Overexpression of membrane metalloendopeptidase inhibits substance P stimulation of cholangiocarcinoma growth

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Meng F, DeMorrow S, Venter J, Frampton G, Han Y, Francis H, Standeford H, Avila S, McDaniel K, McMillin M, Afroze S, Guerrier M, Quezada M, Ray D, Kennedy L, Hargrove L, Glaser S, Alpini G. Overexpression of membrane metalloendopeptidase inhibits substance P stimulation of cholangiocarcinoma growth. Am J Physiol Gastrointest Liver Physiol 306: G759–G768, 2014. First published March 6, 2014; doi:10.1152/ajpgi.00018.2014.—Substance P (SP) promotes cholangiocyte growth during cholestasis by activating its receptor, NK1R. SP is a proteolytic product of tachykinin (Tac1) and is deactivated by membrane metalloendopeptidase (MME). This study aimed to evaluate the functional role of SP in the regulation of cholangiocarcinoma (CCA) growth. NK1R, Tac1, and MME expression and SP secretion were assessed in human CCA cells and nonmalignant cholangiocytes. The proliferative effects of SP (in the absence/presence of the NK1R inhibitor, L-733,060) and of L-733,060 were evaluated. In vivo, the effect of L-733,060 treatment or MME overexpression on tumor growth was evaluated by using a xenograft model of CCA in nu/nu nude mice. The expression of Tac1, MME, NK1R, PCNA, CK-19, and VEGF-A was analyzed in the resulting tumors. Human CCA cell lines had increased expression of Tac1 and NK1R, along with reduced levels of MME compared with nonmalignant cholangiocytes, resulting in a subsequent increase in SP secretion. SP treatment increased CCA cell proliferation in vitro, which was blocked by L-733,060. Treatment with L-733,060 alone inhibited CCA proliferation in vitro and in vivo. Xenograft tumors derived from MME-overexpressed human Mz-ChA-1 CCA cells had a slower growth rate than those derived from control cells. Expression of PCNA, CK-19, and VEGF-A decreased, whereas MME expression increased in the xenograft tumors treated with L-733,060 or MME-overexpressed xenograft tumors compared with controls. The study suggests that SP secreted by CCA promotes CCA growth via autocrine pathway. Blockade of SP secretion and NK1R signaling may be important for the management of CCA.

autocrine; biliary cancer; neuroendocrine; sensory innervation; VEGF

CHOLANGIOCYTES, which line the intrahepatic and extrahepatic bile ducts (28), are the target of chronic cholestatic liver diseases (i.e., cholangiopathies) that are characterized by biliary inflammation and dysregulation of the balance be-

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vous system and peripheral organ system (35, 39). Previous research in our team supports these extrasensory functions as the knockout of α-CGRP and NK1R reduce biliary hyperplasia in mice with extrahepatic cholestasis, giving support that CGRP and SP may have the capability to induce biliary hyperplasia (19, 20).

SP synthesis is regulated by the expression of tachykinin (Tac1, the gene encoding SP) and membrane metalloendopeptidase (MME), the enzymes responsible for the synthesis and degradation of SP, respectively (11, 47). No information exists regarding the autocrine and paracrine role of SP in the regulation of CCA growth.

Given the observation that CCAs display neuroendocrine phenotypes (2), the aims of our study were to demonstrate that 1) CCA and nonmalignant biliary epithelial cells express NK1R; 2) SP stimulates CCA growth by a receptor-mediated mechanism in vitro; 3) there is enhanced expression of Tac1 and reduced expression of MME in CCA, leading to increased synthesis of SP.

Fig. 1. A: by immunofluorescence, HIBEpiC and cholangiocarcinoma (CCA) cell lines show immunoreactivity for NK1R. Negative controls using the blocking peptide for NK1R showed no reaction. Bar = 50 μm. B and C: evaluation of the expression of NK1R in CCA and nonmalignant cell lines by real-time PCR and immunobots. The expression of NK1R was observed in nonmalignant cholangiocytes as well as in all the CCA cell lines used. Data are mean ± SE of 4 experiments. D: evaluation of the expression of NK1R, Tac1, and membrane metalloendopeptidase (MME) in CCA and nonmalignant cell lines by FACS analysis. The expression of NK1R and Tac1 increased, whereas the expression of MME decreased in CCA cell lines compared with HIBEpiC cells. Data are mean ± SE of 4 experiments. *P < 0.05 vs. HIBEpiC cells; **P < 0.05 vs. corresponding NK1R evaluations; #P < 0.05 vs. corresponding NK1R and Tac evaluations.
and augmented CCA growth; and 4) reduction of CCA SP synthesis (by molecular stable silencing) and interaction of SP with NK1R (by NK1R antagonists) reduces CCA growth both in vitro and in xenograft tumor models in vivo.

