Melatonin exerts by an autocrine loop antiproliferative effects in cholangiocarcinoma; its synthesis is reduced favoring cholangiocarcinoma growth

Yuyan Han,1,6,8 Sharon DeMorrow,2,3 Pietro Invernizzi,5 Qing Jing,6 Shannon Glaser,2,3 Anastasia Renzi,3 Fanyin Meng,2,3,4 Julie Venter,3 Francesca Bernuzzi,5,10 Mellanie White,3 Heather Francis,2,3,4 Ana Lleo,5,10 Marco Marzioni,2 Paolo Onori,8 Domenico Alvaro,9 Guido Torzilli,11 Eugenio Gaudio,12 and Gianfranco Alpini1,2,3

1Division Research, Central Texas Veterans Health Care System, 2Scott & White Digestive Disease Research Center, 3Department of Medicine, Division Gastroenterology, and 4Division of Research and Education, Scott & White Hospital and Texas A&M Health Science Center, College of Medicine, Temple, Texas; 5Center for Autoimmune Liver Diseases, Division of Internal Medicine, Istituto Di Ricovero e Cura a Carattere Scientifico, Istituto Clinico Humanitas, Rozzano, Italy; 6Department of School of Life Science and Technology, Tongji University, Shanghai, China; 7Gastroenterology, Università Politecnica delle Marche, Ospedali Riuniti General Hospital of Ancona, Ancona; 8Department of Experimental Medicine, State University of L’Aquila, L’Aquila; 9Department of Scienze e Biotecnologie Medico-Chirurgiche, University of Rome, Sapienza, Polo Pontino, Latina; 10Department of Translational Medicine, and 11Liver Surgery Unit, Humanitas Cancer Center, Department of Translational Medicine, Università degli Studi di Milano, Rozzano; and 12Department of Anatomical, Histological, Forensic Medicine and Orthopedics Sciences, “La Sapienza”, Rome, Italy

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Han Y, DeMorrow S, Invernizzi P, Jing Q, Glaser S, Renzi A, Meng F, Venter J, Bernuzzi F, White M, Francis H, Lleo A, Marzioni M, Onori P, Alvaro D, Torzilli G, Gaudio E, Alpini G. Melatonin exerts by an autocrine loop antiproliferative effects in cholangiocarcinoma; its synthesis is reduced favoring cholangiocarcinoma growth. Am J Physiol Gastrointest Liver Physiol 301: G623–G633, 2011. First published July 21, 2011; doi:10.1152/ajpgi.00118.2011.—Cholangiocarcinoma (CCA) is a devastating biliary cancer. Melatonin is synthesized in the pineal gland and peripheral organs from serotonin by two enzymes, serotonin N-acetyltransferase (AANAT) and acetylserotonin O-methyltransferase (ASMT). Cholangiocytes secrete neuroendocrine factors, including serotonin-regulating CCA growth by autocrine mechanisms. Melatonin exerts its effects by interaction with melatonin receptor type 1A/1B (MT1/MT2) receptors. We propose that 1) in CCA, there is decreased expression of AANAT and ASMT and secretion of melatonin, changes that stimulate CCA growth; and 2) in vitro overexpression of AANAT decreases CCA growth. We evaluated the 1) expression of AANAT, ASMT, melatonin, and MT1/MT2 in human nonmalignant and CCA lines and control and CCA biopsy samples; 2) melatonin levels in nonmalignant and CCA lines, and bile and serum from controls and patients with intrahepatic CCA; 3) effect of melatonin on the expression of AANAT/ASMT and MT1/MT2 in CCA lines and biopsy samples; 4) melatonin effects on the secretion and expression of melatonin in CCA lines and biopsy samples. Melatonin secretion decreased in the supernatant of CCA lines and bile of CCA patients. Melatonin decreased xenograft CCA tumor growth in nude mice by increased AANAT/ASMT and melatonin, along with reduced MT1/MT2 expression. Overexpression of AANAT in Mz-Cha-1 cells inhibited proliferation and MT1/MT2 expression and increased apoptosis. There is dysregulation of the AANAT/ASMT/melatonin → melatonin receptor axis in CCA, which inhibited melatonin secretion and subsequently enhanced CCA growth.

biliary epithelium; biliary neoplasm; neuroendocrine regulation

CHOLANGIOCARCINOMA (CCA) is a devastating malignancy of intrahepatic and extrahepatic bile ducts, which is steadily increasing worldwide in incidence, morbidity, and mortality (3, 8). CCA is often clinically silent until it becomes an advanced disease with obstructive symptoms (2, 46). The response of this neoplasm to conventional chemotherapy is poor, with surgical resection being the only effective therapeutic approach, but unfortunately, only partially to a minority of patients (46). Studies aimed to better understand the intracellular mechanisms of CCA growth are necessary as background for future pharmacological therapeutic strategies.

