Neuropeptide Y inhibits cholangiocarcinoma cell growth and invasion

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DeMorrow S, Onori P, Venter J, Invernizzi P, Frampton G, White M, Franchitto A, Kopriva S, Bernuzzi F, Francis H, Coufal M, Glaser S, Fava G, Meng F, Alvaro D, Carpino G, Gaudio E, Alpini G. Neuropeptide Y inhibits cholangiocarcinoma cell growth and invasion. Am J Physiol Cell Physiol 300: C1078–C1089, 2011. First published January 26, 2011; doi:10.1152/ajpcell.00358.2010.—No information exists on the role of neuropeptide Y (NPY) in cholangiocarcinoma growth. Therefore, we evaluated the expression and secretion of NPY and its subsequent effects on cholangiocarcinoma growth and invasion. Cholangiocarcinoma cell lines and nonmalignant cholangiocytes were used to assess NPY mRNA expression and protein secretion. NPY expression was assessed by immunohistochemistry in human liver biopsies. Cell proliferation and migration were evaluated in vitro by MTS assays and matrigel invasion chambers, respectively, after treatment with NPY or a neutralizing NPY antibody. The effect of NPY or NPY depletion on tumor growth was assessed in vivo after treatment with NPY or the neutralizing NPY antibody in a xenograft model of cholangiocarcinoma. NPY secretion was upregulated in cholangiocarcinoma compared with normal cholangiocytes. Administration of exogenous NPY decreased proliferation and cell invasion in all cholangiocarcinoma cell lines studied and reduced tumor cell growth in vivo. In vitro, the effects of NPY on proliferation were blocked by specific inhibitors for NPY receptor Y2, but not Y1 or Y5, and were associated with an increase in intracellular d-myos-inositol 1,4,5-trisphosphate and PKCα activation. Blocking of NPY activity using a neutralizing antibody promoted cholangiocarcinoma growth in vitro and in vivo and increased the invasiveness of cholangiocarcinoma in vitro. Increased NPY immunoreactivity in human tumor tissue occurred predominantly in the center of the tumor, with less expression toward the invasion front of the tumor. We demonstrated that NPY expression is upregulated in cholangiocarcinoma, which exerts local control on tumor cell proliferation and invasion. Modulation of NPY secretion may be important for the management of cholangiocarcinoma. Neuropeptide Y (NPY) is a neurotransmitter, mainly found in the brain but also present in neurons throughout the gastrointestinal tract, around the walls (tunica adventitia and tunica media) of hepatic vessels, and in high concentrations in the biliary tree (25, 32). Other studies have shown that immunohistochemical NPY reactivity is present not only in intrahepatic nerve fibers and ganglion cells but also in cholangiocytes (15). Limited information exists regarding the role of NPY in the regulation of biliary function. NPY has been shown to play a role in the neural control of biliary motility and secretion (14, 53). NPY has inhibitory effects on cancer cell lines of various origins (51), but nothing is known about the effects of NPY on cholangiocarcinoma growth.

NPY exerts its many functions through six main receptor subtypes (Y1 through Y6) (7). These receptors are a class of G protein-coupled receptors that can either inhibit 3′,5′-cyclic adenosine monophosphate (cAMP) synthesis (22, 35) or increase d-myos-inositol 1,4,5-trisphosphate (IP3)/Ca2+-signaling (1, 39). We have previously shown that both cAMP- and IP3/Ca2+-signaling modulate cholangiocarcinoma growth (3, 6, 57). Cholangiocarcinoma arises from the neoplastic transformation of the epithelial cells (i.e., cholangiocytes) that line the intra- and extrahepatic bile ducts (6, 57). Typically, cholangiocarcinomas are adenocarcinomas and have a poor prognosis and limited treatment options. This is due, at least in part, to the late presentation of symptoms and the relative resistance to current treatment options (57, 60). The incidence of this cancer is increasing in Western societies, and the mortality rates of the intrahepatic cancer are increasing (57). Therefore, research into the mechanisms by which cholangiocarcinoma growth and disease progression are regulated is imperative in an attempt to design more effective treatment options for this cancer.

We have previously demonstrated that cholangiocarcinoma secretes factors capable of regulating its own proliferation (2, 8). For example, cholangiocarcinoma synthesizes and secretes higher amounts of serotonin (2) and dopamine (8), which can be detected in bile (but not serum) of patients with cholangiocarcinoma (2, 8). Both of these neuroendocrine modulators exert growth-promoting effects on cholangiocarcinoma (2, 8), and blocking their synthesis slowed the rate of cholangiocarcinoma tumor growth in vitro and in an in vivo xenograft model of cholangiocarcinoma.