MATERIALS AND METHODS

Materials. All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Synthetic SP (SZ1062) and the SP EIA kit (EZ-061-05, recognizing human, rat, and mouse species) were purchased from Phoenix Pharmaceuticals (Belmont, CA). The rabbit polyclonal antibody (NA4300-0100) and the blocking peptide (neurokinin 1 receptor rat COOH-terminal pentadecapeptide, as negative control, NP1442-0100) for NK1R were purchased from Biomol International (Plymouth Meeting, PA). The NK1R antagonist (L-733,060) was purchased from Sigma-Aldrich. The mouse monoclonal antibody (ab34199) to CD10 was purchased from Abcam (Cambridge, MA). CD10 (also known as the common acute lymphocytic leukemia antigen, CALLA) is a cell surface enzyme with MME activity that inactivates a variety of biologically active peptides including SP (6).

The selected specific primers were purchased from SABiosciences (Qiagen; Valencia, CA) and designed by using sequences with the following NCBI GenBank Accession numbers: NM_001058 (human NK1R); NM_013998 (human Tac1); NM_000902 (human MME); NM_182649 (human PCNA); NM_002276 (human CK-19); NM_003376 (human VEGF-A); and NM_002046(human glycer-aldehyde-3-phosphate dehydrogenase, GAPDH).

Human CCA cell lines and nonmalignant cholangiocytes. We used six human CCA cell lines (Mz-ChA-1, SG231, HuCC-T1, TFK-1, HuH-28, and CCLP1) of different origins and the normal human intrahepatic biliary epithelial cell line, HIBEpiC (ScienCell Research Laboratories, Carlsbad, CA) (14). Mz-ChA-1 cells (from human gallbladder) (30) were a gift from Dr. G. Fitz (University of Texas Southwestern Medical Center, Dallas, TX). HuH-28 and TFK-1 cells (from human extrahepatic bile ducts) (32, 42) were acquired from Cancer Cell Repository (Tohoku University, Sendai, Japan). These cell lines were maintained at standard conditions (27). HuCC-T1, CCLP1, and SG231 cells (from intrahepatic bile ducts) were a kind gift from Dr. A. J. Demetris (University of Pittsburgh, Pittsburgh, PA) and were cultured as described (53). HIBEpiC cells were cultured as recommended by the supplier (14).

Expression of NK1R in cell lines. The expression of NK1R was evaluated by immunofluorescence (14), real-time PCR (using 1 μg total RNA) (14), immunoblot (using 10 μg of protein from whole cell lysate) (14) and FACS analysis (22) in the CCA cell lines and HIBEpiC cells.

Immunofluorescence for NK1R in the selected cell lines was performed as described (14). Following staining, cells were mounted onto microscope slides with Prolong Antifade Gold containing 4',6-diamidino-2-phenylindole (DAPI) as a nuclear counterstain (Molecular Probes, Eugene, OR). Negative controls were also included. Sections were visualized via an Olympus IX-71 inverted confocal microscope (Tokyo, Japan).

RNA was isolated from the selected cell lines by the RNeasy Kit (Qiagen) and reverse transcribed with the Reaction Ready First Strand cDNA synthesis kit (SABiosciences, Frederick, MD). PCR reactions were used as templates for the PCR assays by using a SYBR Green PCR master mix and specific primers designed against the selected gene analyzed in the real-time thermal cycler (Agilent MX3005P System, Woburn, MA). mRNA expression was analyzed using the 2^(-ΔΔCt) method. 

Fig. 2. A and B: by real-time PCR, there was an increase in the expression of Tac1 and decreased expression of MME mRNA in most of the cholangiocarcinoma (CCA) used compared with HIBEpiC cells. Data are mean ± SE of 3 experiments. *P < 0.05 vs. HIBEpiC cells. C: CCA cell lines secreted higher levels of substance P (SP) compared with HIBEpiC cells. Data are mean ± SE of 6 experiments.
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A

TFK-1

Mz-ChA-1

SG231

CCLP-1

HUCC-T1

HUH-28

B

Mz-ChA-1

C

Mz-ChA-1

HuH-28

HuCC-T1

SG231

CCLP-1

TFK-1

D

24 hr

48 hr

E

Number

Number

Number

Number

Channels (FL3-A)

Channels (FL3-A)

Channels (FL3-A)

Channels (FL3-A)

Debris

Aggregates

Apoptosis

Dip G1

Dip G2

Dip S
thermocycler, Santa Clara, CA). A ΔΔCT (delta delta of the threshold cycle) analysis was performed using HIBEpiC cells as controls (14). Data are expressed as the fold-change of relative mRNA levels ± SE. Immunoblots were normalized by α-tubulin (the housekeeping gene). Band intensity was determined by scanning video densitometry via the phospho-imager, Storm 860 (GE Healthcare, Piscataway, NJ), and the ImageQuant TL software version 2003.02 (GE Healthcare).

