γ-Aminobutyric Acid Inhibits Cholangiocarcinoma Growth by Cyclic AMP–Dependent Regulation of the Protein Kinase A/Extracellular Signal-Regulated Kinase 1/2 Pathway

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Abstract
We studied the effect of the inhibitory neurotransmitter, γ-aminobutyric acid (GABA), in the regulation of cholangiocarcinoma growth. We determined the in vitro effect of GABA on the proliferation of the cholangiocarcinoma cell lines (Mz-ChA-1, HuH-28, and TFK-1) and evaluated the intracellular pathways involved. The effect of GABA on migration of Mz-ChA-1 cells was also evaluated. In vivo, Mz-ChA-1 cells were s.c. injected in athymic mice, and the effects of GABA on tumor size, tumor cell proliferation, apoptosis, collagen quantity, and the expression of vascular endothelial growth factor-A (VEGF-A) and VEGF-C (cancer growth regulators) were measured after 82 days. GABA decreased in vitro cholangiocarcinoma growth in a time-dependent and dose-dependent manner, by both cyclic AMP/protein kinase A– and GABA receptors prevented GABA inhibition of cholangiocarcinoma proliferation. GABA inhibited Mz-ChA-1 cell migration and, in vivo, significantly decreased tumor volume, tumor cell proliferation, and VEGF-A/C expression whereas increasing apoptosis compared with controls. An increase in collagen was evident in GABA-treated tumors. GABA decreases biliary cancer proliferation and reduces the metastatic potential of cholangiocarcinoma. GABA may represent a therapeutic agent for patients affected by malignancies of the biliary tract.

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Introduction
Cholangiocarcinoma is characterized by the malignant proliferation of cholangiocytes, which line intrahepatic and extrahepatic biliary ducts. Biliary tumors display high malignance and poor prognosis (1). No established treatments exist for this tumor. γ-Aminobutyric acid (GABA) is the most important inhibitory neurotransmitter in the central nervous system (CNS; ref. 2). GABA is also present in the peripheral nervous system and several organs and tissues (2–4). In addition to the CNS, the liver represents the most important site of synthesis and metabolism of GABA (5). Hepatocytes absorb GABA from the portal circulation (6), express GABA receptors (7), and contain enzymes responsible for GABA synthesis (e.g., monoamine oxidase, diamine oxidase, and glutamic acid decarboxylase; ref. 8) and catabolism (e.g., GABA-transaminase; ref. 9). GABA action is mediated by two classes of receptors. The ionotropic GABAA and GABAC receptors are pentameric chloride channels and are constituted, respectively, by combinations of several subunit types (α1, α2, β1, β2, γ1, β3, δ, ε, θ, and π) and by only single or multiple subunits (ρ1, 3). The metabotropic GABAB receptor is associated with G proteins and exists as R1a, R1b, and R2 isoforms (10). GABA has inhibitory effects on gastric cancer (11), colon carcinoma (12), and hepatocarcinoma (13); GABA content and GAD activity are increased in neoplastic tissues, such as colon (14) and gastric cancer (15). The role of GABA in the regulation of cholangiocarcinoma growth is unknown. We posed these questions: (a) Do malignant cholangiocytes express GABA receptors? (b) Does GABA regulate cholangiocarcinoma growth? (c) Do all three GABA receptor subtypes play a role in mediating this GABA effect on malignant cholangiocytes? (d) Is GABA regulation of cholangiocarcinoma growth associated with increased γ-myo-inositol-1,4,5-triphosphate/Ca2+–dependent pathways, leading to down-regulation of extracellular signal-regulated kinase 1/2 phosphorylation. Blocking of GABAA, GABAC, and GABAB receptors prevented GABA inhibition of cholangiocarcinoma proliferation. GABA inhibited Mz-ChA-1 cell migration and, in vivo, significantly decreased tumor volume, tumor cell proliferation, and VEGF-A/C expression whereas increasing apoptosis compared with controls. An increase in collagen was evident in GABA-treated tumors. GABA decreases biliary cancer proliferation and reduces the metastatic potential of cholangiocarcinoma. GABA may represent a therapeutic agent for patients affected by malignancies of the biliary tract.

