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Original Research

Dietary Melatonin and Omega-3 Fatty Acids Induce Human Cancer Xenograft Regression In Vivo in Rats by Suppressing Linoleic Acid Uptake and Metabolism

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Melatonin, the circadian nighttime neurohormone, and eicosapentaenoic acid (EPA) and docosahexaenoic acids (DHA), which are omega-3 fatty acids (FA) found in high concentrations in fish oil (FO) and plants, abrogate the oncogenic effects of linoleic acid (LA), an omega-6 FA, on the growth of rodent tumors and human breast, prostate, and head and neck squamous cell carcinoma (HNSCC) xenografts in vivo. Here we determined and compared the long-term effects of these inhibitory agents on tumor regression and LA uptake and metabolism to the mitogenic agent 13-[S]-hydroxyoctadecadienoic acid (13-[S]-HODE) in human prostate cancer 3 (PC3) and FaDu HNSCC xenografts in tumor-bearing male nude rats. Rats in this study were split into 3 groups and fed one of 2 diets: one diet containing 5% corn oil (CO, high LA), 5% CO oil and melatonin (2 µg/mL) or an alternative diet 5% FO (low LA). Rats whose diet contained melatonin had a faster rate of regression of PC3 prostate cancer xenografts than those receiving the FO diet, while both in the melatonin and FO groups induced the same rate of regression of HNSCC xenografts. The results also demonstrated that dietary intake of melatonin or FO significantly inhibited tumor LA uptake, cAMP content, 13-[S]-HODE formation, [^{3}H]-thymidine incorporation into tumor DNA, and tumor DNA content. Therefore, long-term ingestion of either melatonin or FO can induce regression of PC3 prostate and HNSCC xenografts via a mechanism involving the suppression of LA uptake and metabolism by the tumor cells.

Abbreviations: A-V, arterial-venous differences; cAMP, cyclic-adenosine monophosphate; AKT, serine-threonine protein kinase; DHA, docosahexaenoic acid; EGFR, epithelial growth factor receptor; EPA, eicosapentaenoic acid; ERK1/2, extracellular signal regulated kinase p44/46 (MAPK, mitogen-activated protein kinase); FA, fatty acid; FFAR, free fatty acid receptor; FO, fish oil; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; G protein, guanine nucleotide binding protein; HNSCC, Head and Neck Squamous Cell Carcinoma; 13-[S]-HODE, 13-[S]-hydroxyoctadecadienoic acid; LAN, light at night; LA, linoleic acid; MEK, mitogen-activated protein kinase kinase; PC3, Prostate Cancer-3; TFA, total fatty acids.

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Although significant progress has been made in cancer prevention, early detection, and treatment, cancer is still the number 2 cause of death in the US.[23] Recent reports indicate that while overall cancer incidence rates are leveling off in males and increasing slightly in females (2013 to 2017),[23,46] an estimated 191,930 new cases of prostate cancer (1 in 9 men) will be diagnosed in 2020, and 33,330 men will die from this disease. Another cancer of interest to our laboratory is head and neck squamous cell carcinoma (HNSCC), the sixth most common malignancy worldwide, which is estimated to be diagnosed in 48,200 men and 17,430 women this year.[46] This carcinoma resembles squamous cells of the upper layer of the skin and may occur in various areas of the body, including mucous membranes of the mouth, nose, and throat. Despite current medical interventions, 2020 mortality rates in men and women are estimated to be 17,430 (22%) and 3,740 (21%), respectively.

Dietary fats play an essential role in human and rodent cancer growth and metabolism.[24] The metabolism and growth of transplantable rodent hepatomas,[5,15,16,37-43] human mammary cancers,[3,4,6-8,44,48] and leiomysarcomas[18] are stimulated by LA, an omega-6 (ω-6; characterized by the presence of a double bond 6 atoms away from the terminal methyl group in their chemical structure) FA. LA is the most abundant FA in the Western diet and is found in high concentrations in corn oil (CO). Originally described in previous literature, the strong growth-stimulatory response of LA is a direct result of its conversion to 13-[S]-HODE in hepatoma 7288CTC and MCF-7 steroid receptor-positive and –negative human breast cancer xenografts.[5-8,45] The LA growth-stimulatory response is suppressed in these tumors by the circadian neurohormone melatonin[3-8,17,18] and omega-3 (ω-3) FAs.[41,44-45] When circulating levels of melatonin are low (i.e. during daytime), LA uptake by cancer cells is maximal due to cAMP enhancement of FA activity via a cAMP-dependent protein kinase.

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Once LA enters the tumor cell, it is rapidly oxidized to 13-[S]-HODE by a 15-lipoxygenase associated with the intracellular domain of the epidermal growth factor (EGF) receptor (EGFR). The elevated 13-[S]-HODE levels, in turn, stimulate EGFR autophosphorylation and tyrosine phosphorylation of downstream signal transduction proteins such as ERK 1/2 (MAPK), resulting in transcriptional activation and increased EGF-responsive mitogenesis.[9,12,20,21,31] Conversely, during the night, when melatonin levels are elevated, melatonin suppresses tumor LA uptake, 13-[S]-HODE production, and tumor growth via inhibitory G-protein-coupled melatonin receptors (MT_1 and/or MT_2).[5,13] Subsequently, previous work proposed the involvement of a similar signal transduction pathway for human MCF-7 breast cancer associated with binding of EPA and DHA to a putative inhibitory G-protein-coupled free fatty acid receptor (FFAR).[44,45] Both signal transduction pathways are associated with an inhibition of adenyl cyclase activity and subsequent reduction in tumor cAMP levels. The decreased cAMP levels result in lower FA transport activity, leading to a suppression of LA uptake by the cancer cells. As less LA enters the tumor cells, less 13-[S]-HODE is produced, resulting in abrogated EGF-responsive mitogenesis. These elegant early studies provided strong support for the oncostatic impact of both melatonin and FO on the growth of rodent hepatoma[3-6,40-43] and human breast tumor xenografts.[6-8,44,45]