Melatonin (N-acetyl-5-methoxytryptamine), the major secretory hormone of the pineal gland (42), is also secreted from several extrapineal tissues, such as gastrointestinal tract and liver (21). Melatonin is synthesized from serotonin by two enzymes, serotonin N-acetyltransferase or arylalkylamine N-acetyltransferase (AANAT) and acetylsertotonin O-methyltransferase (ASMT). Cholangiocytes secrete neuroendocrine factors, including serotonin-regulating CCA growth by autocrine mechanisms. Melatonin exerts its effects by interaction with melatonin receptor type 1A/1B (MT1/MT2) receptors. We propose that 1) in CCA, there is decreased expression of AANAT and ASMT and secretion of melatonin, changes that stimulate CCA growth; and 2) in vitro overexpression of AANAT decreases CCA growth. We evaluated the 1) expression of AANAT, ASMT, melatonin, and MT1/MT2 in human nonmalignant and CCA lines and control and CCA biopsy samples; 2) melatonin levels in nonmalignant and CCA lines, and bile and serum from controls and patients with intrahepatic CCA; 3) effect of melatonin on the expression of AANAT/ASMT and MT1/MT2 in CCA lines and biopsy samples; 4) melatonin effects on the secretion and expression of melatonin in CCA lines and biopsy samples. Melatonin secretion decreased in the supernatant of CCA lines and bile of CCA patients. Melatonin decreased xenograft CCA tumor growth in nude mice by increased AANAT/ASMT and melatonin, along with reduced MT1/MT2 expression. Overexpression of AANAT in Mz-Cha-1 cells inhibited proliferation and MT1/MT2 expression and increased apoptosis. There is dysregulation of the AANAT/ASMT/melatonin → melatonin receptor axis in CCA, which inhibited melatonin secretion and subsequently enhanced CCA growth.

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On the basis of this background, we hypothesize that 1) in CCA, there is a decrease in the expression of AANAT and ASMT and the secretion of melatonin, changes that induce an increase in CCA growth by an autocrine mechanism; and 2) increases in the biliary expression of melatonin (by in vivo administration of melatonin or in vitro overexpression of AANAT in CCA cells) decreases CCA growth.

MATERIALS AND METHODS

Reagents were purchased from Sigma (St. Louis, MO), unless differently indicated. The antibodies against AANAT (FL-207), ASMT (C-20), MEL-1A-R (V-15), and MEL-1B-R (T-18) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), unless differently indicated. The rabbit polyclonal antibody against melatonin (ab35137) was purchased from Abcam (Cambridge, MA). The ELISA kits for detecting melatonin levels in nonmalignant and CCA cell lines, and serum and bile from controls and CCA patients, were purchased from GenWay Biotech.

In Vitro Studies

Cell lines. We used six human CCA cell lines (Mz-ChA-1, HuH-28, TFK-1, CCLP1, SG231, and HUCC-T1) with different biliary origin. Mz-ChA-1 cells (from human gallbladder) (28) were a gift from Dr. G. Fitz (University of Texas Southwestern Medical Center, Dallas, TX). HuH-28 cells (from human intrahepatic bile ducts) (30) and TFK-1 cells (from human extrahepatic bile ducts) (47) were acquired from Cancer Cell Repository, Tohoku University, Japan. These cells were maintained at standard conditions (25). CCLP1 (52), HuCC-T1 (35), and SG231 (54) (from intrahepatic bile ducts) were a gift from Dr. A. J. Demetris (University of Pittsburgh) and cultured as described (35, 52, 54). The human immortalized, nonmalignant cholangiocyte cell line, H69 (from Dr. G. J. Gores, Mayo Clinic, MN), was cultured as described (19).

Expression of AANAT and ASMT in nonmalignant and CCA lines and tissue arrays. The expression of AANAT and ASMT was evaluated by immunofluorescence (1) in H69 cells and CCA lines using specific primary antibodies. Images were taken in a coded fashion with an Olympus Fluoview 500 Laser scan microscope with a DP70 digital camera (Tokyo, Japan). Negative controls were performed with the omission of the respective primary antibodies.