Neuropeptide Y (NPY) is a neurotransmitter, mainly found in the brain but also present in neurons throughout the gastrointestinal tract, around the walls (tunica adventitia and tunica media) of hepatic vessels, and in high concentrations in the biliary tree (25, 32). Other studies have shown that immunohistochemical NPY reactivity is present not only in intrahepatic nerve fibers and ganglion cells but also in cholangiocytes (15). Limited information exists regarding the role of NPY in the regulation of biliary function. NPY has been shown to play a role in the neural control of biliary motility and secretion (14, 53). NPY has inhibitory effects on cancer cell lines of various origins (51), but nothing is known about the effects of NPY on cholangiocarcinoma growth.
For example, while both the α₂-adrenergic receptor agonist, UK14,304, and secretin inhibit cholangiocarcinoma growth by cAMP-dependent mechanism (28, 44), other molecules such as tauroursodeoxycholate, the H3 histamine receptor agonist, RAMH, and gastrin inhibit cholangiocarcinoma proliferation by activation of the IP₃/Ca²⁺-dependent PKCα isoform (3, 19, 27). Furthermore, the neurotransmitter, γ-aminobutyric acid, inhibits the growth of biliary cancer by activation of both cAMP- and IP₃/Ca²⁺-dependent signaling (17).

Thus, the aims of our studies were to 1) assess the expression of NPY and the NPY receptors in normal liver and cholangiocarcinoma tissue samples and nonmalignant and cholangiocarcinoma cell lines; 2) determine the effects of NPY treatment on cholangiocarcinoma cell growth and elucidate the intracellular mechanism by which this occurs; and 3) evaluate the effects of NPY depletion on cholangiocarcinoma cell growth in vitro.

**MATERIALS AND METHODS**

**Materials**

Reagents were purchased from Sigma Chemical (St. Louis, MO) unless otherwise indicated. The nuclear dye 4,6-diamidino-2-phenylindole (DAPI) was obtained from Molecular Probes (Eugene, OR). The antibody against proliferating cellular nuclear antigen (PCNA) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antibodies recognizing the different subtypes of NPY receptors (Y1 to Y6) were purchased from Santa Cruz Biotechnology. The affinity-purified goat polyclonal antibody (A-17, against Y1) was raised against a peptide mapping near the NH₂ terminus of Y1 of human origin. The affinity-purified goat polyclonal antibody (L-17, against Y2) was raised against a peptide mapping near the COOH terminus of Y2 of human origin. The synthetic peptide, corresponding to NH₂-terminal amino acids 1-14 of Y3, was purchased from Tocris Biosciences (Ellisville, MO). The affinity-purified goat polyclonal antibody (C-20, against Y4) was raised against a peptide mapping at the COOH terminus of Y4 of human origin. The affinity-purified goat polyclonal antibody (N-20, against Y5) was raised against a peptide mapping within an extracellular domain of Y5 of human origin. The affinity-purified goat polyclonal antibody (P-20, against Y6) was raised against a peptide mapping within an internal region of Y6 of mouse origin. All the purchased antibodies are recommended for the detection of all NPY receptor subtypes of mouse, rat, and human origin by immunobLOTS and immunofluorescence. The anti-NPY antibody was developed in rabbits using synthetic NPY (porcine) conjugated to keyhole limpet hemocyanin as the immunogen (Sigma Chemical). The highly selective and potent antagonist of Y1, BVD 10 (4), was purchased from Tocris Biosciences. The potent, selective, and competitive nonpeptide antagonist for Y2, BIIE 0246 (12), was purchased from Tocris Biosciences. The selective, nonpeptide Y5 antagonist, CGP 71683 hydrochloride (13), was purchased from Tocris Biosciences. Specific inhibitors for Y3, Y4, and Y6 are not commercially available. The radioimmunoassay (RIA) kits for the measurement of intracellular cAMP ([125I] Biotrak Assay System, RPA509) and IP₃, ([3H] Biotrak Assay System, TRK1000) levels were purchased from GE Healthcare (Piscataway, NJ).

**Cell Lines**

The in vitro experiments were performed in six human cholangiocarcinoma cell lines (Mz-ChA-1, HuH28, HuCC-T1, CCLP1, SG231, and TFK-1) with different origins. Mz-ChA-1 cells, from human gallbladder (29), were a gift from Dr. G. Fitz (University of Texas Southwestern Medical Center, Dallas, TX). HuH28 cells, from human intrahepatic bile duct (31), and TFK-1 cells, from extrahepatic cholangiocarcinoma (52), were acquired from Cancer Cell Repository, Tohoku University (Tohoku, Japan). These cells were maintained at standard conditions as previously described (10, 27). CCLP1-1 (56), HuCC-T1 (40), and SG231 (58) (from intrahepatic bile ducts) were a kind gift from Dr. A. J. Demetris (University of Pittsburgh, Pittsburgh, PA) and were cultured as previously described (40, 56, 58). The human immortalized, nonmalignant cholangiocyte cell line, H69 (from Dr. G. J. Gores, Mayo Clinic, Rochester, MN), was cultured as previously described (21). Human intrahepatic biliary epithelial cells (HIBECs) were purchased from Sciencell (Carlsbad, CA) and cultured as previously described (8).

