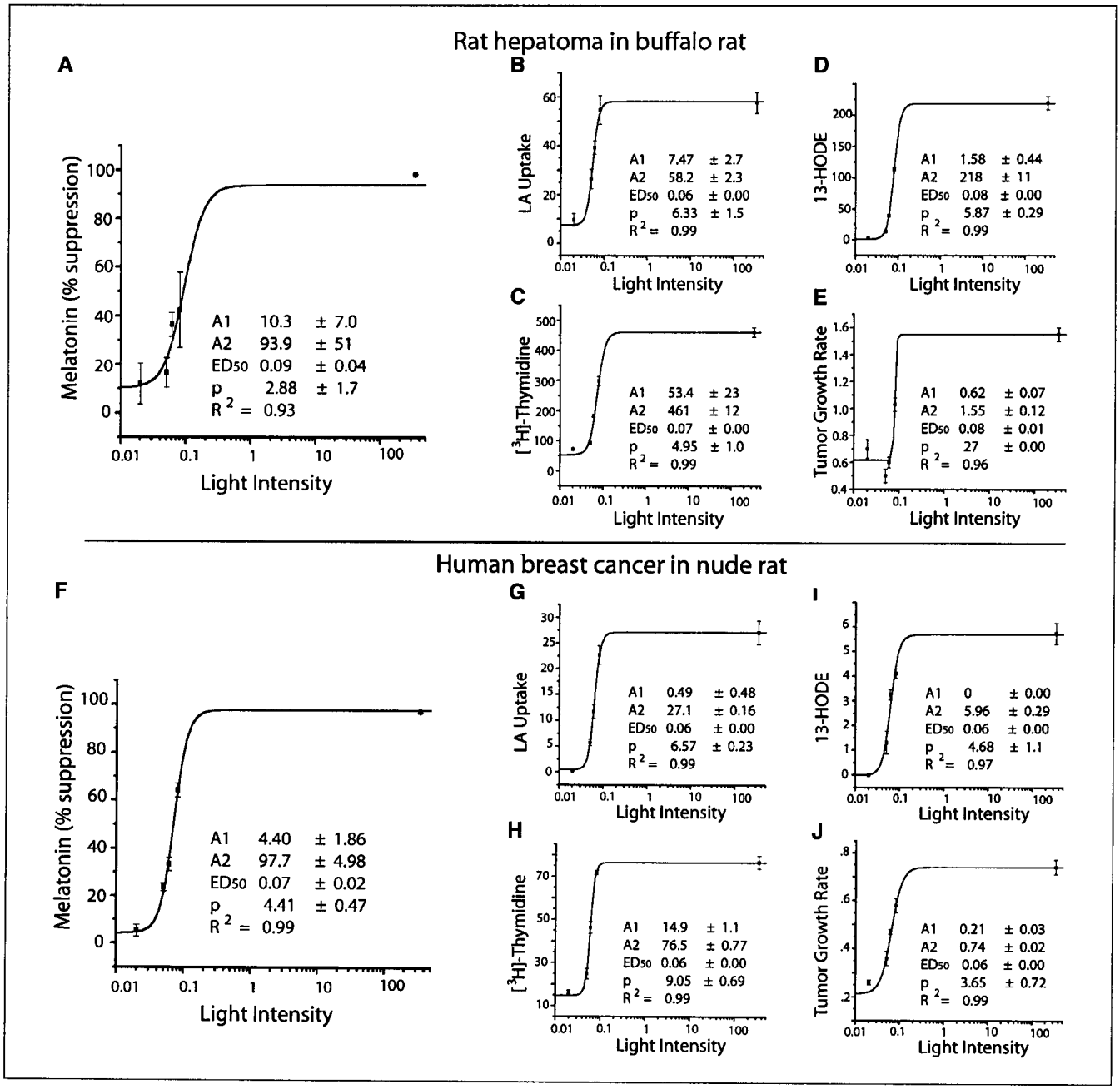

Figure 4. Four-variable parabolic curves. Relationship between increasing intensities of white fluorescent light and suppression of plasma melatonin levels in either male rats (*A*) or female nude rats (*F*) following 2 weeks of light exposure during the entire dark phase (12 hours) of a 12-hour light/12-hour dark cycle. Plasma samples were collected via decapitation when melatonin peaks at the mid-dark phase (2400 hours). The fluence-response relationship between light intensity ($\mu W/cm^2$) and percent change (suppression) in melatonin levels compared with the dark control levels was determined by the fluence-response curve formula: $Y = [(A_1 - A_2) / 1 + (X/X_{50})^P] + A_2$, where A_1 = theoretical initial Y value for curve; A_2 = theoretical final Y value for curve; ED_{50} = X value of curve midpoint Y value; P = power function describing curve slope; R^2 = correlation coefficient. *Points,* means (n = 6); *bars,* SE. Effects of light exposure on male rats bearing hepatoma 7288CTC (*B-E*) or female nude rats bearing SR$^-$ MCF-7 human breast cancer xenografts (*G-J*) to increasing intensities of light during the entire dark phase (12 hours) of a 12-hour light/12-hour dark cycle on tumor linoleic acid metabolism and growth activity. Each of the four-variable parabolic curves shows the fluence-reponse relationship between light intensity ($\mu W/cm^2$) and tumor linoleic acid uptake (% supply), 13-HODE formation (ng/min/g), [^{3}H]thymidine incorporation into DNA(dpm/μg DNA; determined at the end of each tumor growth period in Fig. 3), and growth rates (g/d; slopes of the regression lines of tumor growth curves of Fig. 3) as determined by the formula above. *Points,* mean (n = 6); *bars,* SE.

Table 2. Tumor levels of total cAMP in response to increasing light intensities during darkness

Light intensity ($\mu W/cm^2$)	Tumor cAMP concentration (nmol/g)	
	Rat hepatoma 7288CTC	SR^- MCF-7 human breast cancer xenografts
Dark	0.18 ± 0.01	0.02 ± 0.0
0.02	0.45 ± 0.06*	0.09 ± 0.01†
0.05	0.50 ± 0.06*	0.27 ± 0.01†