For real-time PCR analysis (11) of AANAT and ASMT, RNA was extracted from the selected cell lines using RNeasy Mini kit (Qiagen, Valencia, CA) and reverse transcribed using the Reaction Ready First Strand cDNA Synthesis kit (SABiosciences, Frederick, MD). These reactions were used as templates for the PCR assays using SYBR Green PCR Master Mix (SABiosciences) in the real-time thermal cycle (ABI Prism 7900HT sequence detection system) using commercially available primers (purchased from SABiosciences) designed against human AANAT (NM_001088) (9), ASMT (NM_004043) (12), and glyceraldehyde-3-phosphate dehydrogenase (housekeeping) (NM_002046) (37) genes. A ΔΔCt (delta delta of the threshold cycle) analysis (34) was performed using H69 as the control sample. Data are expressed as relative mRNA levels ± SE (n = 3).

FACS analysis (38) for AANAT and ASMT was performed in H69 and Mz-ChA-1 cells using a C6 flow cytometer and analyzed by CFlow Software (Accuri Cytometers, Ann Arbor, MI). At least 20,000 events in the light scatter (side scatter/forward scatter) were acquired. The expression of the AANAT and ASMT was validated and gated on fluorescence 1 area (FL1-A)/count plots. The relative quantity of AANAT and ASMT (mean selected protein fluorescence) was expressed as mean FL1-A (samples)/mean FL1-A (secondary antibodies only). The standard errors were calculated as CV (FL1-A) × (mean FL1-A/SQR(count-1)), where CV is coefficient of variation, and SQR is square root.

The immunoreactivity for AANAT and ASMT was assessed in commercially available Accumax tissue arrays (Isu Abixs, Seoul, Korea) by immunohistochemistry (1). These tissue arrays contain 48 well-characterized CCA biopsy samples from a variety of tumor differentiation grades, as well as four control liver biopsy samples. Staining intensity was assessed (in a blinded fashion) on a scale from 1 to 4 (1 = no staining, 4 = intense staining), and the abundance of positively stained cells was given a score from 1 to 5 (1 = no cells stained, 5 = 100% stained). The staining index was calculated by the staining intensity multiplied by the staining abundance, which gives a range from 1 to 20 (1). Immunohistochemistry observations were taken with a BX-40 light microscope (Olympus) with a video-cam (Spot Insight, Diagnostic Instrument).

Evaluation of melatonin immunoreactivity in tissue arrays and measurement of melatonin secretion in cell lines and serum and bile from patients. We measured melatonin immunoreactivity by immunohistochemistry (1) in human biopsy samples from controls and CCA patients from commercially available tissue arrays (Isu Abixs) using a specific monoclonal antibody (ab35137, Abcam) against melatonin (see above). Melatonin levels in the supernatant of H69 and Mz-ChA-1 lines were measured as follows using two different approaches. In the first approach, cells (1 × 107 cells/ml) were incubated in the dark for 24 h at 37°C, and the amount of melatonin released into the media was assayed using a commercially available melatonin ELISA kit (55), according to the manufacturer’s instructions (GenWay Biotech). In the second approach, to determine by ELISA kits (39) the amount of melatonin secreted in the basolateral and apical domains of H69 cells, we plated the cell line on collagen-coated filters of tissue culture inserts to produce a confluent monolayer (60). After 48 h of incubation, the supernatant of the basolateral and apical inserts was collected for evaluation of melatonin levels by ELISA kits (39). We did not use Mz-ChA-1, since these cells do not form polarized cell systems (61).

We collected serum and bile from patients with intrahepatic CCA (n = 15 for serum and n = 13 for bile) and healthy, nonmalignant controls (n = 20 for serum, and n = 18 for bile). Data collection in human serum samples was performed in the laboratory of Dr. Pietro Invernizzi (coauthor of this paper, Department of Internal Medicine, IRCCS Istituto Clinico Humanitas, Rozzano, Milan, Italy). The collection of the samples was approved by the Ethical Committee of the IRCCS Istituto Clinico Humanitas. Serum and bile samples were immediately frozen at −80°C until used for the evaluation of melatonin levels. The melatonin levels in plasma and bile were measured, respectively, via ELISA kit (GenWay Biotech) (55). The serum and bile human samples were obtained from a tissue bank with de-identified