In our previous experiments using MCF-7 human breast cancer xenografts,[44,45] HSNCC xenografts[17] and rodent hepatoma 7288CTC,[41-44] tumors were treated with EPA, the most abundant ω-3 FA in FO, or melatonin for 1 to 2 h during perfusion in situ. In these studies, we observed that both treatments led to inhibition of saturated, monounsaturated, and ω-6 polyunsaturated FA uptake, 13-HODE from LA formation, and [^{3}H]-thymidine incorporation into DNA. These inhibitions were reversed by pertussis toxin (PTX) and 8-bromo-cyclic adenosine monophosphate (8-Br-cAMP), suggesting that EPA, like melatonin, is a ligand that interacts with inhibitory G protein-coupled receptors to decrease the tumor content of cAMP, thereby blocking LA uptake, 13-[13]-HODE release, and [^{3}H]-thymidine incorporation. Although in situ perfusion is a short-term experiment, another study showed that long-term feeding of dietary FO (containing both EPA and DHA), as compared with 5% CO, suppressed the growth of rat hepatoma 7288CTC in vivo by deactivating a specific, LA-dependent, growth-promoting signaling pathway.[47]

In the present study, we hypothesized that long-term treatment of animals bearing either human prostate cancer or HNSCC xenografts with a 5% CO diet containing either melatonin (2 µg/mL) or 5% FO, would induce tumor regression as compared with the tumor growth stimulatory effects of a diet with only 5% CO. In view of the ability of melatonin and ω-3 FA to directly suppress tumor LA uptake and the consequent metabolism of LA to mitogenically active 13-[S]-HODE, we further tested whether chronic treatment with dietary melatonin or FO would induce tumor regression via a mechanism consistent with melatonin- or FO-suppressed LA uptake and conversion to 13-[S]-HODE. To do this, we employed a unique tissue-isolated tumor model for the growth of these human tumors in nude rats; this model was developed in our laboratory and described in previous reports.[3-8,15,17,18]

Materials and Methods

Reagents. HPLC-grade chloroform, methanol, heptane, hexane, and Sep-Pak 18 cartridges for HPLC extraction of samples were purchased from Fisher Chemical (Pittsburgh, PA). Melatonin (catalog no. M5250), rapeseed oil (catalog number 07756), and methyl ester standards (catalog number 1269119), borontrifluoride methanol; and perchloric acid and trichloroacetic acids were purchased from Sigma Scientific (St Louis, MO). The HPLC standards, (±)5-hydroxyeicosatetraenoic acid (catalog number 34210), 13(S)-HODE (catalog number 38610), and ultrapure water (catalog number 400000) were purchased from Cayman Chemical (Ann Arbor, MI).

Animal, housing conditions, and diet. Adult male, homozygous, athymic, inbred nude rats (Hsd:RH-*Foxn1*^{rnu}) used in this study were purchased from Harlan Sprague–Dawley (Indianapolis, IN). The specific-pathogen-free strain (n = 48 rats; 3 to 4 wk old at purchase) were housed in environmentally controlled rooms (23 °C, 45% to 50% humidity) in an individual ventilated caging system (Thoren Caging Systems, Hazelton, PA; n = 2 animals/cage) on an LD 12:12 diurnal lighting cycle (lights on, 0600; 300 lx); rats were completely devoid of any exposure to ocular light at night.[16] All rats were maintained in an AAALAC-accredited facility in accordance with the *Guide or the Care and Use of Laboratory Animals*.[25] All procedures for animal use were approved by the Bassett Research Institute IACUC and the Tulane University IACUC. These experiments were conducted at the Bassett Research Institute (Cooperstown, NY), and this manuscript was written at Tulane University School of Medicine (New Orleans, LA). In addition, this study adhered to NIH principles and guidelines regarding strict application of the scientific method to ensure robust and unbiased experimental design, methodologies, analysis, rigor, and reproducibility, and transparency in reporting.[14]

Rats were maintained in autoclaved cages containing hardwood maple bedding (no.7090, Sanichips, Harlan Teklad, Madison, WI; 2 bedding changes weekly). To ensure that all rats remained free from infection with bacterial and viral agents, serum samples from sentinel animals housed only on the combined soiled bedding from other study cages in the same housing unit were tested quarterly and during this study by enzyme-linked immunosorbent assay for rat coronavirus, Sendai virus, pneumonia virus of mice, sialodacryoadenitis virus, Kilham rat virus, Toolan H1 virus, reovirus type 3, *Mycoplasma* pulmoni, lymphocytic choriomeningitis virus, mouse adenoviruses 1 and 2, Hantaan virus, Encephalitozoon cuniculi, cilia-associated respiratory bacillus, parvovirus NS1, rat parvoviruses, and rat murine virus, and rat theilovirus (Comprehensive Health Monitoring Program, Charles River Laboratories, Kingston, NY) as well as external and internal parasites.

After a 1-wk acclimation period, all rats were randomized and provided free access to acidified water and a semipurified 5% corn oil (CO) diet. Rats were maintained on this diet for a period of 2 wk. The constituents (in g/Kg diet) for both the 5% CO and 5% FO isocaloric diets (in Kcal/g) are listed in Table 1. Diets were prepared weekly and contained water (300 mL) and 0.35 g butylated hydroxytoulene (BHT, Sigma Chemical, St Louis, MO) per 1500 g diet. BHT is a lipophilic derivative of phenol that is widely used to prevent free radical-mediated oxidation in fluids and foods. The US FDA considers BHT to be "generally recognized as safe" and allows small amounts to be added to foods such as the prepared semi-purified diets used in this study. The National Cancer Institute determined that BHT was noncarcinogenic in an animal model.[32]