0.06	0.53 ± 0.03*	0.26 ± 0.03†
0.08	0.48 ± 0.03*	0.31 ± 0.04†
Constant light, 345	0.72 ± 0.04‡	0.33 ± 0.03§

NOTE: Effects of exposure of rats bearing either hepatoma 7288CTC (male rats) or SR^- MCF-7 human breast cancer xenografts (nude female rats) to increasing intensities of light during the entire dark phase (12 hours) of a 12-hour light/12-hour dark cycle on tumor cAMP concentrations. At the end of the growth period for each of the light treatment groups, tumors were freeze-clamped under liquid N_2 and then assayed for cAMP via an ELISA. See Figs. 2 and 3 legends for further details. Value are means ± SE; n = 6 rats (tumors per group).
*$P < 0.05$, hepatoma vs dark control.
†$P < 0.05$, breast vs dark control (except 0.08 $\mu W/cm^2$).
‡$P < 0.05$, hepatoma vs all other groups.
§$P < 0.05$, breast vs all other groups (except 0.08 $\mu W/cm^2$).

pacemaker mechanism (17–19). Melatonin exerts an oncostatic action on a variety of neoplasms in experimental models of cancer *in vitro* and *in vivo*, most notably in those involving mammary carcinogenesis (6–8, 37, 48). Based on human breast cancer perfusion studies described here, however, we now present the first evidence establishing the fact that physiologic, nocturnal circulating levels of melatonin in humans suppress human breast cancer proliferative activity *in vivo* as they do in rat hepatoma 7288CTC. This occurs via an MT_1 melatonin receptor–mediated reduction in cAMP formation, a blockade of linoleic acid uptake/metabolism, via 15-lipoxygenase-1, to 13-HODE leading to a down-regulation in the activation of the MEK/ERK1/2 growth signaling pathway, which is related to both the epidermal growth factor and insulin-like growth factor-I receptor systems (49–51). The fact that similar results were obtained in tissue-isolated rat hepatoma 7288CTC and SR^- and SR^+ human breast cancer xenografts suggests that this is a more general mechanism by which nocturnal melatonin concentrations inhibit cancer growth *in vivo*, irrespective of tumor type and/or steroid receptor status.