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Materials and Methods
Materials
Reagents were purchased from Sigma (St. Louis, MO) and antibodies for immunohistochemistry and immunoblotting from Santa Cruz Biotechnology (Santa Cruz, CA), unless differently indicated. The GABAA receptor antagonist, bicuculline, was purchased from Tocris Cookson, Inc. (Ellisville, MO). The cleaved caspase-3 antibody was purchased from Cell Signaling (Beverly, MA). RIA kits for the determination of intracellular cAMP and γ-myo-inositol-1,4,5-triphosphate (IP3) and cyclic AMP (cAMP) synthesis, which leads to changes in phosphorylation of protein kinase A (PKA)/extracellular signal-regulated kinase 1/2 (ERK1/2)? Is GABA does GABA reduce the migration of cholangiocarcinoma cells? (f) Does GABA inhibit cholangiocarcinoma growth in an in vivo model of biliary cancer?

Cell Culture
We used three human cholangiocarcinoma cell lines (Mz-ChA-1, HuH-28, and TFK-1) with different origins. Mz-ChA-1 cells, from human gallbladder...
Expression of γ-Aminobutyric Acid Receptors

Immunofluorescence. We detected GABA_A β-3 and GABA_A R1 receptor subunits in H-69, Mz-CHA-1, HuH-28, and TFK-1 cells. We selected these particular isoforms because GABA_A β-3 and GABA_A R1 subunits were already detected in the liver, and no studies about their expression and function on cholangiocytes were available (7, 21). There are no commercially available antibodies against the GABA_A receptor. Cells were seeded into six-well dishes containing a sterile coverslip on the bottom of each well. Cells were allowed to adhere overnight, washed once in cold PBS, fixed to the coverslip with 4% bovine serum albumin (BSA in PBS) for 1 hour. GABA_A and GABA_A receptor antibodies were diluted 1:100 in 1% BSA/PBST, added to the coverslips, and incubated overnight at 4°C. Cells were washed 3 × 10 minutes in PBST, and a 1:100 dilution (in 1% BSA/PBST) of Cy3-conjugated secondary antibodies (Jackson Immunoresearch, West Grove, PA) was added for 2 hours at room temperature. Cells were washed again and mounted into microscope slides with Antifade gold containing 4′,6-diamidino-2-phenylindole as a counterstain (Molecular Probes, Eugene, OR). Negative controls were done with the omission of the respective primary antibodies. Images were taken on an Olympus IX71 fluorescence microscope with a DP70 digital camera. For the merged pictures, images from each channel were overlaid electronically using the Adobe Photoshop software.

Immunoblots. Following trypsinization, H-69, Mz-CHA-1, TFK-1, and HuH-28 cells (1 × 10^6) were resuspended in lysis buffer as described (19, 22) and sonicated six times (30-second bursts). We did immunoblots to detect GABA_A β-3 and GABA_A R1 receptor subunits as described (19, 22).

Molecular analysis. We evaluated the expression of GABA_A receptor by reverse transcription–PCR (RT-PCR) using total RNA from H-69, Mz-CHA-1, HuH-28, and TFK-1 cholangiocarcinoma cells. cDNA synthesis was done as described (23). For PCR, we used primers specific for human GABA_A (sense 5′-CAGTGTAGCGTGGCCAAACA-3′, corresponding to nucleotides 1144-1164; antisense 5′-AATGGCAGGTTATCCGTGA-3′, corresponding to nucleotides 1306-1326; refs. 23, 24). PCR was done at the same conditions described (23). Ten microliters of each PCR product were separated on a 1.5% agarose gel and stained with ethidium bromide. The intensity of the bands was determined by scanning video densitometry using the Chemiluminescent imaging system (Alpha Innotech Corp., San Leandro, CA). The images were acquired by Cellquest software (10,000 events evaluated).

Effect of γ-Aminobutyric Acid on Malignant Cholangiocarcinoma Growth

γ-Aminobutyric acid modulation of cholangiocarcinoma growth associated with changes in the protein kinase A/mitogen-activated protein kinase (extracellular signal-regulated kinase) pathway? During incubation, medium was replaced every 24 hours, and fresh GABA at the specified concentrations was added. After incubation of Mz-CHA-1 cells with 0.2% BSA (basal) or GABA (100 µM/L) for 48 hours, culture plates were kept on ice for 30 minutes, then cells were scraped and lysed. Proteins (2.5 μg/lane) were resolved, and immunoblots were done (19, 22). Membranes were subsequently stripped and reprobed to evaluate the expression of the corresponding total proteins.