**Real-Time PCR for NPY and Y Receptors**

The mRNA expression of NPY and its receptors was assessed in the selected cell lines by real-time PCR (2, 10) using commercially available primers against NPY (accession no. NM_000905; reference position +451; expected product size 93 bp), Y1 (accession no. NM_000909; reference position +1,016; expected product size 172 bp), Y2 (accession no. NM_000910; reference position +533; expected product size 164 bp), Y3 (accession no. NM_003467; reference position +192; expected product size 150 bp), Y4 (accession no. NM_005972; reference position +440; expected product size 161 bp), Y5 (accession no. NM_006174; reference position +312; expected product size 116 bp), and Y6 (accession no. NR_002713; reference position +480; expected product size 174 bp; SA Bioscience, Frederick, MD). A ΔΔCT analysis was performed (34) using normal cholangiocytes as the control sample and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the housekeeping gene. Data are expressed as relative mRNA levels ± SE of gene expression-to-GAPDH ratio (n = 3).

**NPY Secretion**

All cell lines (H69, HIBEC, Mz-ChA-1, HuH28, HuCC-T1, SG231, TFK-1, and CCLP-1) were trypsinized, and the resulting cell pellet was resuspended in 1 × Hanks’ buffered saline buffer (1 × 10⁷ cells/ml). Cells were incubated for 6 h at 37°C, and the amount of NPY released into the media was assayed using a commercially available NPY enzyme-linked immunoassay (ELA) kit (Bachem Americas; Torrance, CA) according to the manufacturer’s instructions. NPY secretion from each cell line was assayed in triplicate, and each data point from nonmalignant cells or cholangiocarcinoma cells was collated and plotted on a scatter plot.

In parallel, NPY secretion was assessed in serum and bile samples obtained from cholangiocarcinoma patients and age-matched controls as described previously (2, 8) by an NPY ELA kit (Bachem Americas). The human sera and bile samples were obtained from an unidentified tissue bank from the laboratory of P. Invernizzi (coauthor of this article). The de-identified samples were analyzed in a coded fashion in the laboratory of Dr. Invernizzi. The human liver samples were obtained from a tissue bank from the laboratory of E. Gaudio (coauthor of this article). The samples were analyzed in a coded fashion in the laboratory of Dr. Gaudio by three board-certified pathologists in a blinded fashion. Written informed consent was obtained from all patients included in the study.

**Cholangiocarcinoma Tissue Array Analysis**

NPY immunoreactivity was assessed in commercially available Accumax tissue arrays (Isu Axbis, Seoul, Korea) by immunohistochemistry as previously described (2, 8). The tissue arrays contain 48 well-characterized cholangiocarcinoma biopsy samples from a variety of tumor differentiation grades as well as four control liver biopsy samples. Semiquantitative analysis was performed by three independent board-certified pathologists, in a blind fashion, using the following parameters. Staining intensity was assessed on a scale from 1 to 4 (1, no staining; 4, intense staining), and the abundance of positively stained cells or cholangiocarcinoma cells was collated and plotted on a scatter plot.

Tohoku University (Tohoku, Japan). These cells were maintained at standard conditions as previously described (10, 27). CCLP-1 (56), HuCC-T1 (40), and SG231 (58) (from intrahepatic bile ducts) were a kind gift from Dr. A. J. Demetris (University of Pittsburgh, Pittsburgh, PA) and were cultured as previously described (40, 56, 58). The human immortalized, nonmalignant cholangiocyte cell line, H69 (from Dr. G. J. Gores, Mayo Clinic, Rochester, MN), was cultured as previously described (21). Human intrahepatic biliary epithelial cells (HIBECs) were purchased from Sciencell (Carlsbad, CA) and cultured as previously described (8).
stained cells was given a score from 1 to 5 (1, no cells stained; 5, 100% stained). The staining index was then calculated by the staining intensity multiplied by the staining abundance that gave a range from 1 to 20.

**Immunoblots for NPY Receptors**

The expression of NPY receptor subtypes was evaluated by immunoblotting in protein (10 μg) from whole cell lysate from the selected normal and cholangiocarcinoma cell lines as previously described (2) using the aforementioned specific antibodies for each receptor subtype.

**Immunofluorescence**

The expression of NPY receptors was assessed in the normal human cell lines, H69 and HIBEC, and the cholangiocarcinoma cell line, Mz-ChA-1, by immunofluorescence as previously described by us (10, 18) using the aforementioned antibodies to all the NPY receptor subtypes (Y1 to Y6). Preimmune sera were substituted for the primary antibodies as a negative control. Coverslips were visualized using an Olympus IX-71 inverted confocal microscope (Tokyo, Japan).