As discussed above, the development and/or growth of experimental mammary or liver cancer is accelerated in response to either pinealectomy or ocular exposure to constant bright light, ostensibly due to the lack of a nocturnal melatonin signal (25). In both rodents and humans, light exposure suppresses pineal melatonin production in an intensity-, wavelength-, and duration-dependent manner (20, 21, 52). The present results represent the first demonstration that the rates of both rat hepatoma and human breast cancer growth increase as a function of a light intensity-dependent suppression of nocturnal circulating melatonin levels. The highly correlated ED_{50} values among the fluence-response curves indicate a remarkable degree of fidelity between light during darkness-induced melatonin suppression and the stimulation of signal transduction activity, linoleic acid uptake/metabolism, and tumor growth. In the case of human breast cancer xenografts, only a 15% to 25% (0.040-0.055 $\mu W/cm^2$) suppression of the circadian amplitude of nocturnal melatonin was required to cause the first significant stimulation of growth and linoleic acid uptake/metabolism to 13-HODE. The lack of significant light-induced changes in either plasma corticosterone or estradiol levels during darkness indicated that the tumor growth results were not due to light during darkness-induced alterations in the levels of adrenocortical or ovarian hormones known to influence breast cancer risk (53, 54). The lack of a significant effect of light during darkness on estradiol levels, and the fact that both rat hepatomas and SR^- breast cancer xenografts responded to light-induced nocturnal melatonin suppression, convincingly argues against the postulate that increased breast cancer risk, resulting from pineal hypofunction, and reduced melatonin output, is due to hyperestrogenism (12). It is important to note that Stevens (5) subsequently modified his original hypothesis (4), involving light-induced nocturnal melatonin suppression and hyperestrogenism, to include an effect of light at night in suppressing the direct antiproliferative effects of physiologic melatonin on human breast cancer growth (7, 8).

Table 3. Serum levels of corticosterone and estradiol in rats exposed to increasing light intensities during darkness

Light intensity ($\mu W/cm^2$)	Nude rat serum corticosterone (ng/mL)	Nude rat serum estradiol (pg/mL)	Buffalo rat serum corticosterone (ng/mL)
Constant light, 345	196.2 ± 53.5	25.1 ± 11.3	53.25 ± 23.7
0.08	265.0 ± 28.17	19.9 ± 10.1	120.83 ± 19.6
0.06	322.7 ± 43.8	27.1 ± 16.8	105.08 ± 25.8
0.05	289.3 ± 41.8	20.3 ± 10.78	155.5 ± 19.1
0.02	266.8 ± 34.7	25.1 ± 5.9	138.75 ± 26.8
Dark	266.1 ± 34.4	19.3 ± 10.7	111.08 ± 29.5

NOTE: See Figs. 3 and 4 legends for further details. Blood samples were taken during the middle of the true dark or subjective dark phase. Mean serum hormone (±SE); n = 6 rats per group. There were no significant differences in hormone levels among any of the groups exposed to different light intensities.