Evaluation of the Effect of γ-Aminobutyric Acid on Mz-CHA-1 Cell Migration

Mz-CHA-1 cells were plated on six-well culture dishes and grown using the Collagen Medical Research Laboratories medium 1066 supplemented with 10% fetal bovine serum. After reaching confluence, cell layers were disrupted by producing a linear wound with a sterile pipette tip (31). The cells were then washed with PBS to remove debris and incubated in the absence or presence of GABA (100 µM/L). Hydroxyurea (5 mM/L) was added to the system to arrest cell proliferation (32). At time 0 and after 24, 48, and 72 hours, samples were examined by a phase-contrast microscope (Olympus, C2, Tokyo, Japan), and the wound size was measured at five random sites perpendicular to the wound. At time 0 and after 96 hours of incubation, Mz-CHA-1 cells were resuspended in PBS, and cell size was measured by flow cytometry (FACSCalibur, Becton Dickinson, San Jose, CA). The images were acquired by Cellquest software (10,000 events evaluated).
Effect of γ-Aminobutyric Acid on Cholangiocarcinoma Tumor Implanted in Nude Mice

Treatment Schedule. Male 8-week-old BALB/c nude (nu/nu) mice were purchased from Taconic Farms (Germantown, NY). The mice were kept in a temperature-controlled environment (20±2°C) with a 12-hour light/dark cycle and with free access to drinking water and to standard mouse chow. Mz-ChA-1 cells (3×10⁶) were suspended in 0.5 mL of extracellular matrix gel and injected s.c. in the left back flank of these animals. Mice were divided into two groups: (a) the first group (n = 4, control) received 0.9% NaCl injection (150 μL) into the implanted tumor; and (b) the second group (n = 4) received GABA injections (1000 mg/kg body weight dissolved in 150 μL of 0.9% NaCl ref. 33) into the tumor. The same operator did the injections every other day starting from "day 0," when the tumors were implanted, for 82 days. Tumor variables were measured twice a week by an electronic caliper, and volume was determined as tumor volume (mm³) = 0.5 × [length (mm) × width (mm) × height (mm)]. The measurements started from the third week, day 23, when the tumor mass was well established. A third operator, in a coded and blinded fashion, evaluated morphometric variables. Latency represents the time for the tumor to increase to 150% of the initial volume. After 82 days, mice were anesthetized with sodium pentobarbital (50 mg/kg IP) and sacrificed according to institutional guidelines. Before sacrifice, serum was obtained, and aspartate aminotransferase (AST), alanine aminotransferase (ALT), and creatinine were measured. Heart, liver, and kidneys were isolated, fixed in formalin, embedded in paraffin, processed for histopathology, and stained with H&E for the detection of tissue damage.

Morphologic Analysis of Tumor Tissues. A third operator dissected tumor tissues from mice. Neoplastic tissues were fixed in formalin, embedded in paraffin, processed for histopathology, stained with H&E for routine examination or with Sirius red for collagen visualization, and examined by light microscopy. Tumor protein selectively expressed by cholangiocytes was evaluated after CK-7 immunohistochemical staining; cell proliferation was assessed by immunohistochemistry for proliferating cell nuclear antigen (PCNA; ref. 34). Tumor sections (n = 4) from each group were stained for CK-7 and PCNA, as described (34, 35), and for GABAα and GABAβ receptors. Following staining, sections were counterstained with hematoxylin and examined with a microscope.

Tumor sections (n = 4) from each selected group of animals were stained by immunohistochemistry with antibodies specific for vascular endothelial growth factor-A (VEGF-A) or VEGF-C, diluted 1:400. Apoptosis was evaluated by staining for cleaved caspase-3 (36). Negative controls were obtained by incubating the tumor sections only with the secondary antibody.

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 ANOVA, when more than two groups were analyzed. P < 0.05 was used to indicate statistical significance.

Results

Expression of γ-aminobutyric acid receptors. GABAα β-3 and GABAβ R1 receptor subunits were detected in H-69, Mz-ChA-1, HuH-28, and TFK-1 cells by immunofluorescence (Fig. 1A). The four cell lines expressed two bands, migrating, respectively, at ~59 and ~70 kDa for the GABAα β-3 and GABAβ R1 receptor subunits on immunoblots (Fig. 1B) and a band for the GABAβc receptor by RT-PCR (Fig. 1C).