FACS analysis (with 20,000 events in the light scatter, SSC/SSC, acquired) for NK1R was performed in the selected cell lines by using a C6 flow cytometer and analyzed by CFlow Software (Accuri Cytometers, Ann Arbor, MI) (22). The expression of NK1R was identified and gated on FL1-A/Count plots. The relative quantity of NK1R (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 FL1-A is green fluorescence channel, Count is number of cells examined, CV is standard deviation, SQR is square root, and Count-1 is number of events counted as cholangiocytes (gate 1; we counted 5,000 events under gate 1 in each group to keep consistency in all groups).

Expression of Tac1 and MME and secretion of SP in CCA and normal cholangiocyte lines. We evaluated the expression of 1) the message (by real-time PCR, see above) (14); and 2) the protein for tachykinin (Tac1, the gene encoding SP) (11) and MME in CCA cell lines and HIBEpiC cells by FACS analysis, see above (41).

CCA and HIBEpiC cells (1 × 10^7 cells/ml) were incubated for 24 h at 37°C and the amount of SP released into the media was assayed by using a commercially available EIA kit according to the manufacturer’s instructions.

Effect of SP on the proliferation of nonmalignant and CCA cells. The proliferation of CCA cells and HIBEpiC cells was evaluated by MTS assays (14). Data were expressed as fold change of proliferation vs. basal. Briefly, cells were plated in a 96-well plate (7,000 cells per well) and incubated overnight at 37°C. Medium was changed to serum free and cells were further incubated for 24 h prior to stimulation. The selected CCA cell lines were stimulated with different doses (10^-6 M to 10^-12 M for 48 h) of vehicle or SP before measuring cell growth by MTS assays (14). We also measured the effect of SP (10^-12 M for 48 h) on the proliferation of Mz-ChA-1 cells (used in the in vivo experiments) in the absence/presence of L-733,060 (25 μM) (37). In parallel, we evaluated the effect of L-733,060 treatment alone (0, 5, 10, 25, 50, and 100 μM, for 48 h) (37) on the proliferation of CCA cells by MTS assays (14). We also evaluated, by FACS analysis, the effect of L-733,060 (25 μM for 24 and 48 h) on the cell cycle of HIBEpiC and Mz-ChA-1 cells (25).

We used human cDNA clone (OriGene Technologies, Rockville, MD) with neomycin resistance to generate and select the stable transfected Mz-ChA-1 cells overexpressing MME (5, 14). Real-time PCR (14) was performed to determine the degree of MME overexpression in Mz-ChA-1 cells. We also evaluated the secretion of SP (by EIA kits) in Mz-MME and Mz-neg control stably transfected Mz-ChA-1 cell lines. In the two cell lines, we evaluated cell proliferation by MTS assays (after incubation for 24, 48, 72, and 7 days) (14).

In vivo studies in nu/nu nude mice. Eight-week-old male Balb/c nu/nu nude mice (~30 g; Charles River, Wilmington, MA) were kept in a temperature-controlled environment (20–22°C) with 12-h light-dark cycles with free access to drinking water and standard chow. All the animal experiments were conducted under protocols approved by the Scott & White and Texas A&M HSC IACUC.

Mz-ChA-1 cells (3 × 10^6) were injected subcutaneously into the flanks of nu/nu nude mice (14). Treatments (by intraperitoneal injections) were performed as follows: 1) mice (n = 4) received 0.9% NaCl (100 μl); and 2) mice (n = 4) were treated with L-733,060 (10 mg/kg ip in 100 μl of NaCl) (4) every other day (4). Injections were performed every other day for 52 days after tumor establishment (day 10). After 52 days, mice were anesthetized with pentobarbital sodium (50 mg/kg ip) and tissues were harvested and used for measuring the selected parameter.