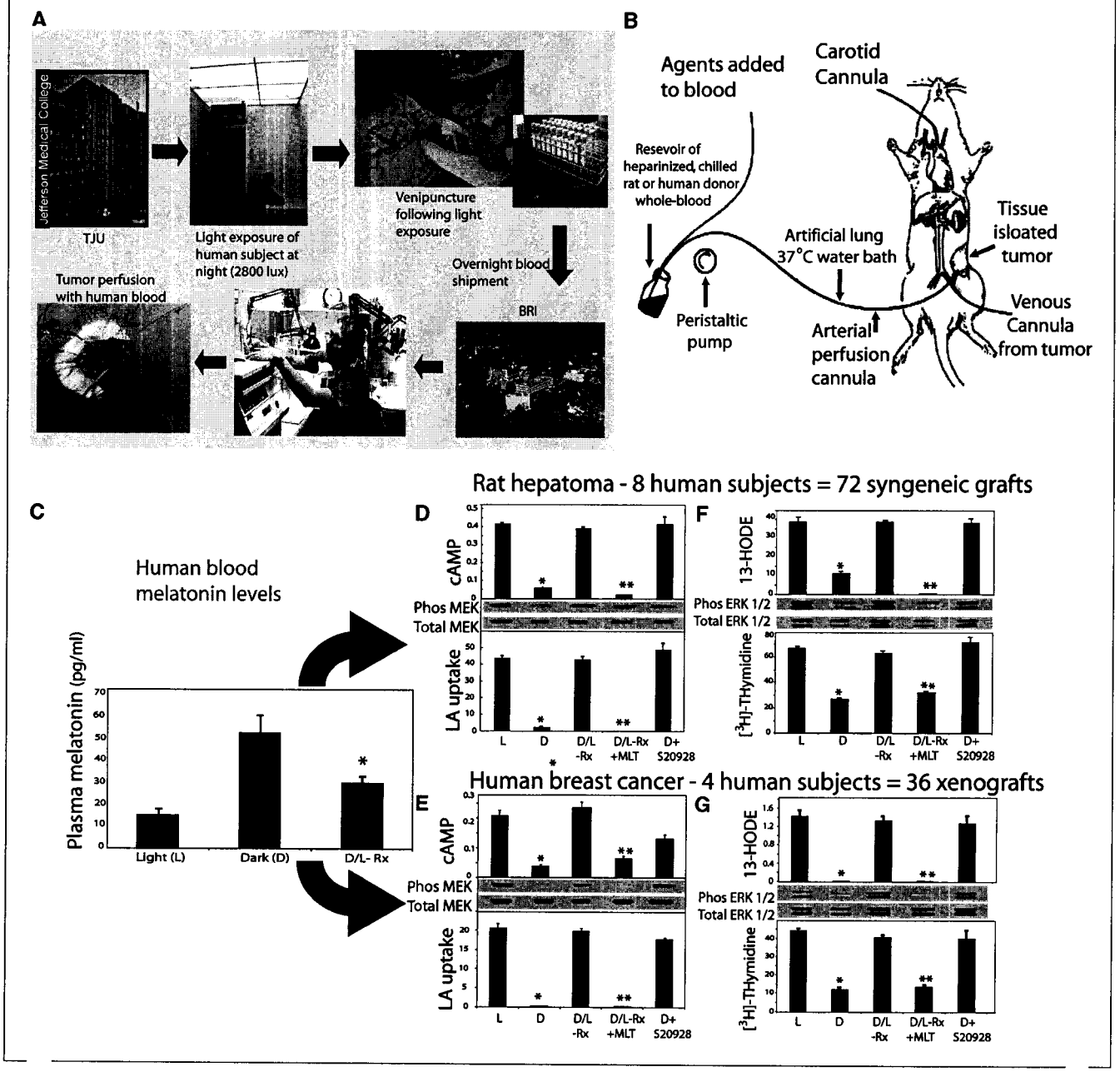

Figure 5. Ocular exposure of human subjects to light at night. *A*, flow chart showing the sequence of exposure of human subjects to light at night and blood collection at Thomas Jefferson University and shipment of samples to the Bassett Research Institute for perfusion through tumors *in situ*. *B*, schematic diagram of setup for perfusion of tissue-isolated tumors (rat hepatoma 7288CTC or SR^- MCF-7 human breast cancer xenografts) *in situ* with human whole blood collected from human volunteers exposed to light at night. *C*, *columns*, mean plasma melatonin levels in 9 of 12 healthy premenopausal female volunteers during the mid-light phase (1200 hours), mid-dark phase (0230 hours), or following 90 minutes of ocular exposure to 2,800 lx of indirect, white fluorescent light (measured at subject eye level) during the mid-dark phase; *bars*, SE. *D-G*, *columns*, mean tumor cAMP levels (nmol/g), linoleic acid (*LA*) uptake (% supply), and MEK activation (*D* and *E*), 13-HODE formation (ng/min/g), [^{3}H]thymidine incorporation into DNA (dpm/μg DNA), and ERK1/2 activation (*F* and *G*) in tissue-isolated rat hepatoma 7288CTC and SR^- MCF-7 human breast cancer xenograft following perfusion *in situ* for 1 hour with human donor whole blood harvested during the light phase (*L*), dark phase (*D*), or following 90 minutes of ocular exposure to 580 μW/cm^2 (i.e., 2,800 lx) of light during the dark phase (*D/L-Rx*); *bars*, SE. Other groups of tumors were perfused with D/L-Rx donor blood to which a physiological nocturnal concentration (500 pmol/L or 116 pg/mL) of synthetic melatonin (*MLT*) was added; other tumors were perfused with D donor blood to which MT_1/MT_2 receptor antagonist S20928 (1 μmol/L) was added; n = 3 human donor blood samples per subject (one blood sample per three tumors) or n = 9 tumors per subject = 108 perfused tumors. *, $P < 0.05$, D versus L, D/L-Rx and D + S20928. **, $P < 0.05$, D/L-Rx + MLT versus D/L-Rx.