**MTS Cell Proliferation Assays**

To assess the effects of NPY on cell proliferation, all cell lines were stimulated with various concentrations of recombinant NPY (10−8 to 10−10 M), and cell proliferation was assessed by CellTiter 96 Cell Proliferation Assay (Promega, Madison, WI) as described previously (10, 18). Absorbance was measured at 490 nm on a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). Data are expressed as the fold change of treated cells compared with BSA-treated cells. In separate sets of experiments, Mz-ChA-1 cells were treated at 37°C for 48 h with saline or NPY (10−7 M) for 48 h in the absence/presence of preincubation with 1) BVD 10−7 M, a selective and competitive nonpeptide antagonist for Y1 (4); 2) BIIE 0246 10−7 M, a selective and competitive nonpeptide antagonist for Y2 (12); or 3) CGP 71683 hydrochloride 10−7 M, a selective, nonpeptide Y5 antagonist (13) before evaluation of proliferation by CellTiter 96 Cell Proliferation Assay (Promega). Conversely, the effects of NPY depletion on cell proliferation were also assessed. Specifically, Mz-ChA-1 cells were treated with a 1:500 or 1:1,000 dilution of a neutralizing NPY antibody (26) for 48 h and cell proliferation was assessed by CellTiter 96 Cell Proliferation Assay (10, 18).

**Evaluation of the Intracellular Mechanisms by Which NPY Regulates Cholangiocarcinoma Growth In Vitro**

**Effect of NPY on intracellular cAMP and IP3 levels.** After trypsinization, Mz-ChA-1 cells were incubated at 37°C for 1 h to regenerate membrane proteins damaged by trypsin (17). Mz-ChA-1 cells (1 × 105 for determination of cAMP levels, and 1 × 106 cells for the measurement of IP3 levels) (17) were incubated at room temperature with 0.2% BSA (basal) or NPY (10−7 M) for 5 min (for cAMP evaluations; 17) or 10 min (for IP3 measurements; 17). Intracellular cAMP and IP3 levels were measured by RIA as previously described (17, 19, 20).

**Effect of NPY on PKCα phosphorylation and translocation.** To begin to assess the involvement of PKCα activation on the antiproliferative effects of NPY, Mz-ChA-1 cells were pretreated with a specific inhibitor for PKCα (Gö6976, 10−6 M) (38) for 1 h before the addition of NPY (10−7 M). Cells were incubated for a further 48 h, after which time the protein lysates were made as previously described (2). PCNA expression was used as an indicator of the proliferative capacity of the cells and was assessed by immunoblots (2). The amount of protein loaded (10 μg) was normalized by immunoblots for β-actin (19). Band intensity was determined by scanning video densitometry using the phospho-imager Storm 860 (GE Healthcare) and the Image-Quant TL software (version 2003.02, GE Healthcare). Furthermore, to assess the phosphorylation of PKCα, Mz-ChA-1 cells were treated with NPY (10−7 M) for 2 h at 37°C. Following stimulation, we assessed the expression of the phosphorylated form for PKCα by immunoblotting using a phosphospecific PKCα antibody (Santa Cruz Biotechnology) and expressed the data as a ratio to total PKCα expression (19).

To detect membrane translocation of PKCα, Mz-ChA-1 cells were plated, stimulated with NPY (10−7 M) for 2 h, and processed for immunofluorescence (10, 18) as described above using the antibody for total PKCα. Negative controls were done with the use of preimmune serum instead of the respective primary antibody. Slides were visualized using an Olympus IX-71 inverted confocal microscope.

**Invasion Assays**

The effects of NPY on the invasive properties of cholangiocarcinoma were performed using growth factor-reduced matrigel invasion chambers (BD Biosciences, San Jose, CA) according to the manufacturer’s instructions. Specifically, Mz-ChA-1 cells were plated in the upper chamber and allowed to adhere overnight. Cells were treated with either NPY (10−7 M) or anti-NPY neutralizing antibody (1:500 dilution) for 24 h. The number of cells that had migrated to the underside of the upper chamber were then counted in 10 fields, and data are expressed as the average (± SE) number of cells as an indication of the invasion index.