After preparation, diet was stored in sealed plastic bags at −20 °C. Diets were analyzed via gas chromatography (n = 6 samples), and FA contents (in mg/g) of both diets are listed in Table 2. Rats were housed 2 per cage and received their food from wide-mouthed feeding jars. The semipurified 5% CO and 5% FO diets contained identical amounts of protein (20%),

carbohydrate (65%), fat (5%), methionine (0.3%), vitamins, minerals and choline bitartrate. The diets were analyzed for FA content with 8 independent gas chromagraphic analyses by procedures previously reported (Table 2).[40-42,47] The semipurified 5% CO diet contained nearly 55% of the ω-6 LA; the semipurified 5% FO diet consisted of Accelerated Action Omega-3 FA's (AAFA-100, San Antonio, TX) and contained the ω-3 FAs eicosapentaenoic acid (*all-cis-eicosa-5,8,11,14,17-penta-enoic* acid, EPA, 55%) and docosahexaenoic (*all-cis*-docosa-4,7,10,13,16,19-hexaenoic acid, DHA, 45%) instead of CO. Mean daily food and water consumption and body weights were measured throughout the study.

Tumor Implantation Procedure. The tumors used in this study were PC3 human prostate cancer and FaDu head/neck squamous cell carcinoma. The PC3 prostate cancer cell line was originally derived in 1969 from bone metastasis in a male patient;[26] the tumor is androgen-independent and verified histopathologically to be a grade IV. The PC3 tumor cell line was acquired from the American Type Culture Collection in Rockville, MD (ATCC CRL-1435) and maintained in our laboratory. The FaDu carcinoma, named for the patient from which the tissue was removed, is classified as a grade II squamous cell carcinoma of the hypopharynx, derived from a patient in Calcutta India in 1968.[34] The etiology of this HNSCC is unknown; however, FaDu is now an established HNSCC cell line, kept in high passage by the ATCC (ATCCHTB-43) from which it was acquired by our laboratory and maintained therein.

When rats weighed approximately 170 g, they were randomly separated into 2 groups (n = 24 per group) and prepared for either PC3 prostate or HNSCC human tumor implantation, as previously described.[6,15-18,38] Briefly, rats were anesthetized lightly by using ketamine (89.1 mg/kg IP)–xylazine (9.9 mg/kg IP) solution (MWI Veterinary Supply, Meridian, IN) delivered through a 25-gauge, 5/8-in. tuberculin syringe and then prepped for an aseptic tumor xenograft implantation. All procedures are performed using aseptic techniques and proper personnel protective equipment in a biologic safety cabinet. The anesthetized rat was placed in a supine position on a heating pad with the area over the lower left abdomen shaved and aseptically cleaned. A small 1-cm incision was made over the abdominal region to expose the left femoral superficial inferior epigastric arteries and veins. The vessels are cleared from the surrounding fat and are ligated approximately 2 cm distal to their origin from the femoral artery and vein. A 3-mm cube of tumor tissue is sutured to the vascular stalk. The tumor implant is enclosed in a sterile parafilm envelope and placed in the inguinal fossa. The tumor grows in "tissue-isolated" fashion in the parafilm envelope with no vascular connections to the host other than the truncated superficial epigastric vessels. Tissue-isolated tumors do not develop the central necroses that frequently occurs in subcutaneous implants, which receive their blood supply from the periphery.[38] Latency-to-onset of tumor growth (first palpable mass after implantation) and estimated tumor growth rates were measured and recorded.[6,15,38] Tumor growth rates (g/day) were generated by linear regression from the estimated tumor weights for the treated and control animals during the investigation. The final tumor weight was determined by weighing at the end of the experiment. Both tumor groups (PC3 and HNSCC) were maintained on the 5% CO diet until tumor xenografts reached an estimated weight of approximately 4 g (PC3 tumors, day 20 after tumor implantation; HNSCC, day 32 after tumor implantation). At that time, each group was randomly separated into 2 additional sub-groups, for a total of 6 groups. Groups I – III were PC3 tumor-bearing rats; Groups IV-VI were HNSCC tumor-bearing rats. Groups I, II, IV and V continued to be maintained on the 5% CO diet, while Groups III and VI were switched from the 5% CO diet to the 5% FO diet (Table 1). In addition, CO diet Groups II and V were given drinking water that contained melatonin (2.0 μg/mL), which resulted in 50 μg/day intake of melatonin based on average daily water intake of approximately 25.0 mL.

Arteriovenous Measurements in Human Cancer Xenografts. When estimated tumor weights were approximately 5.5 g in Groups I and IV, on days 22 and 34 after tumor implantation, respectively, and approximately 2.0 g prior to complete tumor regression in Groups II (Day 27), III (Day 29), V (Day 38), and VI (Day 40), rats were prepared for tumor arterial and venous blood collection.[15,38] Cannulation of the tumor vessels was carried out between 0600 h and 0800 h. Rats were anesthetized

Table 1. Dietary ingredients of 5% Corn Oil (CO) and 5% Fish Oil (FO) diets

Constituents	5% Corn oil diet (Groups I and III)	5% Fish oil diet (Groups II and IV)
AAFA-100 (Fish oil)	0	75[a]
Corn oil	75	0[b]
Casein	312	312
Cornstarch	701	701
Dextrose	177	177
DL-Methionine	4.7	4.7
Choline bitartrate	3.2	3.2
Cellulose	77.8	77.8
Vitamin mix	16.5	16.5
Mineral mix	57.8	57.8
TOTAL	1500	1500
KCAL/G	4.07	4.07
WATER	300 mL	300 mL

[a]$P < 0.001$ compared with the value for 5% CO diet.
[b]$P < 0.001$ compared with the value for 5% CO diet.