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Fig. 1. A: by immunofluorescence, serotonin N-acetyltransferase (AANAT) and acetylserotonin O-methyltransferase (ASMT) are expressed by all cholangiocarcinoma (CCA) lines, as well as H69. Specific immunoreactivity is seen in red, and cells were counterstained with DAPI (blue). Bar = 50 μm. B: mRNA expression of AANAT and ASMT decreased significantly in all CCA lines compared with H69. Values are means ± SE of 4 evaluations. *P < 0.05 vs. the values of nonmalignant cholangiocytes. C: by FACS analysis, the protein expression for AANAT and ASMT decreased in Mz-ChA-1 cells compared with H69 cells. Values are means ± SE of 4 experiments. *P < 0.05 vs. the values of nonmalignant human cholangiocytes. D: the immunohistochemical expression of AANAT and ASMT significantly decreased in biopsy liver samples from CCA patients compared with controls. Values are means ± SE of 90 evaluations for CCA patients, and 4 for normal nonmalignant controls. *P < 0.05 vs. the values of liver biopsy samples from nonmalignant controls. MT1 and MT2, melatonin receptor types 1A and 1B, respectively.
clinical samples from the laboratory of Dr. Invernizzi. The samples were analyzed in a coded fashion in the laboratory of Dr. Invernizzi.

Melatonin receptor expression. We next evaluated the expression of MT1 and MT2 by 1) immunofluorescence (11), real-time PCR (11), and FACS analysis (38) in the selected cell lines; and 2) immunohistochemistry (1) of tissue arrays of human liver biopsy samples from control and CCA patients. For real-time PCR, we used commercially available primers designed against human MT1 (NM_005958, SABiosciences) (45), MT2 (NM_005959, SABiosciences) (44), and glyceraldehyde-3-phosphate dehydrogenase (NM_002046, SABiosciences) (37) genes. A ΔΔCt analysis (34) was performed using H69 as the control sample. Data are expressed as relative mRNA levels ± SE (n = 3).

In Vivo Studies

Male BALB/c 6-wk-old nude (nu/nu) mice (Taconic Farms, Hudson, NY) were kept in a temperature-controlled environment (20–22°C) with 12:12-h light-dark cycles, fed standard chow ad libitum, and with free access to drinking water. Mz-ChA-1 cells (5 × 10⁶) were suspended in 0.2 ml of extracellular matrix gel and injected subcutaneously in the rear flanks of the nude mice (16). After the establishment of the tumor (1 wk with a tumor size of ~5 mm³), mice (n = 4) were treated with vehicle (5% ethanol in water) or melatonin (4 mg/kg body wt, dissolved in 5% aqueous ethanol solution) (51) by intraperitoneal injections three times a week. Tumor variables were measured three times a week by an electronic caliper, and volume was determined as follows: tumor volume (mm³) = 0.5 × [length (mm) × width (mm) × height (mm)]. The measurements started from the first week when the tumor mass was established. After 43 days of treatment, they were anesthetized with pentobarbital sodium (50 mg/kg body wt ip), and tumor tissues were harvested. Heart, liver, and kidney tissues were collected for evaluation of damage by hematoxylin-eosin staining of paraffin-embedded sections (4- to 5-μm thick). All animal experiments were performed in accordance with a protocol approved by the Scott & White and Texas A&M Health Science Center Institutional Animal Care and Use Committee and conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (publication no. 85–23, revised 1996). Tumors were allowed to grow until maximum allowable tumor burden was reached, as set forth by the Scott & White and Texas A&M Health Science Center Institutional Animal Care and Use Committee tumor burden policy. Tumor tissues were fixed for 4 h in 10% buffered formalin and embedded in low-temperature fusion paraffin. Subsequently, sections (4–5 μm) were stained 1) with hematoxylin-eosin for evaluation of necrosis (16); and 2) for proliferating cell nuclear antigen (PCNA) (a marker of proliferation) (4), AANAT, ASMT, melatonin, MT1, and MT2 by immunohistochemistry (16). Negative controls were obtained by staining the tumor sections with nonimmune serum with the omission of the primary antibody. Semiquantitative analysis for these markers was evaluated by image analysis software, Image-Pro Plus (Media Cybernetics, Silver Spring, MD) (33). Three randomly chosen fields were calculated for statistics.