**Nude Mice Treatment**

The effects of NPY or anti-NPY antibody on cholangiocarcinoma growth were assessed in vivo using a xenograft model of cholangiocarcinoma as previously described by us (9) with prior approval from the Scott & White and Texas A&M Health Science Center Institutional Animal Care and Use Committees. Briefly, Mz-ChA-1 cells (5 × 106) were suspended in 0.25 ml of extracellular matrix gel and injected subcutaneously in the flanks of these animals. After the establishment of the tumors, mice received 0.5 μg per tumor per day of NPY, or 50 μl per tumor per day of a 1:500 dilution anti-NPY antibody injected three times per week, and tumor dimensions were measured using electronic callipers (8, 9). After 60 days, mice were anesthetized with pentobarbital sodium (50 mg/kg body wt ip) and euthanized according to institutional guidelines. Serum was collected, and aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured using a Dimension RxL Max Integrated Chemistry system (Dade Behring, Deerfield, IL) by the Scott & White Hospital Chemistry Department.

Tumor tissues were dissected from the flank of these mice. Neoplastic tissues were fixed in formalin, embedded in paraffin, processed for histopathology, and stained for hematoxylin and eosin for routine examination and Masson’s trichrome for collagen. For immunohistochemistry, glass slides were deparaffinized, and endogenous peroxidase activity was blocked by a 30-min incubation in methanolic hydrogen peroxide (2.5%). The endogenous biotin was blocked by a biotin blocking system (code X0590; DAKO, Copenhagen, Denmark) according to the instructions supplied by the vendor. Sections were then hydrated in graded alcohol and rinsed in 1× phosphate-buffered saline (PBS, pH 7.4) before application of the primary antibody. Sections were incubated overnight at 4°C with polyclonal antibodies for NPY (E-17, 1:50 dilution; Santa Cruz Biotechnology), and PCNA (PC10, 1:100 dilution; DAKO). Samples were then rinsed with 1× PBS for 5 min, incubated for 10 min at room temperature with secondary biotinylated antibody (LSAB Plus System, DAKO, Milan, Italy), then with DAKO ABC (LSAB Plus system), and finally developed with 3–3′ diaminobenzidine.

To demonstrate the specificity of the immunoreaction, negative controls [the primary antibody was replaced (same dilution) with normal serum from the same species] were performed for all immu-
noreactions. Apoptosis was measured by quantitative terminal deoxy-
nucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) kit
(Apoptag; Chemicon International). Sections were analyzed in a
coded manner using BX-51 light microscopy (Olympus, Tokyo,
Japan) with a video cam (Spot Insight; Diagnostic Instrument, Sterling
Heights, MI) and processed with an Image Analysis System (Delta
Sistemi, Rome, Italy).

**NPY Immunohistochemistry in Human Tumor Samples**

NPY immunoreactivity was assessed in excised tumor samples
from cholangiocarcinoma patients, where the invasion front of the
cancer was clearly visible. Immunohistochemical staining for NPY
was performed as outlined above.

**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 by ANOVA when more than two groups were analyzed,
followed by an appropriate post hoc test. \( P < 0.05 \) was used to
indicate statistical significance.

**RESULTS**

**Cholangiocarcinomas Express and Synthesize More NPY
Than Normal Cholangiocytes**

NPY mRNA expression was assessed in six cholangiocarcinoma
cell lines and in two nonmalignant cholangiocyte cell lines and was found to be elevated in all cholangiocarcinoma
cell lines studied (Fig. 1A). The increase in NPY expression
was also reflected by increased NPY secretion from all chol-
angiocarcinoma cell lines studied compared with nonmalignant
cholangiocytes (Fig. 1B). In addition, immunohistochemical
analysis of human liver biopsy samples indicated that there is
also increased NPY immunoreactivity in cholangiocarcinoma
samples compared with controls as assessed in a coded fashion
by three independent board-certified pathologists (Fig. 1C).

Analysis of serum and bile samples from cholangiocarcinoma
patients vs. age-matched normal controls revealed no signifi-
cant difference in NPY levels (data not shown), suggesting that
the expression and secretion of NPY from cholangiocarcinoma cells is a local event.

Increased Local Release of NPY Decreases Cholangiocarcinoma Growth and Invasion

We first determined the presence of the NPY receptors in malignant and normal cholangiocyte cell lines. By real-time PCR, all NPY receptor subtypes were expressed by H69 and HIBEC and Mz-ChA-1 cells. Data are expressed as averages ± SE (n = 3). By immunoblot analysis, NPY receptors were expressed by Mz-ChA-1 and H69 cells. The comparability of the protein used was evaluated by immunoblotting for β-actin, the housekeeping gene.

Fig. 2. Evaluation of NPY receptors in nonmalignant cholangiocytes and Mz-ChA-1 cells by real-time PCR (A) and immunoblotting (B). A: by real-time PCR, all NPY receptor subtypes were expressed by H69 and HIBEC and Mz-ChA-1 cells. Data are expressed as averages ± SE (n = 3). B: by immunoblot analysis, NPY receptors were expressed by Mz-ChA-1 and H69 cells. The comparability of the protein used was evaluated by immunoblotting for β-actin, the housekeeping gene.