Table 2. Fatty Acid Content of Experimental Diets

Fatty acid	Diet I (5% Corn oil) mg/g	Diet II (5% Fish oil) mg/g
C14:0	0.05 ± 0.01	0.15 ± 0.03
C16:0	3.45 ± 0.22	1.26 ± 0.18[b]
C16:1	0.05 ± 0.01	0.40 ± 0.06
C18:0	0.81 ± 0.16	1.41 ± 0.67
C18:1 ω-9	8.33 ± 0.56	4.78 ± 0.39[b]
C18:2 ω-6 (LA)	**16.35 ± 0.13**	**0.72 ± 0.16**[b]
C18:3 ω-3	ND[a]	0.06 ± 0.01[b]
C20:4 ω-6	ND	0.72 ± 0.14[b]
C20:5 ω-5 (EPA)	ND	**14.58 ± 0.86**[b]
C22:6 ω-3	ND	0.26 ± 0.07[b]
Total	**30.21 ± 3.08**	**25.10 ± 1.82**[c]
	(n = 8)	(n = 8)

[a]ND, not detectable.
[b]$P < 0.05$ compared with the value for 5% CO diet.
[c]$P > 0.05$ compared with the value for 5% CO diet.

lightly as described above and prepped aseptically. During the entire procedure, rats were maintained in the supine position on a heating pad with an overhead hanging heat lamp (body core temperature, 37 °C). Rats received 100 U of sodium heparin (1000 U/mL; Abbott Hospital Products, North Chicago, Ill) in the left carotid artery for arteriovenous measurements, as previously described.[15,38] A small incision (1.5 cm) was made over the tumor site in the left inguinal fossa. Tumors were examined to ensure the presence of only one vascular connection to the epigastric vessels. The tumor vein was cannulated using a butterfly ST Infusion catheter (number 4573; Abbott), and blood flowed passively into a collection vial on ice for 9 min to provide a 1-mL sample. A corresponding sample was collected from the carotid artery. Samples were capped and placed in ice at the time of collection for immediate analysis. After each blood collection, PC3 prostate and HNSCC tumors were freeze-clamped by using a liquid nitrogen-chilled aluminum clamp and stored at −80 °C for determination of protein content,[30] cyclic-adenosine monophosphate (cAMP) content, [^{3}H]-thymidine incorporation into tumor DNA, tumor DNA content, and expression of total and phosphorylated serine-threonine protein kinase (AKT), extracellular signal-regulated kinase p44/46 (ERK1/2), mitogen-activated protein kinase kinase (MEK).

Melatonin analyses. Arterial plasma melatonin levels were measured by radioimmunoassay using the sensitive melatonin rodent ^{125}I radioimmunoassay kit (catalog number 01-RK-MEL2, Alpco, Salem, NH; lot number 1437.6.U, prepared by Bühlmann Laboratories, Schönenbuch, Switzerland) and analyzed using a Cobra 5005 Automated Gamma Counter (Parkard, Palo Alto, CA), as previously described.[6,18] The minimal detection level for the assay was 1 to 2 pg/mL plasma.

Fatty acid extraction and analyses. Arterial and venous plasma total fatty acids (TFA) and tumor TFA were extracted from 0.1 mL arterial and venous blood and from 0.25 mL of 20% tumor homogenates, respectively, after the addition of the internal standard heptadecanoic acid (C17:0); samples were then methylated and analyzed by gas chromatography as previously described.[3-8,15-17,38-45] The TFA uptake represents the total of the 7 major FAs in rat plasma (myristic, palmitic, palmitoleic stearic, oleic, linoleic, and arachidonic acids). Plasma concentrations are given as μg/mL ± 1 SD, unless otherwise indicated. Uptake was the difference between supply (arterial) and outflow (venous).[39,40] Both supply and uptake rates are expressed in units of μg (or mg) fatty acid/min/g wet weight tumor. These units were used because the mass of FA uptake is related directly to the tumor mass. The 4 major lipid classes (triacylglycerols, phospholipids, FFAs, and cholesterol esters) were extracted from 0.25 mL of the 20% tumor homogenates prepared in 0.15 mol/L NaCl (as above) and were separated by thin-layer chromatography as previously described.[39,40] Heptadecanoic acid, tripentadecanoate, diheptadecanoyl phosphatidyl choline, and cholesterol heptadecanoate were added to the samples as internal standards prior to extraction. Detection of the tumor TFAs and lipid classes, elution, saponification, and separation of the FA methyl esters were carried out as previously described.[39,40] Briefly, the FA methyl esters were measured using a Hewlett-Packard Model 5890A gas chromatograph equipped with a flame-ionization detector, an electronic integrator (Model 3396A) and autoinjector (Model 7673A). Separations were performed with a 0.25 mm × 30 m capillary column (Model 2330, Supelco Bellefonte, PA) at 190 °C with helium as the carrier gas (linear gas rate: 19 cm/sec; split, 100:1). Temperatures of the injection port and detector were 220 °C. FA methyl esters were identified by their retention time compared with known standards. Contents of TFA in the lipid fractions are reported as μg/g of tumor wet weight.

Extraction and analyses of 13-HODE. Arterial and venous plasma samples (0.2 mL) from tumors collected during the study arteriovenous measurements were extracted and analyzed for 13-(S)-HODE by high-performance liquid chromatography.[43] The internal standard was 1 μg of 15-hydroxy-9-oxoprostatrienoic acid (5-HETE, Cayman). Each sample was acidified with 50 μl glacial acetic acid (Fisher Scientific, Pittsburgh, PA) and extracted on a C_{18}-PrepSep column (Fisher Scientific), prewashed with 10 mL methanol and 10 mL water. The lipids were eluted with 4 mL methanol and evaporated to dryness under nitrogen. The residue was taken up in 30% methanol, and the metabolites were separated using a C_{18}-Ultraspere column (5 μm; 4.6 × 250 mm); Altex Scientific, Beckman Instruments, Berkeley, CA) using an ISCO model 2350 pump (Lincoln, NE) with 80% methanol/20% water/0.01% acetic acid as the mobile phase at a flow rate of 1.0 mL/min. The eluent was monitored at 235 nm and 279 nm using an ISCO variable wavelength detector, and the peaks were integrated and quantified using the ISCO ChemResearch Software program. The extinction coefficients used were 23,000 for 13-HODE and 27,000 for 5-HETE. UV absorption spectra of samples and standards were recorded using a Hewlett-Packard (Palo Alto, CA) 8452A diode array spectrophotometer with methanol as solvent and water as blank.