Fig. 2. A: melatonin immunoreactivity is significantly decreased in liver biopsy samples from CCA patients compared with nonmalignant controls. Values are means ± SE of 90 evaluations for CCA patients and 4 for nonmalignant control evaluations. *P < 0.05 vs. the values of liver biopsy samples from nonmalignant controls. B: the secretion of melatonin in Mz-ChA-1 lines compared with H69 cells; notably, the secretion of melatonin was significantly higher in the apical domain of H69. Values are means ± SE of 3 evaluations. *P < 0.05 vs. the values of nonmalignant human cholangiocytes. #P < 0.05 vs. the values of basolateral level. C: there were decreased melatonin levels in the bile (but not serum) of CCA patients compared with controls. Values are means ± SE of x evaluations. *P < 0.05 vs. the corresponding values from normal healthy controls.
Fig. 3. A: by immunofluorescence, all CCA cell lines, as well as H69, expressed MT1 and MT2. Bar = 50 μm. Specific immunoreactivity is seen in red, and cells were counterstained with DAPI (blue). The gene (B) and protein (C) expression of MT1 and MT2 significantly increased in Mz-ChA-1 cells compared with H69. Values are means ± SE of 4 experiments. *P < 0.05 vs. the corresponding values of H69 cells. D: the expression of MT1 and MT2 increased in liver biopsy samples from CCA patients compared with nonmalignant controls. Values are means ± SE of 90 evaluations for CCA patients and 4 for nonmalignant control evaluations. *P < 0.05 vs. the corresponding values of control lines and control patients, respectively.
Overexpression of AANAT in Mz-ChA-1 Cells

The expression vector pCMV6-XL containing human AANAT cDNA was purchased from Origene (Rockville, MD); the control vector was pCMV6-XL. The 624-bp ORF of AANAT containing a COOH-terminal MYC/DDK tag was cloned into the pCMV6-XL entry, and the expression protein was 23.2 kDa. The transfection and the selections of clones were performed as described (27). The plasmid (10 μg) was transfected by nucleofection (27) into Mz-ChA-1 cells, according to the manufacturer’s instructions. Mz-ChA-1 cells (1 × 10⁶ cells) per reaction were resuspended in 100 μl of nucleofector solution (Lonza, Basel, Switzerland). Ten micrograms of AANAT cDNA plasmid DNA (Origene) were mixed with 100 μl of cell suspension and transferred into a cuvette. The cuvette was inserted into the nucleofector (Lonza), and cells were pulsed with program U-017. After pulse, the cells were rinsed and transferred to a six-well plate. Culture medium was replaced 24 h after nucleofection. Stable overexpressing AANAT Mz-ChA-1 cells were selected based on neomycin resistance, and individual colonies were ring-cloned. The overexpression of AANAT in Mz-ChA-1 cells was verified by real-time PCR analysis (15).

Fig. 4. After 34 days of melatonin administration, there was a significant decrease in tumor size in nude mice treated with melatonin compared with mice treated with vehicle. Representative pictures of the tumors from vehicle- and melatonin-treated mice and a summary graph of each data point are also shown. Values are means ± SE of tumor size evaluations from 4 mice per each group of animals. *P < 0.05 vs. the corresponding values of tumor volume of nude mice treated with vehicle. B: there was increased necrosis in sections from melatonin-treated mice compared with control mice. There was a decrease in CCA proliferation [by proliferating cell nuclear antigen (PCNA)] and an increase in CCA apoptosis (by cleaved caspase-3) in tumor sections from melatonin-treated mice compared with vehicle-treated mice. There was an increase in the expression of AANAT, ASMT, and melatonin, and a decrease in MT1/MT2 expression, in nude mice treated with melatonin compared with controls. For semiquantitative analysis of these parameters, see Table 2. Original magnification, ×125.
Melatonin in vivo

Table 1. Measurement of liver and body weight, and liver to body weight ratio, in nude mice treated with vehicle or melatonin in vivo

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle</th>
<th>Melatonin</th>
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<tbody>
<tr>
<td>Body weight, g</td>
<td>34.2 ± 1.48</td>
<td>35.25 ± 0.73</td>
</tr>
<tr>
<td>Liver weight, g</td>
<td>2.67 ± 0.22</td>
<td>2.82 ± 0.13</td>
</tr>
<tr>
<td>Liver-to-body weight ratio, %</td>
<td>7.78 ± 0.3</td>
<td>8.01 ± 0.21</td>
</tr>
</tbody>
</table>

Values are means ± SE of n = 3 mice for vehicle and 4 mice for the melatonin group.