Effect of γ-aminobutyric acid on cholangiocarcinoma growth. GABA (1-100 μmol/L) decreased the proliferation of Mz-ChA-1 cells at 24 and 48 hours (Fig. 2A). The significant inhibitory effect of GABA (100 μmol/L) persisted up to 72 hours (Fig. 2B). GABA inhibited HuH-28 and TFK-1 cell proliferation up to 72 hours of incubation (P < 0.05 versus basal values; data not shown). Immunoblots showed decreased PCNA protein expression in Mz-ChA-1 cells incubated for 48 hours with GABA at increasing concentrations (1-100 μmol/L; Fig. 2C). The inhibitory effect of GABA (100 μmol/L) persisted up to 72 hours (Fig. 2D). Similar results were obtained using HuH-28 and TFK-1 cell lines (data not shown). MTS assays and immunoblots for PCNA showed that GABA effects were significantly blocked by bicuculline, phaclofen, and TPMPA (Fig. 3A-C). As shown in other cell lines (37), 0.1% DMSO had no inhibitory effect on Mz-ChA-1 cell proliferation.
when compared with untreated cells. The inhibitory effect on cholangiocarcinoma proliferation was more evident with higher GABA concentrations (up to 3 mmol/L; Fig. 3B), even if, as previously shown (19), lower dose of gastrin (10^{-7} mol/L) inhibited in vitro cholangiocarcinoma growth at higher extent (40% with respect to basal values). The inhibition of GABA intracellular catabolism, thus favoring stable levels of neuropeptide, potentiates GABA inhibitory effect on cell proliferation (Fig. 3B). Rp-cAMP and BAPTA/AM partially impaired GABA inhibitory effect on cholangiocarcinoma growth when added individually (Fig. 3C). However, when added together, Rp-cAMP1168 and BAPTA/AM completely blocked GABA’s inhibitory effect on cholangiocarcinoma growth (Fig. 3C). These results indicate that similar to other studies in cholangiocarcinoma (22), the increase of intracellular cAMP levels, the subsequent activation of PKA, and also changes of [Ca^{2+}]i levels (19) by GABA stimulation play a role in GABA inhibition of cholangiocarcinoma growth.

Intracellular pathways associated with the inhibitory effect of γ-aminobutyric acid on cholangiocarcinoma growth. In Mz-ChA-1 cells, GABA (100 μmol/L) significantly increased cAMP levels compared with controls (168.3 ± 3.2 versus 141.7 ± 3.3 fmol/100,000 cells, P < 0.05). This increase was prevented by preincubation with the GABA_A, GABA_B, and GABA_C receptor antagonists (115.5 ± 5.1, 115.1 ± 4.0, and 123.7 ± 0.8 fmol/10^5 cells, respectively; P < 0.05 versus GABA-treated cells).

Enhanced IP_3 levels were evident following incubation of Mz-ChA-1 cells with 100 μmol/L GABA (1.51 ± 0.08 versus 2.33

**Figure 2.** Dose-dependent and time-dependent effect of GABA on Mz-ChA-1 cell proliferation. A-C, GABA at (1-100 μmol/L) inhibits DNA synthesis of MzChA-1 cells at 24 and 48 hours of incubation. The inhibitory effect was assessed by MTS assay (A) and immunoblots for PCNA protein expression (C). The decrease in proliferation was significant using GABA at 10 and 100 μmol/L. Furthermore, MTS assay (B) and PCNA protein expression (D) show that GABA (100 μmol/L) decreases Mz-CHA-1 cell proliferation in a time-dependent fashion. Columns, means; bars, SE. *, P < 0.05 versus corresponding basal value.
In Mz-ChA-1 cells, GABA (100 μmol/L) increased the protein expression of phosphorylated PKA (p-PKA) and decreased ERK1/2 phosphorylation, despite no change of total isoforms, with respect to controls (Fig. 4). GABA did not alter the phosphorylation of p38 and c-Jun NH2-terminal kinase (JNK; data not shown).

**Evaluation of the effect of γ-aminobutyric acid on the migration of Mz-ChA-1 cells.** The addition of GABA (100 μmol/L) to Mz-ChA-1 cells in culture increases the time necessary for wound closure. After 72 and 96 hours of incubation, there was a significant difference in wound size between the two groups of stimulated cells (Fig. 5B). After 96 hours, incubation with GABA induced an incomplete wound closure (Fig. 5B), and no difference in cell volume was evident in GABA-treated cells with respect to controls (Fig. 5A). The data supports the hypothesis that GABA inhibits Mz-ChA-1 cell migration.