In separate experiments, Mz-MME (cells stably overexpressing MME) and Mz-neg were cultured and prepared for injection into the flanks of nu/nu nude mice as described (14). Tumor measurements began after tumor establishment (day 10) (14). After 21 days, both groups of mice were anesthetized with pentobarbital sodium (50 mg/kg body wt ip) and tissues were harvested.

Fig. 3. A: SP (10^-6 M to 10^-12 M for 48 h) increases the proliferation of CCA lines compared with their basal values. Data are mean ± SE of 4 experiments. *P < 0.05 vs. basal values. B: the stimulatory effect of SP (at 10^-11 M) on CCA growth was blocked by L-733,060 (25 μM); at the same dose L-733,060 inhibited Mz-ChA-1 growth. Data are mean ± SE of 4 experiments. *P < 0.05 vs. nonmalignant controls. C: when CCA cells were treated with L-733,060 (at 5, 10, 25, 50, and 100 μM for 48 h) there was decreased cell proliferation compared with the corresponding basal values. Data are mean ± SE of 4 experiments. *P < 0.05 vs. the corresponding basal values. D: at the dose of 25 μM, L-733,060 decreased the proliferation of selected CCA cell lines following incubation for 24 and 48 h. *P < 0.05 vs. the corresponding basal values. E: by FACS analysis, we also show that L-733,060 decreased the proliferative activity of Mz-ChA-1 but not HIBEpiC cells. Data are mean ± SE of 4 experiments.
Fig. 5. A: L-733,060 had small effects on tumor growth at early time periods, but significantly decreased tumor volume throughout the measurement time period (up to day 21) compared with the vehicle-treated tumors. Data are mean ± SE of values from 4 nu/nu nude mice. *P < 0.05 vs. the corresponding values of nu/nu nude mice treated with vehicle. B: when implanted into the flanks of nu/nu nude mice, the tumors derived from Mz-MME cells grew to a lower extent (up to 21 days) than those originating from Mz-neg. Data are mean ± SE of values from 4 nu/nu nude mice in each treatment group. *P < 0.05 vs. the corresponding values of tumors originating from Mz-neg.

Tumor parameters were measured twice a week with an electronic caliper and volume was determined as follows: tumor volume (mm³) = 0.5 × [length (mm) × width (mm) × height (mm)]. The tumors harvested were used for measuring the expression of Tac1, MME, NK1R, PCNA, CK-19, and VEGF-A (an important trophic factor for CCA growth) (10) in total tumor samples by real-time PCR (14).

Statistical analysis. All data are expressed as mean ± SE. Differences between groups were analyzed by the 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. A value of P < 0.05 was considered significant.

RESULTS

Expression of NK1R, Tac1, and MME in biliary cell lines and secretion of SP in biliary cell lines. By immunofluorescence, all the CCA cell lines used and HIBEpiC cells showed immunoreactivity for NK1R (red); negative controls with the use of the blocking peptide for NK1R showed no reaction (Fig. 1A). By real-time PCR, immunoblots, and FACS analysis, the expression of NK1R was observed in nonmalignant cholangiocytes as well as in all the CCA cell lines used (Fig. 1, B–D). By FACS analysis, we demonstrated that the expression of NK1R increased in all the CCA cell lines compared with HIBEpiC cells (Fig. 1D).

By real-time PCR, there was increased expression of Tac1 (the gene encoding SP) (11) and decreased expression of MME mRNA in selected CCA cells used compared with HIBEpiC cells (Fig. 2, A and B). By FACS analysis, the protein expression of Tac1 increased, whereas the expression of MME decreased in all CCA cells used compared with HIBEpiC (Fig. 1D). Furthermore, all of the CCA cell lines secreted higher levels of SP compared with HIBEpiC cells (Fig. 2C).

SP stimulates the proliferation of CCA cells. SP (at the doses ranging from 10⁻⁶ M to 10⁻¹² M for 48 h) increased the proliferation of CCA cell lines compared with their basal values (Fig. 3A). However, no significant effects for SP were observed in nonmalignant cholangiocytes (data not shown). The stimulatory effect of SP (at 10⁻¹¹ M at 48 h) on Mz-Cha-1 growth was blocked by the NK1R antagonist, L-733,060 (25 μM) (Fig. 3B); at the same dose L-733,060 inhibited Mz-Cha-1 growth (Fig. 3B). When CCA cell lines were treated with L-733,060 (at 5, 10, 25, 50, and 100 μM) there was a dose-dependent decrease in cell proliferation compared with the relative basal values (Fig. 3C). At the dose of 25 μM, L-733,060 induced a significant decrease in the proliferation of selected CCA cell lines following incubation for 24 and 48 h (Fig. 3D). By FACS analysis, we also show that L-733,060 decreased the proliferative activity of Mz-Cha-1 but not HIBEpiC cells (Fig. 3E). The S phase of Mz-Cha-1 cells (treated with vehicle, basal value) displayed higher proliferation than HIBEpiC cells due to the high mitosis (Fig. 3E). L-733,060 induced arrest of the Go/G1 phase, and decreased the S phase of Mz-Cha-1 cells, indicating disruption of cell cycle progression in Mz-Cha-1 cells through inhibition of SP signaling; L-733,060 induced a significant increase in apoptosis in Mz-Cha-1 but not in HIBEpiC cells (Fig. 3E).