In Mz-ChA-1 cells (stably overexpressing AANAT or the control vector), we measured 1) cell proliferation by real-time PCR analysis for PCNA (15) and alamarBlue cell proliferation assay (Invitrogen, Rockville, MD) (50), according to the manufacturer’s instructions; 2) cell apoptosis with annexin V-FITC apoptosis detection kit (ab14085) (BD Biosciences) by FACS analysis (57); and 3) MT1/MT2 expression by FACS (38).

For the alamarBlue cell proliferation assay (50), 5000 cells per well were seeded in 96-well plates. Subsequently, cells were incubated for 0, 24, and 48 h after the cells were attached. Briefly, 10 μl of medium alamarBlue reagent was added to 100 μl of cell culture and incubated in 37°C for 2 h before detecting the fluorescence intensity under the fluorescence excitation of 540 nm and the fluorescence emission of 590 nm. Data were expressed as the fold change of different time points compared with 0 h.

For the annexin V-FITC apoptosis detection kit I (BD Biosciences) (57), cells were harvested and processed according to the manufacturer’s instructions. Unstained cells, stained cells with propidium iodide (PI; without annexin V), and stained cells with annexin V (without PI) were used as controls to divide the events into four parts. Data were expressed as the percentage of events in different staining situations: healthy cells (lower left, unstained, FITC−PI−), early apoptosis (lower right, FITC+PI−), late apoptosis (upper right, FITC+PI+), and dead cells (upper left, FITC−PI+).

Statistical Analysis

All data are expressed as means ± SE. Differences between groups were analyzed by Student’s unpaired t-test when two groups were analyzed, and ANOVA when more than two groups were analyzed, followed by an appropriate post hoc test.

RESULTS

The Expression of AANAT and ASMT Is Downregulated in CCA

By immunofluorescence, AANAT and ASMT are expressed by H69 and all CCA lines (Fig. 1A). mRNA expression of AANAT and ASMT was significantly decreased in all CCA lines compared with H69 (Fig. 1B). By FACS analysis, the protein expression for AANAT and ASMT decreased in Mz-ChA-1 cells compared with H69 cells (Fig. 1C). The immunohistochemical expression of AANAT and ASMT significantly decreased in biopsies of CCA patients compared with controls (Fig. 1D).

The Expression and Secretion of Melatonin Is Reduced in CCA

Melatonin immunoreactivity decreased in biopsies of CCA patients compared with controls (Fig. 2A). The secretion of melatonin decreased markedly in Mz-ChA-1 lines compared with H69 cells (Fig. 2B). Moreover, the secretion of melatonin was mostly active in the apical domain of H69 cells (Fig. 2B).

Consistent with apical melatonin secretion by CCA, melatonin levels decreased in the bile (but not serum) of patients with intrahepatic CCA compared with controls (Fig. 2C).

Expression of Melatonin Receptors in CCA

By immunofluorescence, all CCA cell lines, as well as H69, expressed MT1 and MT2 (Fig. 3A). The gene and protein expression of MT1 and MT2 was significantly higher in Mz-ChA-1 cells compared with H69 (Fig. 3, B and C) and in liver biopsy samples from CCA patients compared with controls (Fig. 3D).

Melatonin Inhibits CCA Growth In Vivo

After 34 days of melatonin administration, there was a significant reduction in tumor size in nude mice treated with melatonin compared with mice treated with vehicle (Fig. 4A). Representative pictures of the tumors from vehicle- and melatonin-treated mice, and a summary graph of each data point are shown in Fig. 4A. No significant difference in body weight, liver weight, and liver-to-body weight ratio was observed between the two groups of mice (Table 1). By histological examination of tumor sections, there was increased necrosis in sections from melatonin-treated mice compared with control mice (Fig. 4B). We demonstrated a decrease in CCA proliferation (by PCNA) and an increase in CCA apoptosis (by cleaved caspase-3) in tumor sections from melatonin-treated mice compared with vehicle-treated mice (Fig. 4B). By immunohistochemistry in tumor sections, we found an increase in the expression of AANAT, ASMT, and melatonin, and a decrease in MT1/MT2 expression in nude mice treated with melatonin compared with controls (Fig. 4B). For semiquantitative data, see Table 2.