**Effect of γ-aminobutyric acid on the growth of tumors implanted in nude mice.** At 82 days, a significant difference in tumor size was found in GABA-treated mice compared with mice injected with vehicle only (control; 517.48 ± 46.05 versus 1,122.89 ± 137.31 mm3; P < 0.02; Fig. 5C-D). No significant difference in body weight was detected between the two groups (Table 1). There was a significant decrease in the tumor latency (25) in control mice versus mice treated with GABA (Table 1). Normal serum values of AST, ALT, and creatinine were detected in the two groups (Table 1).

Immunohistochemistry for PCNA shows a decrease of proliferation of the tumor cells from GABA-treated mice compared with tumor cells from controls (Fig. 6). A decrease of positivity for CK-7 and for VEGF-A and VEGF-C of GABA-treated versus control tumors is shown (Fig. 6).

This represents the first evidence for the presence of VEGF-A and VEGF-C proteins in Mz-ChA-1 cells. Immunohistochemistry for cleaved caspase-3 (Fig. 6) shows an increase in apoptotic tumor cells in GABA-treated versus control tumors. In mice, the morphologic evaluation of xenografts (Fig. 6, H&E) showed encapsulated tumors with several acinar or tubular structures resembling those of other adenocarcinomas and fibrotic tissue that is more prominent in GABA-treated animals (Fig. 6, Sirius red). An amorphous matrix negative after staining with Sirius red, Gomory (collagen), and Congo red (amyloid) was often observed between groups of cells in all the tumors. Randomly distributed necrotic areas were common without difference between control and treated tumors, whereas apoptotic bodies, evaluated either after stained caspase-3 or H&E staining (Fig. 6), were significantly higher in GABA-treated tumors. Moreover, the expression of GABA_A and GABA_B receptors is maintained in GABA-treated mice.
treated, with respect to the control tumors, at the end of the treatment (Fig. 6). No organ damage was found by immunohistochemical analysis in the two groups of animals (data not shown).

Discussion

We found that H-69 cholangiocytes and Mz-ChA-1, TFK-1, and HuH-28 cholangiocarcinoma cells express GABA<sub>A</sub>, GABA<sub>B</sub>, and GABA<sub>C</sub> receptors. We show that GABA inhibits Mz-ChA-1, HuH-28, and TFK-1 cholangiocarcinoma growth, and GABA inhibition of cholangiocarcinoma growth is associated with increased intracellular IP<sub>3</sub> and cAMP levels, PKA phosphorylation, and dephosphorylation of ERK1/2 but not of p38 and JNK1/2. GABA inhibition of cholangiocarcinoma growth was mediated by interaction with GABA<sub>A</sub>, GABA<sub>B</sub>, and GABA<sub>C</sub> receptors. In a nude mouse model, we showed that GABA inhibits cholangiocarcinoma growth in vivo.

In several types of cancers, the increase in cAMP levels is accompanied by a decrease in cell growth (22, 38, 39). Enhanced cAMP levels in human meningioma cells inhibit growth (38), and up-regulation of the cAMP/PKA pathway causes apoptosis leading to a decrease in proliferation of malignant gliomas (39). Stimulation of α<sub>2</sub>-adrenergic receptors by UK1,304 decreases cholangiocarcinoma growth by enhancing cAMP levels, which inhibit mitogen-activated protein kinase activity (22).

We showed that GABA inhibits cholangiocarcinoma growth by interacting with the three GABA receptors subtypes (sensitivity to GABA<sub>A</sub>, GABA<sub>B</sub>, and GABA<sub>C</sub> receptor antagonists). Maintaining stable levels of GABA by blocking GABA intracellular catabolism, cholangiocarcinoma growth was inhibited to a greater extent. The inhibition of cholangiocarcinoma growth by GABA was partially blocked by incubation with RpcAMP116816 and BAPTA/AM but was totally blocked by incubation with RpcAMP116816 and BAPTA/AM added together to the medium. These findings support the hypothesis that GABA, through GABA<sub>A</sub>, GABA<sub>B</sub>, and GABA<sub>C</sub> receptor stimulation, activates several intracellular pathways, involving cAMP and IP<sub>3</sub>/Ca<sup>2+</sup>, which, interacting with each other, form a complex loop leading to the decrease of cholangiocarcinoma cell proliferation. The importance of the crosstalk between cAMP- and Ca<sup>2+</sup>/IP<sub>3</sub>-related pathways has been described in cholangiocytes (40, 41). We propose that cAMP- and Ca<sup>2+</sup>/IP<sub>3</sub>-dependent pathways are both activated in GABA-treated cholangiocarcinoma cells and have a synergistic effect in the inhibition of cholangiocarcinoma proliferation.