Determination of [^{3}H]-thymidine into tumor DNA and DNA content. [^{3}H]-thymidine incorporation into tumor tissue DNA and DNA content were determined as described previously.[6,8,38,41] Briefly, [^{3}H]-thymidine incorporation was performed by injecting 20 μL saline containing 0.05 μg/gm [methyl-^{3}H]-thymine into the right external jugular of the rat 20 min prior to the end of the experiment.[15,39] Radioactivity incorporated into tumor DNA was measured using a Beckmann LS100 liquid scintillation counter (Brea, CA) with internal standardization. Incorporation was reported as dpm/μg tumor. Tumor DNA content (mg/g tumor) was measured fluorometrically in 20% (w/w saline) homogenates using a Turner Scientific 100 Fluorometer (Jacksonville, IL) and Hoescht dye 33258, as described in Technical Bulletin 119 (Hoefer Scientific Instruments, San Francisco, CA)

Determination of intratumor cAMP content. PC3 and HNSCC tumor samples were freeze-clamped at the end of the arteriovenous measurements. A portion of the tumors were pulverized under liquid nitrogen, and cAMP was analyzed in duplicate 100-mg portions using the Biotrak Enzyme Immunoassay System (RPN 225, Amersham Biosciences, Piscataway, NJ) as previously described.[6,17,45] Results are reported as nmol/g tumor wet weight.

Statistical analysis. Unless otherwise noted, all data are presented as mean ± 1 SD. Statistical differences among group means were determined by using one-way ANOVA followed by Bonferroni multiple comparison tests. Differences in the slopes of regression lines (that is, tumor growth rates) among groups were determined by regression analyses and tests for parallelism (Student *t* test). Differences were considered to be statistically significant at $P < 0.05$. Student *t* test, one-way ANOVA followed by Bonferroni post hoc test, and linear regression analyses were all carried out using GraphPad Prism 8 software (La Jolla, CA).

Results

Diets, dietary and water intake, and body weights. Table 1 shows the dietary ingredients of the semipurified control (5% CO) and experimental (5% FO) diets employed in this study.

The constituents (in grams per 1500 g batch) are presented for each diet, along with measures of energy content in Kcal/g. The principal difference between the diets was the 75 g fish oil content in the 5% FO diet, compared with the 75 g corn oil content in the 5% CO control diet. Total caloric content was identical for both diets. The mean content (as % FA/Total FA; n = 8) of the following FAs for 5% CO and 5% FO diets respectively were (Table 2): myristic (C14:0), 0.17 ± 0.03% and 0.60 ± 0.12%; palmitic (C16:0), 11 ± 1% and 5 ± 1%; palmitoleic (C16:1, ω-9), 0.17 ± 0.03% and 1.59 ± 0.23%; stearic (C18:0), 2.7 ± 0.5% and 5.6 ± 2.7%; oleic (C18:1, ω-9), 28 ± 2% and 19 ± 2%; LA (C18:2, ω-6), 54 ± 0% and 3 ± 1; linolenic (C18:3, ω-3), 0 ± 0% and 0 ± 0%; arachidonic (C20:4, ω-6), 0 ± 0% and 2.9 ± 0.6%; EPA (C20:5, ω-3), 0 ± 0% and 58 ± 3%; DHA (C22:6, ω-3) 0 ± 0% and 1.0 ± 0.3%. The daily dietary and water intake and final mean body weights at day of harvest measured for each rat over the entire course of the experiment (n = 216 measurements/group) was not significantly different between the PC3 and HNSCC tumor-bearing groups and are reported here as combined means of 24 ± 4 g/day, 25 ± 3 mL/day, and 322 ± 33 g, respectively.

Tumor Regression Rates. The latency-to-onset of tumor appearance after xenograft implantation, which measured the time between implantation and the first palpable mass (approximately 10 mm^3) for the PC3 prostate and HNSCC tumor-bearing groups, was 11 d and 22 d, respectively (Figure 1 A and B . Tumor growth rates during the initial phase of the investigation, when all rats were maintained on 5% CO diet, for Groups I-III (PC3) and IV-VI (FadDu HNSCC) were 0.61 ± 0.03 g/day and 0.43 ± 0.02 g/day, respectively. This tumor xenograft rate of growth was sustained for the 5% CO Control Groups I (PC3) and IV (HNSCC), respectively, over the full duration of the tumor growth study. After initiation of treatment for Groups II and III (PC3, Day 20), and Groups V and VI (HNSCC, Day 32), when estimated tumor weight was approximately 4.0 g, tumor xenografts in these groups began to regress in size within a 2-d period. During the treatment phase, daily melatonin intake for Groups II and V amounted to 52 ± 8 µg/day and 49 ± 7 µg/day, respectively. Estimated rates of tumor regression for PC3 Groups II and III, respectively, were -0.25 ± 0.04 g/day and −0.18 ± 0.02 g/day; rates of tumor regression for HNSCC Groups V and VI were −0.14 ± 0.00 g/day and −0.14 ± 0.00 g/day, respectively.

A-V Difference Measurements. Arterial and venous determinations for pH, hematocrit and blood flow rates did not vary among the groups during tumor vein cannulation and whole blood collections and are represented here as the mean values of the combined measurements. Mean values for arterial and venous whole blood pH were 7.43 ± 0.03 and 7.30 ± 0.02, respectively; mean values for arterial and venous hematocrit were 44.4 ± 0.61% and 46.2 ± 0.61%, respectively; the tumor venous blood flow rate was 0.124 ± 0.002 mL/min. Final excised tumor wet weights in Groups I – VI were, respectively, 5.6 ± 0.3 g (I), 2.3 ± 0.2 g (II), 2.9 ± 0.3 g (III), 5.3 ± 0.1 g (IV), 2.0 ± 0.1 g (V), and 2.0 ± 0.2 g (VI). Tumor protein content for Groups II and III (PC3) were significantly different (P < 0.05) at 217 ± 12 mg/g (II), 273 ± 14 mg/g (III), respectively, as compared with Group I at 393 ± 20 mg/g. FaDu HNSCC tumor protein content for Groups V and VI were significantly different (P < 0.05) at 95 ± 10 mg/g (V), 119 ± 7 mg/g (VI), respectively, as compared with Group IV at 167 ± 9 mg/g.