Effect of Overexpression of AANAT on the Proliferation, Apoptosis, and MT1/MT2 Expression of Mz-ChA-1 Cells

Since 1) CCA lines proliferate at a higher rate because they express lower amount of AANAT and secrete less melatonin compared with nonmalignant cholangiocytes, and 2) melatonin inhibits Mz-ChA-1 growth in athymic mice by upregulation of AANAT, we tested the hypothesis that in vitro overexpression of AANAT in Mz-ChA-1 reduces (similar to what is shown in vivo in nude mice) the proliferation and MT1/MT2 expression and increases apoptosis in these cells. Stable transfection of AANAT cDNA into Mz-ChA-1 cells increased the mRNA

Table 2. Semiquantitative data for the expression of necrosis, PCNA, cleaved caspase-3, melatonin, AANAT, ASMT, MT1, and MT2 in nude mice treated with vehicle or melatonin

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle</th>
<th>Melatonin</th>
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<tbody>
<tr>
<td>Necrosis, %</td>
<td>&lt;5†</td>
<td>&gt;75†</td>
</tr>
<tr>
<td>PCNA, %total nucleus</td>
<td>0.77 ± 0.05</td>
<td>0.25 ± 0.09</td>
</tr>
<tr>
<td>Cleaved caspase-3, μm²</td>
<td>151.36 ± 53.59</td>
<td>4,490.66 ± 1,053.52*</td>
</tr>
<tr>
<td>Melatonin, μm²</td>
<td>10,464 ± 2,034.44</td>
<td>41,241.48 ± 7,634.43*</td>
</tr>
<tr>
<td>AANAT, μm²</td>
<td>567.22 ± 131.95</td>
<td>1,584.52 ± 127*</td>
</tr>
<tr>
<td>ASMT, μm²</td>
<td>6,173.15 ± 1,008.19</td>
<td>46,051.09 ± 13,063.04*</td>
</tr>
<tr>
<td>MT1, μm²</td>
<td>6,362.05 ± 672.01</td>
<td>1,847.76 ± 419.9*</td>
</tr>
<tr>
<td>MT2, μm²</td>
<td>5,081.64 ± 607.34</td>
<td>1,417.96 ± 425.71*</td>
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Values are means ± SE of four randomly chosen fields. †The percentage is based on the whole field of section. *P < 0.05 vs. vehicle.
expression of AANAT in these cells compared with Mz-ChA-1 cells transfected with the control vector (Fig. 5A). In Mz-ChA-1 cells (overexpressing AANAT), there was 1) a reduction in CCA proliferation (Fig. 5, B and C); 2) an increase in apoptosis (Fig. 5D); and 3) a decrease in the expression of MT1/MT2 (Fig. 5E) compared with Mz-ChA-1 cells transfected with the control vector (Fig. 5, B–E).

DISCUSSION

In this study, we found that melatonin synthesis is down regulated in CCA, and that melatonin inhibits CCA growth by enhanced biliary expression of AANAT, the key enzyme regulating melatonin synthesis (17). The data introduce the novel concept that decreased melatonin synthesis in CCA suppresses melatonin antiproliferative actions via an autocrine loop. We first demonstrated that 1) the expression of AANAT and ASMT and melatonin immunoreactivity are downregulated in CCA lines, as well as liver biopsies of CCA patients, compared with controls. There was a decrease in melatonin levels in CCA lines (compared with H69 cells) and in bile (but not serum) of patients with intrahepatic CCA compared with healthy controls. The expression of MT1 and MT2 increased in CCA lines and liver biopsy samples from CCA patients compared with nonmalignant controls. Also, melatonin inhibited the growth of Mz-ChA-1 cells when implanted into the flanks of nude mice. In vivo analysis of the implanted tumors revealed that melatonin decreased CCA proliferation, increased the expression of AANAT and ASMT and melatonin, but decreased the immunoreactivity of MT1 and MT2 that was associated with enhanced apoptosis and necrosis of CCA cells. In vitro overexpression of AANAT in Mz-ChA-1 cells decreases the proliferation and MT1/MT2 expression and increases apoptosis in these cells. The data suggests that 1) decreased melatonin expression/secretion is a key feature of CCA, leading to increase in tumor growth by an autocrine fashion; 2) evaluation of decreased melatonin levels in bile [e.g., during endoscopic retrograde cholangiopancreatography (ERCP)] can be an important diagnostic marker for the early detection of preneoplastic biliary diseases; and 3) the modulation of this metabolic pathway (targeting AANAT) may be useful for the development of effective therapies to CCA.