Substances, such as gastrin, inhibit in vitro cholangiocarcinoma growth to a greater extent than GABA. Nevertheless, the inhibitory effect of GABA on cholangiocarcinoma cell proliferation was confirmed by the in vivo study in nude mice. Injections of GABA into cholangiocarcinoma tissue implanted s.c. in nu/nu athymic mice induced a decrease in cholangiocarcinoma proliferation. This effect was macroscopically evident by the significant reduction of the tumor mass in the GABA-treated mice at 82 days. We speculate that the inhibition of the tumor growth by GABA is also due to a blocking of the production or the release of trophic factors (e.g., VEGF-A and VEGF-C), which are determinant for the vascularization, the development, and the growth of neoplasias. At 27 days after tumor implantation, the inhibitory effect of GABA (40%) on cholangiocarcinoma growth in vivo was similar to lanreotide, a somatostatin analogue (42). Moreover, the immunohistochemical analysis of the malignant specimens showed decreased protein expression of PCNA, VEGF-A, and VEGF-C, confirming the inhibitory effect of GABA on cholangiocarcinoma growth. The inhibition of cancer growth induced by GABA was accompanied by increased apoptosis in malignant cells and abundant fibrosis. In addition, an amorphous matrix of unknown significance was often observed between groups of cells in all tumors. Previous studies suggest that GABA stimulates collagen synthesis and proliferation of human fibroblasts (43). Furthermore, the fibrogenic pathways could be activated in response to apoptotic and necrotic processes, more evident in GABA-treated tumors (44) with respect to the controls. Studies have shown the relationship between the GABAergic system and tumors originating from different organs (e.g., colon, breast, stomach, and ovary). High levels of diamine oxidase, an enzyme producing GABA, are described in at least nine...
human malignancies (45, 46). Ovarian cancers are associated with elevated GABA levels, which are reflected in high urinary neurotransmitter levels, supporting the hypothesis that the measurement of GABA, combined with other cancer-associated markers, may be useful as a noninvasive indicator for cancer diagnosis or during follow-up to evaluate the tumor response to therapy (47).

In malignant conditions, an increase of diamines, polyamines, and activity of diamine oxidase could enhance GABA production (46). We suppose that the increase in content, production, and secretion of GABA could represent a cell response and an immune defense mechanism against tumor development. However, even if malignant cells produce high GABA concentrations, such levels are much less (46, 47) than the doses of neuropeptide required to inhibit cancer cells proliferation. Thus, we suppose that the increased production of GABA by tumoral cells is insufficient to block tumor growth, because inadequate concentrations of the neuropeptide are achieved.

![Figure 5](image)

**Figure 5.** GABA reduces in vitro wound healing. Mz-ChA-1 cells were grown to confluence, and the monolayers were disrupted by generating a straight scrape wound. The cells were incubated in the absence or presence of GABA (100 μmol/L) and observed at time 0 and after 24, 48, 72, and 96 hours. A, after 96 hours, there was no significant alteration in volume of the cells incubated with GABA with respect to controls. B, an increase in time necessary to wound closure was observed following GABA stimulation. Columns, means of at least three separate experiments; bars, SE. *, P < 0.01 versus control. C-D, malignant xenograft growth was evaluated in mice treated with periodic injections of GABA or NaCl (controls) into the tumors. C, at 82 days, a significant difference in tumor size was macroscopically evident in GABA-treated mice compared with controls. D, at 82 days, the cholangiocarcinoma xenograft volume of mice periodically injected with GABA (1,000 mg/kg) was 517.48 ± 46.05 versus 1,122.89 ± 137.31 mm³ for the control mice. Points, mean tumor size (mm³); bars, SE.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control solution</th>
<th>GABA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>5.32 ± 0.47</td>
<td>5.35 ± 1.1 (NS)</td>
</tr>
<tr>
<td>Tumor latency (d)</td>
<td>117.2 ± 2.80</td>
<td>347.2 ± 3.9¹</td>
</tr>
<tr>
<td>Tumor size (mm³)</td>
<td>1,122.89 ± 137.31</td>
<td>517.48 ± 46.05¹</td>
</tr>
<tr>
<td>ALT (n.v. 28-132 μg/L)</td>
<td>32.3 ± 6.9</td>
<td>52.7 ± 11.1</td>
</tr>
<tr>
<td>AST (n.v. 59-247 μg/L)</td>
<td>55.2 ± 17.3</td>
<td>62.0 ± 11.9</td>
</tr>
<tr>
<td>Creatinine (n.v. 0.2-0.8 mg/dL)</td>
<td>0.2 ± 0.02</td>
<td>0.2 ± 0.02</td>
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</table>