To validate the overexpression of MME in Mz-Cha-1 cells (used in our in vivo studies in nu/nu nude mice) we have shown that Mz-MME cells express higher levels of the message for MME compared with vector transfected cells (Fig. 4A). By MTS assays, there was reduced proliferation of Mz-MME compared with Mz-neg cell lines (Fig. 4B).

Effects of administration of L-733,060 and overexpression of MME on the proliferation and expression of VEGF-A in Mz-Cha-1 cells implanted in nu/nu nude mice. The NK1R inhibitor, L-733,060, had small effects on tumor growth at early time periods but at day 21 significantly decreased xeno-
Here, we present evidence that SP exerts growth-promoting effects on CCA cells in vitro, and blocking SP activity subsequently inhibits CCA cell proliferation in vitro and tumor growth in vivo. The mechanism by which SP is growth promoting in CCA is unknown; nevertheless, some of the functional effects of the upregulation of SP signaling in other cancers have been elucidated. For example, SP and NK1R are upregulated in HER2-positive breast tumors and are found to transactivate HER2 and EGFR; also, the inhibition of NK1R strongly decreased steady-state expression EGFR and HER2 (16). Similarly, transactivation of EGFR is thought to be partly responsible for the mitogenic actions of SP in glioblastoma cells (9). In addition, SP-mediated activation of the Ca²⁺/Src/PKCα/ERK1/2 pathway has been shown to enhance cancer cell proliferation (54). Given that both EGFR activation and ERK1/2 activation are known to increase CCA cell proliferation and tumor growth (15, 44, 52, 55), it is conceivable that SP may be exerting its mitogenic effects via a combination of these pathways. SP had been shown to mediate the antiapoptotic and proliferative responses of human colonocytes via activation of Akt signaling mechanisms (31). Previous studies have shown that VEGF plays an important role in regulating both normal and neoplastic cholangiocyte proliferation (10, 17, 18). SP has been shown to stimulate VEGF expression levels promoting angiogenesis and wound healing (29). This finding may indicate an additional mechanism by which inhibition of SP signaling may decrease VEGF expression and subsequently CCA proliferation. Further studies are warranted to elucidate this potential mechanism mediated by SP.

The upregulation of SP/NK1R have also been observed in chronic liver diseases (19, 50). Indeed, increased SP levels can be detected in the serum of both cholestatic patients and experimental cholestatic rodents (50). Within the liver, SP has been shown to increase cholangiocyte proliferation during cholestasis via the cAMP-dependent phosphorylation of protein kinase A (19). Furthermore, NK1R expression is observed in cholangiocytes and upregulated in a model of extrahepatic biliary obstruction. In addition, NK1R knockout mice have a dampened proliferative reaction to biliary hyperplasia (19). Consistent with these observations, we found that treatment of HBEPiC cells with recombinant SP also increased proliferation in vitro.

A study has shown that MME, the neutral endopeptidase responsible for cleaving and deactivating SP, is expressed in the bile canaliculi and interlobular ducts (7). The expression of MME is downregulated during liver cirrhosis and hepatocellular carcinoma (38). Furthermore, suppression of MME immunoreactivity has previously been described in extrahepatic bile duct cancers (49). The data presented here demonstrate a suppression of MME expression in CCA, which supports the notion that MME expression may be downregulated in various types of liver cancers. Consistent with this concept, high MME expression in the tumor center or invasion front, but not in the lymph node metastases, was a favorable prognostic indicator in human bladder cancer (46). In contrast, the expression of MME has been shown to decrease in noninvasive low-grade cancers compared with nonneoplastic tissue, but the expression increases again in invasive high-grade tumors (26). This suggests that perhaps MME expression may be a reflection of the grade and invasiveness of the tumor in question. Indeed, MME expression was associated with a more aggressive phenotype in
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