It would be impossible, both logistically and ethically, to test the light at night/breast cancer hypothesis in humans by chronically exposing healthy women to bright light each night under controlled laboratory conditions. Thus, the perfusion of tissue-isolated tumors *in situ* with whole blood collected from healthy human female volunteers represents a unique approach to directly assess the effect of the human nocturnal, circadian melatonin output signal on proliferative, metabolic, and signal transduction

activity in human breast cancer xenografts. In fact, in our opinion, this is the only strategy available to evaluate the potential consequences of circadian disruption of the human melatonin signal, by ocular exposure of human subjects to bright, white light at night, on human tumor growth activity. Consistent with the results of perfusion *in situ* of rat hepatomas (22, 23) and human breast cancer xenografts (see above) with rat donor whole blood to which exogenous melatonin was added at a nocturnal physiologic concentration, the present findings show that physiologically melatonin-rich blood, collected during the night from human subjects, markedly inhibited signal transduction activity (i.e., cAMP, MEK, ERK1/2), linoleic acid uptake/metabolism to 13-HODE and proliferative activity (i.e., [^{3}H]thymidine incorporation into DNA, DNA content) in both rat hepatoma and human breast cancer xenografts (i.e., both SR^- and SR^+ tumors). Each of these oncostatic effects were completely eliminated upon suppression of the nocturnal, circadian melatonin signal following the exposure of human subjects to 90 minutes of 2,800 lx (580 $\mu W/cm^2$) of indirect, white fluorescent light. These tumor-suppressive effects of nighttime-collected, melatonin-rich blood were also totally blocked by the nonselective melatonin receptor (MT_1/MT_2) antagonist S20928 (36), indicating that they were, in fact, due to the presence of physiologically elevated levels of circulating melatonin acting via a melatonin receptor–mediated process. This is further supported by the ability of melatonin, added at a physiologic nocturnal concentration, to melatonin-deficient blood collected following light exposure at night, to restore the tumor-inhibitory responses.

To our knowledge, the present findings are the first to establish a role for the nocturnal, physiologic melatonin signal from the pineal in the prevention and progression of an overt human disease. More specifically, melatonin is now the first soluble, nocturnal anticancer signal to be identified in humans that directly links the central circadian clock with some of the important mechanisms regulating human breast carcinogenesis and possibly the progression of other malignancies as well. These findings also provide the first definitive nexus between the exposure of healthy premenopausal female human subjects to bright white light at night and the enhancement of human breast oncogenesis via disruption (i.e., suppression) of the circadian, oncostatic melatonin signal. The suppression of circadian melatonin production by ocular exposure to bright white light at night, leading to augmented nocturnal tumor uptake of dietary linoleic acid and its conversion to mitogenically active 13-HODE, can now be afforded serious consideration as a new risk factor for human breast cancer (4, 5) and a significant public health issue (55). The high nocturnal dietary intake of fat, particularly linoleic acid, reported for night shift workers (56, 57), coupled with melatonin suppression by exposure to light at night provide a firm mechanistic basis upon which to explain, in part, the increased risk of breast cancer in some women who work night shifts for many years (9–11). Thus, strategies to preserve the integrity of the circadian melatonin signal (i.e., avoidance of bright light at night, intelligent lighting design, circadian-timed physiologic melatonin supplementation) coupled with modifications in nocturnal dietary fat intake may offer a unique approach to the prevention of breast cancer, and perhaps other melatonin-sensitive cancers, in our increasingly 24-hour society.

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