We tested whether tumor xenografts exhibited differences in cAMP levels, TFA and LA uptake, 13-HODE release, [^{3}H]-thymidine incorporation into tumor DNA and tumor DNA content in association with altered arterial plasma melatonin or FO levels at the time of harvest (Table 3). Tumor cAMP levels, TFA and LA uptake, 13-HODE release, [^{3}H]-thymidine incorporation into tumor DNA, and tumor DNA content in Groups II and III (PC3), respectively, were significantly different from Control Group I (P < 0.001) by 77%, 115% and 130%, 100%, 60%, and 70%; and 84%, 84% and 80%, 99%, 49%, and 67%. Tumor cAMP levels, TFA and LA uptake, 13-HODE release, [^{3}H]-thymidine incorporation into tumor DNA, and tumor DNA content in Groups IV and IV (HNSCC), respectively, were significantly different from Control Group III (P < 0.001) by 65%, 101% and 113%, 100%, 79%, and 46%; and, 77%, 68% and 43%, 99%, 71%, and 39%.

Melatonin Analyses. Daytime (light phase) (0600 h to 0800 h) arterial plasma concentrations for rats in Groups I-VI are

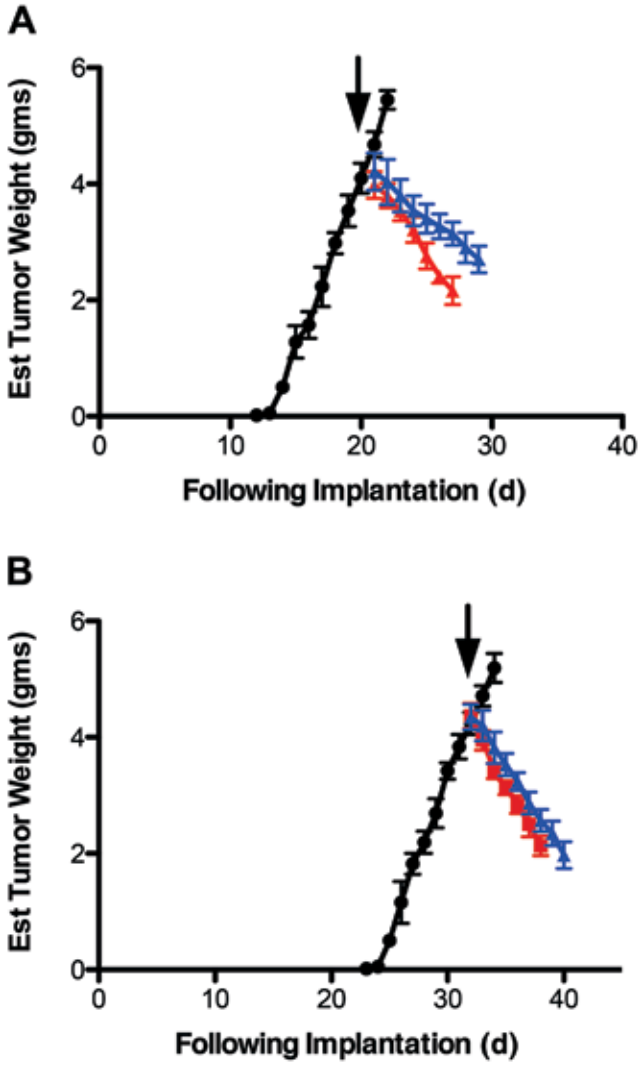

Figure 1. Tumor growth rates (g/d; mean ± 1 SD; n = 8 per group) of PC3 prostate-bearing (A) and FaDu HNSCC-bearing (B) adult male nude rats maintained on either 5% corn oil (CO) diet (black circles, Groups I and IV), 5% CO diet + 50 µg/mL melatonin-water/day (red triangles, Groups II and V), or 5% fish oil (FO) diet (blue triangles, Groups III and VI). Latency-to-onset of tumor growth was significantly different (P < 0.05) of FaDu HNSCC tumor xenografts (Day 22) compared with PC3 prostate tumor xenografts (Day 11). Black arrows indicate first day of treatment; tumor growth rates of Groups I and IV were significantly different (P < 0.05); tumor growth rates of Groups II, III, V, and VI were significantly different from Groups I and III (P < 0.05); tumor growth rates of Groups II and III, and V and VI, respectively, were significantly different (P < 0.05).

Table 3. Effects of dietary regimens of rats bearing either the human PC3 prostate (Groups I-IV) or FaDu HNSCC (Groups V-VI) xenografts on tumor cAMP levels, TFA and LA uptake, 13-HODE release, tumor DNA [^{3}H]thymidine incorporation and DNA Content.