Fig. 3. NPY receptor localization was assessed by immunofluorescence in H69 and Mz-ChA-1 cells. Specific receptor immunoreactivity is shown in red. Nuclei were counterstained with DAPI (blue). Negative controls were performed by substitution of the primary antibodies with preimmune serum. Scale bar, 20 μm.
cinoma cell lines with NPY (given as a single dose of $10^{-6}$ to $10^{-10}$ M) caused a significant ($P < 0.05$) decrease in cell proliferation after 48 h as demonstrated by cell proliferation assay (Fig. 4A). Repeated daily administration of NPY had no additional antiproliferative effects beyond that after a single dose, suggesting that the recombinant NPY is stable in these culture conditions (data not shown). In separate sets of experiments, we demonstrated that 1) NPY decreased the growth of Mz-ChA-1 cells (Fig. 4B) and 2) the antiproliferative effects of NPY ($10^{-7}$ M) on cholangiocarcinoma growth was prevented by the specific inhibitor of Y2, BIIE 0246 (12), but not inhibitors for Y1 and Y5 (Fig. 4B), suggesting that Y2 are involved in the inhibitory effects of NPY on cholangiocarcinoma growth. Alone, inhibitors of Y1 and Y5 did not affect the growth of Mz-ChA-1 cell growth (not shown). We did not evaluate the effects of Y3, Y4, and Y6 on NPY inhibition of cholangiocarcinoma growth since these receptor inhibitors are not available.

NPY receptor activation can result in changes to either cAMP levels or to intracellular Ca$^{2+}$/IP$_3$ levels in other cell types (1, 22, 35, 39). Therefore, we assessed the effects of NPY on intracellular IP$_3$ and cAMP. NPY ($10^{-7}$ M) significantly increased intracellular IP$_3$ levels in Mz-ChA-1 cells compared with the basal levels (Fig. 5A) and had no effect on intracellular cAMP (data not shown), suggesting that NPY is acting through an IP$_3$/Ca$^{2+}$-dependent signaling pathway.

We have previously demonstrated that one of the major downstream effectors of Ca$^{2+}$ signaling in the regulation of cholangiocarcinoma growth is not available.
The growth of cholangiocarcinoma cells is PKCα-sensitive (3, 19, 20, 27, 28). Pretreatment of Mz-ChA-1 cells with the specific inhibitor of PKCα before the addition of NPY (10^{-7} M) prevented the NPY-induced decrease in PCNA protein expression (as a marker of proliferative capacity; Fig. 5B), suggesting that PKCα is involved in the effects of NPY on cholangiocarcinoma growth. Indeed, treatment of Mz-ChA-1 cells with NPY (10^{-7} M) increased the phosphorylation of PKCα (pPKCα) compared with the basal levels. pPKCα, total PKCα. *P < 0.05 vs. its corresponding basal value (4 immunoblots from cumulative preparations of cholangiocytes). In D, by immunofluorescence, there was a distinct positive stain for PKCα under basal conditions localized in the cytoplasm, whereas after NPY stimulation, there was translocation of PKCα from the cytosolic region to the membrane domain of the cells. Scale bar, 50 μm.

In support of the in vitro data, treating an in vivo xenograft model of cholangiocarcinoma tumors with NPY significantly suppressed tumor growth (Fig. 6A). In addition, the latency of tumor growth (i.e., time taken for tumor volume to increase to 150% of the original size) was increased after NPY treatment compared with vehicle treatment (Fig. 6A). Analysis of liver enzymes in the serum revealed that there was no significant difference in AST levels (vehicle, 80.67 ± 8.25 U/l vs. NPY, 108.67 ± 21.38 U/l) and ALT levels (vehicle, 28.33 ± 3.08 U/l vs. NPY, 35.0 ± 2.54 U/l) between NPY-treated and vehicle-treated animals, both of which fell within normal range, suggesting that the NPY treatment was well tolerated and did not cause any liver damage. Histological analysis of liver, heart,
and kidney also indicated no significant organ damage caused by the chronic NPY treatment (data not shown).

**Histological analysis** of the excised tumors revealed that all tumor cells within tumors from NPY-treated and vehicle-treated animals were positive for cytokeratin-19, a specific marker for cholangiocytes (2, 8), indicating biliary phenotypes (data not shown). Using PCNA immunoreactivity as a marker of proliferative capacity, NPY treatment decreased the number of PCNA-positive nuclei per field compared with vehicle treatment (Fig. 6B). In parallel, using TUNEL staining as a marker of apoptosis, NPY treatment significantly increased the percentage of TUNEL-positive nuclei per field compared with vehicle treatment (Fig. 6B).