A number of studies in other cell types (6, 14, 41, 49) support our finding that decreased AANAT and ASMT expression and subsequent reduced melatonin secretion stimulate CCA growth. In fact, mRNA levels of both AANAT and ASMT decreased in pineal parenchymal tumors compared with normal pineal gland (14). Also, reduced melatonin levels have been linked to higher incidence of breast, prostate, endometrial, and colorectal cancer (41, 49). Circulating melatonin levels (produced by pineal glands and peripheral tissues) are depressed in patients with primary tumors of different histology, including endocrine-dependent (e.g., endometrial and prostate cancer) and endocrine-independent tumors (e.g., gastric and colorectal cancer) (6). An inverse relationship exists...
between melatonin secretion and the growth rate of the tumors (6). Consistent with the presence of a local melatonin loop (regulating CCA growth), we have shown (1) higher secretion of melatonin in the apical domain of H69 cells and lower levels of melatonin in Mz-ChA-1 cells; and (2) decreased melatonin levels in bile (but not serum) of CCA patients compared with healthy controls. These findings are consistent with studies showing that (1) cholangiocytes from human gallbladder are exposed apically to high concentration of melatonin in bile (5, 22, 56); and (2) melatonin levels in bile are two to three orders of magnitude higher than those in serum (56). Since high levels of melatonin in bile may be essential to prevent oxidative damage to cholangiocytes (5, 56), the decrease in the levels of melatonin in bile observed in CCA patients (who display biliary damage) supports our findings. Since the concentration of melatonin in bile is high in normal conditions (5, 22, 56) and reduced in CCA patients, we propose that evaluation of biliary melatonin levels (e.g., during ERCP) may be an important tool for the early diagnosis of premalignant biliary diseases.

The increase in MT1 and MT2 observed in CCA is likely due to a compensatory mechanism by this tumor to retard the progression of cell growth in the presence of decreased AANAT expression and melatonin secretion. This concept is supported by a number of previous studies (31, 48). Indeed, upregulation of MT1 receptors has been observed in the hippocampus of Alzheimer’s disease patients, possibly as a compensatory response to impaired melatonin levels to augment melatonin’s neuroprotective effects (48). Consistent with our findings, a recent study has demonstrated that MT1 receptor expression is upregulated in breast cancer; and (2) melatonin inhibition of breast cancer growth is associated with reduction of MT1 expression (31). Also, a recent study has demonstrated the presence of ASMT and MT1 in normal and malignant human gallbladder (5). The decrease in the expression of MT1 and MT2 following melatonin in vivo administration is likely due to the desensitization of these receptors, as suggested by other studies (29). Supporting the presence of an autocrine loop, the decrease in MT1/MT2 expression is likely due to the increased expression of AANAT following melatonin administration. On the basis of these findings, we propose that an autocrine loop (AANAT/ASMT → melatonin → MT1/MT2 axis) plays an important role in the progression of CCA. Indeed, melatonin effects on cancer growth have been shown to be receptor dependent (62), although receptor-independent pathways should be evaluated in future experiments.

Our in vivo finding in nude mice implicates a positive feedback loop, where melatonin treatment stimulates CCA to produce more melatonin (through increased AANAT and ASMT) to inhibit the cancer cell growth and induce apoptosis and necrosis. Indeed, studies have shown that melatonin inhibits the growth of tumor cells in vitro, including prostate and breast cancer cells (20, 24). In addition to inhibiting the growth of a rat hepatoma cell line in vivo (7), melatonin has been able to inhibit the growth of pancreatic AR42J tumor cells by activation of caspase-3 (18). Supporting our finding, melatonin potentiates the effects of doxorubicin, which inhibits the mitosis and enhances the apoptosis of HepG2 and Bel-7402 hepatoma cell lines.

In conclusion, our study demonstrates a dysregulation of the AANAT → melatonin receptor axis in CCA, which causes decreased secretion of melatonin with subsequent enhanced growth of CCA. Also, evaluation of decreased melatonin levels in bile (during ERCP) can be an important diagnostic marker for the early detection of preneoplastic biliary diseases. The study has important clinical application, since therapeutic targeting of AANAT can be an important approach for the management of CCA. Since melatonin regulates core clock circuitry (20, 24), undergoing studies from our laboratory aim to elucidate the role of clock genes during melatonin regulation of biliary neoplasms.

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DISCLOSURES

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