Treatment (8/group)	cAMP (nmol/g tissue)	Fatty acid uptake (μg/min/g)	LA uptake (μg/min/g)	13-HODE (ng/min/g) Arterial	13-HODE (ng/min/g) Venous	[3H] Thymidine Incorporation (dpms/ μg/DNA)	DNA content (mg/g)
PC3 Prostate Tumors							
Group I							
(5% CO)	0.61 ± 0.08[b]	3.55 ± 0.62[b]	1.06 ± 0.16[b]	ND[a]	20.67[b] ± 2.58	53.5 ± 12.8[b]	3.55 ± 0.62[b]
Group II							
(5% CO + MLT)	0.14 ± 0.05	−0.55 ± 0.25[c]	−0.08 ± 0.08[c]	ND[a]	ND[a,h]	21.5 ± 3.9	1.07 ± 0.58
Group III							
(5% FO)	0.10 ± 0.04	0.55 ± 0.85[d,e]	0.21 ± 0.08[d]	ND[a]	0.162 ± 0.051[f]	27.4 ± 3.70[f]	1.18 ± 0.91[f]
FaDu HNSCC Tumors							
Group IV							
(5% CO)	0.43 ± 0.02[d]	3.99 ± 0.59[d]	1.06 ± 0.17[d]	ND[a]	18.99[d] ± 1.59	56.8 ± 4.7[d]	4.15 ± 0.15[d]
Group V							
(5% CO + MLT)	0.15 ± 0.08	−0.05 ± 0.45[e]	−0.14 ± 0.11[e]	ND[a]	ND[a,e]	12.0 ± 1.0[e]	2.23 ± 0.07[e]
Group VI							
(5% FO)	0.10 ± 0.02	1.28 ± 0.76	0.43 ± 0.19	ND[a]	0.102 ± 0.038	16.6 ± 2.2	2.53 ± 0.07

Values represent means ± 1 SD (n = 8/group).
[a]ND, not detectable.
[b]$P < 0.05$ compared with value for II and III.
[c]$P < 0.05$ compared with value for III.
[d]$P < 0.05$ compared with value for V and VI.
[e]$P < 0.05$ compared with VI.

shown in Figure 2. Arterial plasma melatonin levels (0800 h) for Groups I-III (PC3) and IV-VI (FaDu HNSCC). Daytime plasma levels in Groups II (330 ± 18 pg/mL) and V (347 ± 12pg/mL), respectively, were nearly 40-fold higher ($P < 0.001$) than normal daytime levels (less than 10 pg/mL), as revealed in Groups I (9 ± 2 pg/mL), II (9 ± 1 pg/mL), IV (8 ± 1 pg/mL) and VI (8 ± 1 pg/mL), indicative of the high nighttime melatonin intake for Groups II and V.

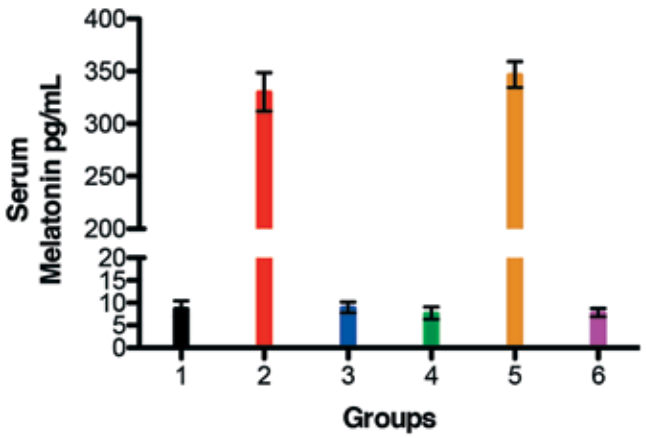

Figure 2. Arterial plasma melatonin levels (pg/mL) in animals (mean ± 1 SD; n = 8 per group) of Groups I-III (PC3 prostate cancer xenografts) and IV-VI (FaDu HNSCC xenografts) at 0800 h during light phase. Groups I, III, IV, and VI reveal normal daytime levels (less than 10 pg/mL) and are not significantly different ($P > 0.05$) from one another, but are significantly lower than Groups II and V ($P < 0.001$); Groups II and V are not significantly different ($P > 0.05$) from one another.

Tumor fatty acid composition and lipid measurements. The FA contents of the tumors in the arteriovenous difference measurements are listed in Table 4. Total contents of the 9 FA in PC3 prostate and HNSCC tumor xenografts in Groups I – VI were, respectively, are listed in Table 4. Total fatty acid contents differed significantly in HNSCC tumors as compared with PC3 prostate tumors ($P < 0.05$) (that is, Group IV was significantly different from Groups I through III, and Group V was significantly different from Groups I through III. Groups II and III (PC3) were significantly different ($P < 0.05$) from Group I, and Groups V and VI (HNSCC) were significantly different form Group III. Tumor linoleic, palmitic, and stearic acid content were 32 ± 3%, 28 ± 3%, and 19 ± 4%, respectively, of the tumor TFA content in Groups I, II, V, and VI (5% CO), as compared with 10 ± 1%, 25 ± 3%, and 17 ± 2% in Groups III and VI (5% FO). Tumor oleic acid, as a percent of tumor TFA content, was significantly higher ($P < 0.05$) in FO Groups III and VI (30 ± 3%) as compared with CO Groups I, II, V, and VI (11 ± 1%), respectively. These effects were consistent with those of whole blood for the respective 5% CO and 5% FO dietary groups. As shown previously, the saturated FA's or oleic acid did not affect tumor [^{3}H]-thymidine incorporation (proliferative response),[41] which was not the case for either LA or EPA, as shown here. Rather the tumor growth stimulative effect observed was due principally to LA (5% CO diet); the tumor growth inhibitory effects were due principally to melatonin and EPA (5% FO diet).

Table 4. Fatty acid composition and lipid content of PC3 prostate and FaDu HNSCC tumor xenografts.