**Inhibition of NPY Function Increases Cholangiocarcinoma Cell Growth and Invasion In Vitro**

Because NPY expression and secretion increased in cholangiocarcinoma, but then had an apparently contradictory, anti-proliferative effect on cell growth, we designed experiments aimed to demonstrate that blocking of NPY activity had growth-promoting effects on cholangiocarcinoma. Indeed, treating Mz-ChA-1 cells with an NPY-specific antibody that has neutralizing activity (26) increased cell proliferation as shown by MTS assays (Fig. 7A). Furthermore, Mz-ChA-1 cells treated with anti-NPY antibody for 24 h significantly (P < 0.05) increased the rate of cell invasion compared with that seen under basal conditions (basal 123.35 ± 1.29, anti-NPY antibody 162.0 ± 3.25).

In addition, treatment of an in vivo xenograft model of cholangiocarcinoma tumors with anti-NPY antibody significantly increased the rate of tumor growth (Fig. 7B) and decreased the latency of tumor growth (Fig. 7B). Analysis of liver enzymes in the serum revealed that there was no significant difference in AST (vehicle, 69.66 ± 8.95 U/l vs. anti-NPY, 53.67 ± 13.79 U/l) and ALT levels (vehicle, 38.67 ± 13.68 U/l vs. anti-NPY, 30.00 ± 8.15 U/l) between anti-NPY antibody-treated and vehicle-treated animals, both of which fell within normal range, suggesting that the inhibition of NPY did not cause any liver damage. Histological analysis of liver, heart, and kidney also indicated no significant organ damage caused by the chronic inhibition of NPY (data not shown).
Immunohistochemical Analysis of Tumor Tissue Reveals a Gradient of NPY Expression

The concept that cholangiocarcinoma cells are overproducing NPY, which in turn slows the growth rate of the tumor, appears somewhat counterintuitive. Therefore, we wished to determine whether the increased expression of NPY occurs uniformly throughout the tumor. Interestingly, the increased expression of NPY occurred predominantly in the center of the tumor (Fig. 8, A and B), particularly near the necrotic areas, with considerably less expression toward the periphery of the tumor (Fig. 8 C). Furthermore, the nonmalignant hepatocytes in the normal liver tissue surrounding the tumor appear to also have an increased expression of the antiproliferative NPY (Fig. 8A).

DISCUSSION

The major findings of the study relate to the local regulation of cholangiocarcinoma cell growth and migration by NPY. We demonstrated that cholangiocarcinoma cells express and produce more NPY than normal cholangiocytes and that there is a gradient of NPY expression within the tumor, with the maximum expression occurring toward the center of the tumor, near the necrotic areas. Furthermore, treatment of cholangiocarcinoma cells with NPY in vitro and in vivo decreases both proliferation and migration. In vitro, NPY inhibition of cholangiocarcinoma growth was prevented by the specific inhibitor of Y2, BIIE 0246 (12), but not inhibitors for Y1 and Y5 (Fig. 4B), suggesting that Y2 are involved in the inhibitory effects of NPY on cholangiocarcinoma growth; inhibitors for Y3, Y4, and Y6 are not available. In addition, we have shown that 1) NPY-induced inhibition of cholangiocarcinoma cell growth was associated with an increase in intracellular IP3 and activation of PKCα and 2) the antiproliferative effects of NPY are blocked by a specific PKCα inhibitor. Taken together these data suggest that the gradient expression of NPY in cholangiocarcinoma may be a key regulatory feature of the local regulation of cholangiocarcinoma growth and progression.

A physiological function of NPY is to regulate food intake and increase fat storage (23). Furthermore, NPY has been shown to be upregulated in obesity (5), which is a risk factor for cholangiocarcinoma (59). Therefore, because of the link between obesity and cholangiocarcinoma, it is conceivable that molecules that are upregulated during obesity, such as NPY, may also play a role in cholangiocarcinoma initiation, growth, and progression. Indeed, recently, we demonstrated that another obesity-related molecule, leptin, was involved in the development and growth of cholangiocarcinoma in a thioacetamide rat model of cholangiocarcinoma (16).