NOTE: Data are mean ± SE of four mice. Abbreviations: NS, not significant; n.v., normal value.
*P < 0.05 vs the corresponding value of control solution-treated mice.
¹P < 0.02 vs the corresponding value of control solution-treated mice.
From this assumption, it can be speculated that malignant tumor growth can be modulated by using agonists of the GABAergic system. GABA has been shown to be an inhibitory regulator for the migration of SW480 colon carcinoma cells (12). In our study, we also showed that GABA has an antimigratory action on Mz-ChA-1 cells; thus, it might reduce the metastatic potential of cholangiocarcinoma.

Because GABA and its receptor agonists (e.g., baclofen) cross the blood-brain barrier, this neurotransmitter may interact with the CNS and perhaps inhibit cancer cell proliferation by nervous system regulation of malignant tissue growth. The inhibitory effect of GABA could also be due to the systemic modulation of the complex hormonal system. GABA and its agonists, interacting with the hypothalamic-hypophyseal axis, regulate the secretion of several hormones (e.g., thyroid hormone, prolactin, GnRH, adrenocorticotropic hormone, and growth hormone; ref. 48) that are known to be tropic factors for malignant cells. These neurotransmitters are thus potentially able to inhibit the growth of tumors expressing such hormone receptors. However, we did not find any evidence related to any change of the hormonal status in GABA-treated mice.

Although studies showed that chronic exposure of cultured cerebral cortical neurons to GABA decreases GABA_A receptor subunit protein (49), other works showed that prolonged exposure of HEK 293 cells stably expressing recombinant α_1β_2γ_2s GABA рецепторы for GABA and muscimol results in up-regulation of receptor number (50). We show that at the end of the treatment, the expression of GABA_A and GABA_B was maintained in GABA-treated tumors.

Prolonged systemic effects of GABA administration are usually associated with decreased body weight. Further evidence that in vivo GABA acts directly on Mz-ChA-1 cells and not through...
systemic interaction with nervous or hormonal systems is given by the fact that we did not find any change in body weight in mice treated with control solutions compared with GABA-treated mice. The absence of systemic toxicity of GABA was confirmed by normal values of serum AST, ALT, and creatinine and by no evidence of organ damage in the two groups of treated animals. In conclusion, our findings raise the possibility of a novel therapeutic approach for the treatment of tumors of the biliary tract based on the modulation of the GABAergic system.

References

Figure 6 Continued. B, cholangiocarcinoma cells forming several acinar or tubular structures (H&E). An amorphous matrix (\#) localized between groups of cancer cells is observed in all the tumors. A damaged area (arrows) characterized by several apoptotic bodies and a few necrotic cells is present in GABA-treated tumors. Sirius red staining shows fibrotic septa (red) surrounding cholangiocarcinoma nodules. In GABA-treated tumor, a wide area of fibrotic tissue is present. \# amorphous matrix. Columns, means of four experiments; bars, SE. * \( P < 0.05 \) versus control solution-treated tumor. The expression of GABA\(_A\) and GABA\(_B\) receptors is maintained in GABA treated with respect to control tumors at the end of the treatment. Original magnification, \( \times 50 \); \( \times 100 \) (cleaved caspase-3) and \( \times 75 \) (GABA receptors).

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27. Volgyi B, Xin D, Bloomfield SA. Feedback inhibition increased proliferative activity and apical bile acid transporter expression in both small and large rat cholangiocytes. Hepatology 2001;34:868–76.


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