	PC3 Human prostate tumors (Mean ± SD, n = 8/group)			FaDu HNSCC tumors (Mean ± SD, n = 8/group)		
Fatty Acid	Group I	Group II	Group III	Group IV	Group V (μg/g)	Group VI
C14:0	38 ± 7	12 ± 1	11 ± 2	25 ± 6	5 ± 1	5 ± 1
C16:0	2687 ± 291	810 ± 63	738 ± 76	1707 ± 209	337 ± 31	377 ± 25
C16:1	240 ± 291	73 ± 6	66 ± 7	154 ± 20	30 ± 3	34 ± 3
C18:0	1876 ± 379	534 ± 52	485 ± 55	1270 ± 278	222 ± 23	248 ± 22
C18:1	1074 ± 113	959 ± 84	870 ± 93	682 ± 79	398 ± 40	446 ± 31
C18:2 (LA)	3159 ± 334	325 ± 27	296 ± 30	3018 ± 232	135 ± 14	151 ± 11
C20:4	418 ± 52	82 ± 10	92 ± 8	650 ± 65	246 ± 66	178 ± 20
C22:5 (EPA)	ND[a]	ND[a]	121 ± 19	ND[a]	ND[a]	234 ± 19
C24:6 (DHA)	ND[a]	ND[a]	22 ± 3	ND[a]	ND[a]	14 ± 3
Total	9741 ± 1010	2929 ± 225[b,d]	2901 ± 225[b,d]	6222 ± 769[b,c]	1216 ± 121[b,c,d]	1519 ± 115[b,c,d]

[a]ND, not detectable.
[b]$P < 0.05$ compared with value for Group I.
[c]$P < 0.05$ compared with value for Groups II and III.
[d]$P < 0.05$ compared with value for Group IV.

Discussion

The results of the present study indicate clearly that the long-term dietary intake of either melatonin or FO increases tumor regression by suppressing LA metabolism of both human prostate and HNSCC cancer xenografts growing in nude rats in vivo, as compared with the tumor growth-stimulatory effects of CO (high LA content), in our unique tissue-isolated tumor model.[14] Previous studies from our laboratory demonstrated that the growth rate in hepatoma 7288CTC,[3,15,38-42] human breast,[6-8] leiomyosarcoma,[18] and HNSCC[17] cancer xenografts perfused in situ depended on the rate of 13(S)-HODE formation from LA. As dietary intake of LA increases, blood concentrations of LA also increase. Subsequent investigations[1,10,17,18,44,45] demonstrated that melatonin and ω-3 FA's suppress the uptake of LA by tumor tissues. The effects of EPA may be mediated by an inhibitory G-protein FFAR.[2,49] However, in the perfused HNSCC tumor xenografts, the actions of melatonin are mediated by well-documented inhibitory G-protein-coupled MT_1 and/or MT_2 receptors, thereby decreasing cAMP levels.[3,6,13,17] This effect leads to decreased tumor TFA and LA uptake, suppression of 13(S)-HODE formation, and a resultant inhibition of [^{3}H]-thymidine incorporation and growth.

Arterial plasma melatonin levels remained nearly 400% higher in Groups II and V as compared with Groups I, III, IV, and VI even at 2 to 3 h after the onset of the light phase. This may help to explain the enhanced melatonin oncostatic influence on tumor regression rates of Groups II (PC3) and V (FaDu HNSCC) by nearly 40% compared with Groups III and VI (FO). Our previous studies[3-8,16-18] demonstrated the potent impact of normal circadian nighttime plasma melatonin levels (80 to 150 pg/mL) on rodent and human tumor growth inhibition. Such nighttime physiologic levels may be obtained in animals with normal water daily water intake of melatonin at concentrations of approximately 0.05μg/mL as compared with the levels of 2.0 μg/mL used in this study.[6] The normal dark phase drinking patterns of rodents may explain why the higher plasma melatonin levels extend a few hours into the light phase.

The influence of diet and dietary fats on the incidence and growth of cancer in both humans and laboratory animals is well established.[23] Increased consumption of diets rich in LA, the most prominent FA in the Western diet, has long been known to stimulate the growth of human and animal tumors.[23,37,39,40,42] Conversely, substantial evidence indicates that the circadian nighttime neurohormone melatonin functions as a highly effective oncostatic agent that influences the initiation, progression, and metastatic stages of cancer.[33,35,36] The results of the current study of tumor regression are compatible with our previous findings on tumor growth. In addition, EPA and DHA (ω-3), the most prominent FAs in FO, vegetables, and the Mediterranean-type diet, also inhibit the growth and metabolism of human and animal cancer.[9,12,19] The intake ω-6 polyunsaturated FAs such as LA in the Western diet is 10 to 15 times greater than that of ω-3 FAs,[28,29] as compared with the Mediterranean-type diet.[24] Furthermore, normal and neoplastic tissues preferentially metabolize these polyunsaturated FAs in the order: ω-3> ω-6 > ω-9. The augmented oral intake of EPA and DHA in FO dramatically increases their abundance in blood lipids,[27] blood cells,[11] and in normal[35] and neoplastic tissues. As referenced above, melatonin binds to tumor melatonin receptors, whereas EPA and DHA may bind to G-protein coupled FFAR's.[44] Two possible candidate inhibitory FFARs are GPR40 and GPR120, which are expressed in a variety of rodent and human tumors.[10,22,49] A novel finding of the present study was that melatonin caused significantly increased tumor regression rates as compared with FO in PC3 tumor xenografts. However, in HNSCC tumor xenografts, melatonin and FO caused identical rates of tumor regression. Subtle differences between the 2 tumor types with regard to their expression/function of melatonin receptors and FFARs and/or downstream signaling pathways may account for differences in the sensitivity of PC3 and HNSCC xenografts to tumor blood and tissue levels of the melatonin, EPA, and DHA ligands for rates of tumor regression.[27] Further studies are warranted to explore this possibility.

In this study, we examined 2 unique environmental factors that regulate the metabolism and growth of human PC3 prostate and HNSCC carcinomas. The circadian neurohormone melatonin and the dietary fats found in FO (primarily the ω-3 FA's EPA and DHA) reduce overall TFA uptake and metabolism by these tumors. Furthermore, both melatonin and FO increase rates of tumor regression by suppressing the rate of LA uptake and its metabolism to mitogenically-active 13-(S)-HODE. These

findings provide a potential scientific basis for the development of a new dietary approach that combines reduced dietary LA intake and increased dietary melatonin or FO supplementation for the treatment or prevention of a variety of human cancers in addition to prostate and head/neck squamous cell carcinomas.

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