In support of our observation that cholangiocarcinoma cells secrete increased levels of NPY, this neuropeptide is secreted...
in a number of other tumors. For example, NPY immunoreactivity has been shown in ~75% of prostate cancer tumor specimens studied (36). Furthermore, NPY treatment of three different prostate cancer cell lines stimulated proliferation in one cell line and decreased proliferation in the other two (49). Both the growth-enhancing and antiproliferative actions of NPY were through Y1, and the difference between the proliferative and antiproliferative effects appears to be via the mechanism by which extracellular signal-regulated kinase 1/2 is activated (49). NPY has also been shown to be upregulated in a number of neuroendocrine tumors, such as pheochromocytomas (11, 24), and neuroblastomas (30, 41), and are thought to be of diagnostic importance; however, its contribution to tumor progression still remains to be clarified. We have demonstrated that cholangiocarcinomas display typical markers of a neuroendocrine phenotype such as the expression of chromogranin A and neuron-specific enolase (2); therefore it is not surprising that cholangiocarcinomas also expressed NPY. In the present study, the diffuse positivity of the neuroendocrine marker, NPY, observed in human ductular cholangiocarcinoma samples is supported by a number of studies. Indeed, Liu et al. (33) have demonstrated higher expression of NPY in nonaggressive prostate epithelial tumors. In the present study, the diffuse positivity of the neuroendocrine marker, NPY, observed in human ductular cholangiocarcinoma samples is supported by a number of studies. Indeed, Liu et al. (33) have demonstrated higher expression of NPY in nonaggressive prostate epithelial tumors. Moreover, these observations about the nonconventional immunolocalization of neuroendocrine proteins are previously discussed in cholestasis and cholangiocarcinoma where the important role of GABA, chromogranin A, glycolipid A2-B4, S-100 protein, and neural cell adhesion molecule are demonstrated by us and other groups (17, 37, 46–48), suggesting that the biliary epithelium is a significant target for the neuroendocrine system. A direct outgrowth of our findings will be to study the expression of NPY in hepatocytes and cholangiocytes in cholangiocarcinoma peritumoral tissue.

While the majority of studies into the effects of NPY on cancer growth and invasion demonstrate a growth-promoting and invasive effect (45, 50, 55), there are a number of studies that have demonstrated antiproliferative effects of NPY. As mentioned above, NPY administration to a number of prostate cancer cell lines inhibited the proliferation through a Y1-dependent mechanism (49). In addition, NPY administration to colon carcinoma cells in vitro reduced the invasion potential of these tumor cells in a concentration-dependent manner (42). The data that we present here support an antiproliferative action of NPY on cholangiocarcinoma cell growth in vitro and in vivo and also an inhibitory effect on cholangiocarcinoma migration and invasion. It is conceivable that the different effects of NPY on cell growth may be due to the number of specific NPY receptors through which NPY may exert its effects. We demonstrated that all receptors are present in cholangiocarcinoma, but because of the lack of reliable specific antagonists for Y3, Y4, and Y6, we were unable to pinpoint the specific NPY receptor that is responsible for the actions of NPY on cholangiocarcinoma, and this is a limitation of the present study. However, since a specific inhibitor of Y2, BIIE 0246 (12), blocks the in vitro inhibitory effect of NPY on cholangiocarcinoma growth, likely Y2 play an important role.
in the inhibitory effect of NPY on cholangiocarcinoma growth. Further experiments (gene silencing of NPY receptors with small interfering RNAs) are necessary to pinpoint the NPY receptors involved in NPY effects on cholangiocarcinoma. As mentioned previously, NPY receptor activation can elicit cAMP- or Ca2+-mediated signal transduction pathways (1, 22, 35, 39). The data presented here support a role for IP3/Ca2+-mediated PKCα activation in the antiproliferative action of NPY since NPY increased IP3 (but not cAMP) levels and induced the activation of PKCα. These findings support the concept that Ca2+-dependent PKCα is a key regulator of the hyperplastic and neoplastic growth of cholangiocytes. Indeed, we have previously shown that the inhibition of cholangiocarcinoma growth (e.g., by gastrin, H3 histamine receptor agonists, α2-adrenergic receptor agonists, and the bile salt, tauroursodeoxycholate) is associated with enhanced phosphorylation of PKCα.

In the present study, we demonstrated a gradient of NPY expression in the cholangiocarcinoma tumor, with the highest expression found in the center of the tumor near the necrotic areas. The concept of a gradient of expression to accommodate the different microenvironments within the tumor is not new. Recently, a large-scale expression analysis of melanoma metastases was performed to identify genes that exhibit differential expression between the invasion front and the central tumor areas (54). The authors identified 248 genes that were differentially expressed within the tumor, 97 of which had higher expression within the center of the tumor compared with the invasion front (54). Some of the genes that were expressed higher at the invasion front, generally, had known functions in cell invasion (54); however, the function, with respect to cancer progression, of the genes predominantly expressed in the central area of the tumor was largely unknown (54). In addition, Ohira et al. (43) recently demonstrated that a gradient of transforming growth factor-β expression is evident in cholangiocarcinoma with little to no expression in the invasion front and strong expression in tumors growing in the bile duct lumen (43). Here we speculate that NPY may be expressed higher in the center of the tumor where it exerts growth-suppressive and antiinvasive effects to allow for the recruitment of adequate stromal support.

In conclusion, the data presented here demonstrate a role of NPY in the local control of cholangiocarcinoma cell growth. Specifically, NPY appears to be expressed to a greater extent in the center of the tumor, where we speculate that it exerts a local antiproliferative and antimigratory effect on cholangiocytes. Modulation of NPY signaling may be a useful target for the design of therapeutic tools for the treatment of this devastating cancer.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES


