

The Biological and Therapeutic Importance of Gastrin Gene Expression in Pancreatic Adenocarcinomas

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ABSTRACT

The *gastrin* gene is expressed widely in pancreatic adenocarcinomas and the study aimed to assess its role in both the resistance of cancer cells to apoptosis and the sensitivity of cells to chemotherapeutic agents.

Two human pancreatic cell lines, PAN1 and BXPC3, expressed gastrin at both the RNA and protein levels and are shown to be representative of human pancreatic adenocarcinomas in terms of gastrin expression. Inhibition of endogenous gastrin production by tumor cells was achieved with neutralizing gastrin antiserum and transfection with a gastrin antisense plasmid.

Gastrin antiserum synergized with both taxotere and gemcitabine in inhibiting the *in vitro* growth of the PAN1 cell line with the inhibitory effect of the antiserum increasing from 12.7% to 70.2% with taxotere ($P < 0.05$) and 28.6% with gemcitabine ($P < 0.01$) after controlling for the effects of the cytotoxics. Synergy was only achieved with taxotere in BXPC3 cells with the inhibitory effect of gastrin antiserum increasing from 22.9% to 50.0% ($P < 0.005$). Cells transfected with gastrin antisense had reduced *in vitro* growth in low serum conditions and were poorly tumorigenic in nude mice at an orthotopic site. Gastrin antisense-transfected PAN1 cells had increased sensitivity to the antiproliferative effects of both gemcitabine (IC_{50} of $>100 \mu\text{g/ml}$ reduced to $0.1 \mu\text{g/ml}$) and taxotere (IC_{50} of $20 \mu\text{g/ml}$ reduced to $<0.01 \mu\text{g/ml}$) when compared with vector controls. The increased sensitivity of PAN1 antisense coincided with increased caspase-3 activity and reduced protein kinase B/Akt phosphorylation in response to both gemcitabine and taxotere.

Gastrin gene circumvention may be an optimal adjunct to chemotherapeutic agents, such as taxotere and gemcitabine, in pancreatic adenocarcinoma.

INTRODUCTION

Pancreatic adenocarcinoma remains a formidable disease responsible for 6% of all cancer deaths and associated with limited treatment options (1). Surgical resection rates are low due to late presentation of the disease, and first-line treatment for patients with locally advanced or metastatic disease is gemcitabine, a nucleoside analog, which has been associated with a clinical response rate of 23.8% (2, 3).

The role of gastrin and the gastrin/CCK-2 receptor (also known as CCK-B) in human pancreatic carcinogenesis has remained a focus of investigation since the first report identifying gastrin as a growth factor of human pancreatic cancer by Smith *et al.* (4). A mouse model in which the CCK-2 receptor was exclusively expressed in the exocrine pancreas revealed that the receptor mediated an increase in pancreatic weight but not carcinogenesis (5). However, when the *ElasCCK-2* transgenic mouse (similar site of CCK-2 receptor expression) was crossed with a transgenic hypergastrinemic mouse (the *INS-Gas* mouse) malignant transformation resulted in 3 of the 20 offspring (6).

The CCK-2 receptor and gastrin are coexpressed in both human pancreatic adenocarcinoma specimens (7, 8) and cell lines (9–12) at both the gene and protein levels. Secretion of gastrin protein was identified in BXPC3 and seven additional pancreatic cell lines together with immunocytochemical confirmation of gastrin expression in human pancreatic cancer specimens but not associated normal tissue (13). In terms of specific gastrin proteins secreted by tumor cells, Goetze *et al.* (7), using a specific radioimmunoassay, confirmed amidated gastrin expression in 14 of 19 carcinomas, whereas a second study detected mainly precursor gastrin forms, progastrin and glycine-extended gastrin (8). Glycine-extended gastrin peptides were also shown to be secreted by the rat pancreatic adenocarcinoma cell line AR42J (14).

The CCK-2 receptor after activation by externally applied gastrin increases expression of key signaling pathways, such as those involving mitogen activated protein kinase (15) in the rat pancreatic cell line AR42J and also protein kinase B/Akt, which imparts resistance to apoptotic stimuli (16). The interaction of the gastrin gene with the latter pathway has not been investigated to date.

Therefore, the aims of the present study were to confirm the biological significance of gastrin gene expression in pancreatic carcinomas in terms of: (a) apoptosis circumvention with focus on protein kinase B/Akt phosphorylation; (b) sensitivity to chemotherapeutic agents; and (c) the potential role of gastrin as a therapeutic target in combination with chemotherapeutics.

MATERIALS AND METHODS

Test Cell Lines. PAN1 is a human pancreatic cell line derived from a poorly differentiated human pancreatic adenocarcinoma within the Academic Unit of Cancer Studies (University of Nottingham, Nottingham, United Kingdom). This cell line is distinct from the PANC1 cell line (European Collection of Animal Cell Cultures no. 87092802). BXPC3, a moderate to poorly differentiated human pancreatic cell line, and HCT116, a poorly differentiated human colon cell line, were obtained from European Collection of Animal Cell Cultures (nos. 93120816 and 91091005, respectively). ST16 is a poorly differentiated human gastric adenocarcinoma cell line derived within the Academic Unit of Cancer Studies. AR42J is a rat pancreatic adenocarcinoma cell line and was obtained from the European Collection of Animal Cell Cultures (no. 93100618). All of the cell lines were routinely cultured in Roswell Park Memorial Institute (RPMI) 1640 culture (Life Technologies, Inc., Paisley, United Kingdom) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS; Sigma, Poole, United Kingdom) at 37°C in 5% CO₂ and humidified conditions. For experimental use, cells from semiconfluent monolayers were harvested with 0.025% EDTA (Sigma).

Human Tumor Specimens and Ethics. Fasting sera were obtained from pancreatic cancer patients preoperatively and stored at -80°C . Tumor tissue was obtained after resection and was either snap frozen in liquid nitrogen within 30 min and stored at -80°C or fixed in formalin and embedded into wax before processing. All of the patients consented to the study, and ethical approval was obtained from the Ethics Committee at the Queen's Medical Centre (University Hospital, Nottingham, United Kingdom).

Extraction of RNA. For both frozen and fixed tissue, total RNA was extracted as described previously (17).

Real-Time PCR. RNA was reverse transcribed from random hexamer primers (Pharmacia) using Superscript RT (Life Technologies, Inc.). Real-time PCR was performed using the 5700 Sequence Detection System (PE Applied

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Biosystems, Warrington, United Kingdom) as described previously (18). The gastrin primer sequences were as follows: U CCACACCTCGTGGCAGAC and L TCCATCCATCCATAGGCTTC.

The relative gene expression for each sample was determined using the formula $2^{-\Delta Ct} = \frac{2^{Ct(\text{glyceraldehyde-3-phosphate dehydrogenase})}}{2^{Ct(\text{gastrin})}}$ and reflected gastrin gene expression normalized to glyceraldehyde-3-phosphate dehydrogenase levels.

Immunohistochemical Evaluation of Progastrin Expression. Cells were cultured in eight-well SuperCell chamber slides (Menzel-Gläser, Braunschweig, Germany) for 24 h with RPMI 1640 (Sigma) plus 10% FBS (Sigma) at a density of $5 \times 10^4 \text{ ml}^{-1}$ then fixed in cold 70% ethanol at subconfluence and stained with polyclonal rabbit antiprogastrin antibodies raised against the NH₂-terminal domain of progastrin (Aphthon Corporation, Woodland, CA; Ref. 19).

Progastrin labeling was assessed by computerised image analysis using custom macroroutines created with Qwin Standard analysis software (Licca Microsystems, Cambridge, United Kingdom). Results are represented as mean percentage of labeling over an average of 15 readings per coverslip or tissue section. Interobserver variation was 6%, and intra-assay variation was <10%.

Radioimmunoassay for Progastrin and Amidated Gastrin. Supernatants were collected from the cell lines as described previously and together with fasting patient sera were analyzed by radioimmunoassay for the presence of amidated gastrin-17 and -34 and progastrin within the laboratories of Prof. Andrea Varro (University of Liverpool, Liverpool, United Kingdom), as described previously (20).

In Vitro Clonogenicity Assays. Subconfluent cells were harvested, resuspended in RPMI 1640 + 10% FBS at concentrations between 1×10^3 and 6×10^3 viable cells/ml and plated into 96-well plates in a final volume of 200 μl in replicates of 5. The total volume within each well was made up to 200 μl with growth medium and the plates incubated at 37°C, 5% CO₂ for 18 h. The medium was aspirated and replaced with 200 μl of RPMI 1640 + 1% FBS. Cell numbers were assessed at 0, 24, 48, and 72 h using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-based assay as described previously (20).

In Vitro Growth Assays with Antigastrin Antibodies and Cytotoxics. The cytotoxics, gemcitabine (Eli Lilly), cisplatin (Rhone-Poulenc Rorer), camptothecin (Faulding Pharmaceuticals), and taxotere (Rhone-Poulenc Rorer) were prepared as stock solutions (prepared at 1 mg/ml in sterile distilled water) and then diluted into assay medium (RPMI 1640 with 1% FBS).

Affinity purified mouse monoclonal antigastrin antibodies were raised against the NH₂ terminus of human gastrin-17 [antigastrin monoclonal antibody (mAb), G17, Aphthon Corporation, CA] and diluted to a final concentration of 100 and 500 $\mu\text{g/ml}$ in assay medium from a stock solution (2 mg/ml in sterile PBS). Purified mouse IgG1 λ antibodies (Sigma) were used as a negative control.

Subconfluent cells were harvested, resuspended in RPMI + 10% FBS at a concentration of 1×10^5 viable cells/ml, and plated into 96-well plates in 100- μl aliquots. The cells were incubated overnight at 37°C. The medium was aspirated and replaced by test compounds/antibodies with the final volume per well being 100 μl . The plates were then incubated for 48 h at 37°C and proliferation assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

Additive and synergistic effects were defined as follows:

$$\text{IF ADDITIVE} \frac{\text{Growth in presence of antigastrin mAb}}{\text{Growth in presence mouse IgG}} = \frac{\text{Growth in presence of antigastrin mAb} + \text{cytotoxic}}{\text{Growth in presence mouse IgG} + \text{cytotoxic}}$$

$$\text{IF SYNERGY} \frac{\text{Growth in presence of antigastrin mAb}}{\text{Growth in presence mouse IgG}} < \frac{\text{Growth in presence of antigastrin mAb} + \text{cytotoxic}}{\text{Growth in presence mouse IgG} + \text{cytotoxic}}$$

Gastrin Antisense Plasmid Manufacture. PCR amplification of the entire coding region of the gastrin gene was carried out on cDNA from LoVo

colorectal cells using the gastrin sense and antisense primers CAGAGACCT-GAGAGGCCACCAG and GTTCTAGGATGGTTAGTTCATC, respectively. The antisense primer was 5' phosphorylated to facilitate cloning in the antisense direction. The product was cloned into the TA cloning vector pCR3.1 Uni (Invitrogen, Paisley, United Kingdom). Bacterial transformants were assayed by PCR and sequenced using forward and reverse primers to ensure that the insert was in the antisense orientation.

Tissue Culture and Transfection. Cells were seeded into 24-well plates, cultured until ~50% confluent and transfected in serum-free medium using TFX-50 liposomes (Promega, Southampton, United Kingdom) according the manufacturer's instructions. The transfection mix of 200 μl containing 1 μg of plasmid and 4.5 μl of liposomes was gently layered on the cells and incubated at 37°C for 60 min. Culture medium was added, and the cells were placed in the incubator for 48 h before selection with 1 mg/ml G418 (Sigma). After 4 weeks of selection the cells formed a monolayer and were confirmed as stable transfectants by PCR.

The establishment of stable antisense and control plasmid (self-ligated) clones was achieved by plating cells at a density of less than one cell per well in a microtiter plate. Clonal colonies were established in selective media and expanded to form stable cultures.

Caspase-3 Assay of Apoptosis. Confluent cells were harvested, washed, and counted. The cells were resuspended into growth medium (RPMI 1640 + 10% FBS) at 1×10^6 viable cells/ml. Six flasks were reseeded per cell line at 3×10^6 cells/flask. Total volume was made up to 15 ml with growth medium. The flasks were incubated overnight at 37°C, 5% CO₂.

Taxotere and gemcitabine solutions were prepared at 20 and 250 $\mu\text{g/ml}$, respectively, in assay medium (RPMI 1640 + 1% FBS + 2 mM L-glutamine) and added individually to each cell line in duplicate. After 4 h of incubation, the cells were harvested and the cell pellets assayed for caspase-3 activity using a Clontech assay kit (Clontech, Cowley, United Kingdom).

Protein Kinase B/Akt Western Blot Analysis. After overnight serum starvation, cells were treated with fresh serum-free medium containing 10 nM gastrin or 1% FBS medium containing gemcitabine or taxotere at their respective concentrations inducing 50% inhibition (IC₅₀ concentrations) for 1 h. After incubation, cells were washed with ice-cold PBS (Oxoid, Basingstoke, United Kingdom) and lysed with sample buffer [62.5 mM Tris-HCl at (pH 6.8; Invitrogen), 2% w/v SDS (Sigma), 10% glycerol (Sigma), 50 mM mercaptoethanol (Sigma), 0.01% w/v bromphenol blue (Sigma), 1 mM sodium fluoride (Sigma), 80 mM sodium B-glycerophosphate (Sigma), 1 mM phenylmethylsulfonyl fluoride (Sigma), and 10 $\mu\text{l/ml}$ Calbiochem Protease Inhibitor mixture III (Calbiochem, San Diego, CA)]. Following the primary antibody manufacturer's instructions, samples were separated via gel electrophoresis on 8–16% Tris-glycine gels (Invitrogen) and transferred to phenylmethylsulfonyl fluoride membrane (Invitrogen). Membranes were blocked and incubated with the primary polyclonal antibodies either Akt or phospho-Akt (Serine473; New England Biolabs, Hitchin, United Kingdom), washed, and then incubated with a horseradish peroxidase-labeled swine antirabbit secondary antibody (Dako, Ely, United Kingdom) diluted to 1:1000. Chemiluminescent visualization and protein detection was carried out using the Amersham enhanced chemiluminescence kit (Amersham, Little Chalfont, Buckinghamshire, United Kingdom) and Kodak X-OMAT film (Sigma).

In Vivo Therapeutic Assays with Antisense Cell Lines. All of the tumors were grown in male MF1 nude mice (4–6 weeks of age) bred within the Academic Unit of Cancer Studies.

For orthotopic implantation, cells were resuspended at 1×10^6 in 20 μl in sterile PBS (pH 7.4). A laparotomy was performed under anesthetic (Hypnorm, Roche: Hypnovel, Jansson) and the tip of the pancreas gently exteriorized before the cell inoculum was injected. The peritoneal wall was closed with suture and the skin with wound clips. Gemcitabine (Eli-Lilly Co. Ltd., Basingstoke, England) was administered at a dose of 16 mg/kg intravenously on days 1, 3, and 6, which was repeated at day 28 (as recommended by the manufacturer) and taxotere at a dose of 20 mg/kg intravenously on day 15 (single dose; Ref. 21). Experimental groups were between 10 and 15 mice.

All of the *in vivo* experimentation was performed according to the United Kingdom Coordinating Committee for Cancer Research guidelines.

Statistics. For evaluation of *in vitro* data, either a Student *t* test or a one-way ANOVA was used. Gene expression was assessed using a Mann

Whitney nonparametric assessment, and *in vivo* data were analyzed by one-way ANOVA using the Minitab statistical package.

RESULTS

Determination of the Relevance of the Test Cell Lines in Terms of Gastrin Expression to Human Patient Pancreatic Adenocarcinomas

Gastrin gene expression was measured in two test pancreatic cell lines, PAN1 and BXPC3, by real-time PCR, and levels were in the range of those shown for a series of resected human pancreatic adenocarcinoma specimens (Fig. 1A).

Protein expression was confirmed by determining progastrin immunoreactivity on the same series of human pancreatic adenocarcinoma specimens and the two test cell lines grown as xenografts. Progastrin expression by PAN1 and BXPC3 was shown to be at the upper and lower ranges, respectively, of the human pancreatic adenocarcinoma specimens (Fig. 1B).

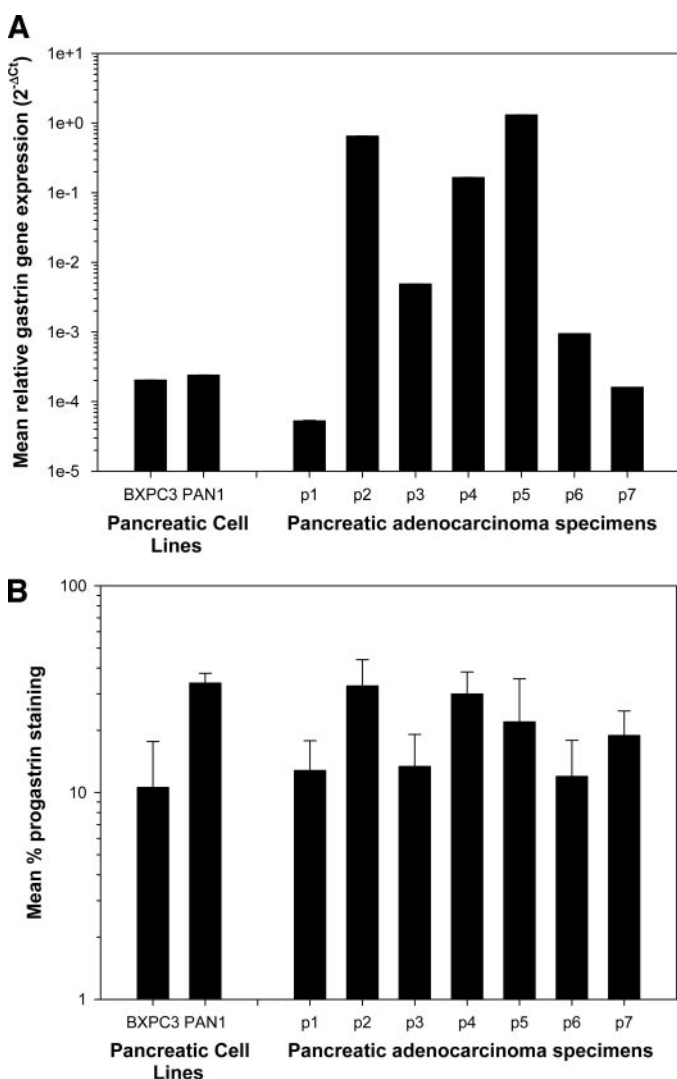


Fig. 1. Gastrin expression of the two-test pancreatic adenocarcinoma cell lines in comparison with a panel of human pancreatic adenocarcinoma specimens from patients with resectable disease. A, gene expression was determined by real-time PCR, and each observation is the mean of two cDNA samples assessed in duplicate (SDs do not show with current scale of graph). B, progastrin immunoreactivity as measured by image analysis. Each observation is the mean percentage of staining (with SDs shown) after assessment of 10 separate fields per section. Percentage of staining was calculated after assessment of 10 separate fields per section by image analysis

Table 1 Serum levels of progastrin and amidated gastrin in patients with resectable and advanced pancreatic adenocarcinoma

	No. of patients (%) with detectable progastrin* (range pM)	No. of patients (%) with detectable amidated [†] gastrin
Resectable pancreatic cancer	0/17 (0)	5/17 (29)
Advanced pancreatic cancer	11/68 (16) [‡]	54/68 (79) [§]

NOTE. *n* = serum samples from 17 resectable and 16 advanced pancreatic patients.

* Detection sensitivity of assay was 40 pM.

[†] Detection sensitivity of assay was 4 pM.

[‡] *P* = 0.048 when compared to resectable cancer, Student's *t* test.

[§] *P* = 0.008 when compared to resectable cancer, Student's *t* test.

Table 2 Synergistic effects of antigastrin mAbs combined with the cytotoxic agents taxotere and gemcitabine

Cell line	% Inhibition [antigastrin mAb + cytotoxics/mouse IgG + cytotoxics] × 100]		% Inhibition [(antigastrin mAb/mouse IgG) × 100]
	Taxotere	Gemcitabine	
PAN1	70.2*	28.6 [†]	12.7 [‡]
BXPC3	50.0 [§]	36.6	22.9 ⁺

NOTE. *n* = combined means of two to three separate assays, five replicates per assay.

* *P* < 0.05, one-way ANOVA when compared to percentage inhibition of antigastrin mAb/mouse IgG.

[†] *P* < 0.01, one-way ANOVA when compared to percentage inhibition of antigastrin mAb/mouse IgG.

[‡] *P* < 0.005 when compared to mouse IgG alone.

[§] *P* < 0.005, one-way ANOVA when compared to percentage inhibition of antigastrin mAb/mouse IgG.

Progastrin and Amidated Gastrin Secretion by Human Pancreatic Tumor Specimens

Tumor material from patients with locally advanced and metastatic pancreatic adenocarcinoma was not available due to the low rate of surgical resection in this patient group. In an attempt to indirectly demonstrate gastrin gene expression, levels of both progastrin and amidated gastrin were determined in the serum of patients with either advanced or resectable disease.

In sera from 68 pancreatic adenocarcinoma patients, significantly greater levels of amidated gastrin and progastrin were detected in patients with advanced disease compared with patients with resectable disease (*P* = 0.008 and 0.046, Student's *t* test; Table 1).

Effect of Neutralizing Gastrin Antibodies on the Basal Growth of Pancreatic Cell Lines Alone and in Combination with Gemcitabine and Taxotere

The effect of 100 and 500 μg/ml antigastrin mAbs on the basal growth of PAN1 and BXPC3 was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide uptake. An antigastrin mAb concentration of 100 μg/ml significantly reduced basal growth of BXPC3 (*P* < 0.0001, ANOVA, 10% inhibition when compared with IgG control) but not PAN1 (data not shown). An antibody concentration of 500 μg/ml significantly inhibited the basal growth of both cell lines (12.7% inhibition with PAN1 and 22.9% with BXPC3, *P* < 0.005 when compared with IgG control; Table 2).

The higher concentration of antigastrin mAbs was combined with the cytotoxics, gemcitabine and taxotere, at their respective IC₅₀s to determine whether synergistic effects on growth could be achieved (Table 2).

With BXPC3, a taxotere concentration of 0.075 μg/ml and gemcitabine concentration of 0.05 μg/ml were defined as the respective IC₅₀ doses. Antigastrin mAbs significantly synergized with taxotere resulting in the inhibitory effect of the antibodies increasing from 22.9% to 50% after correction for the effect of the cytotoxic

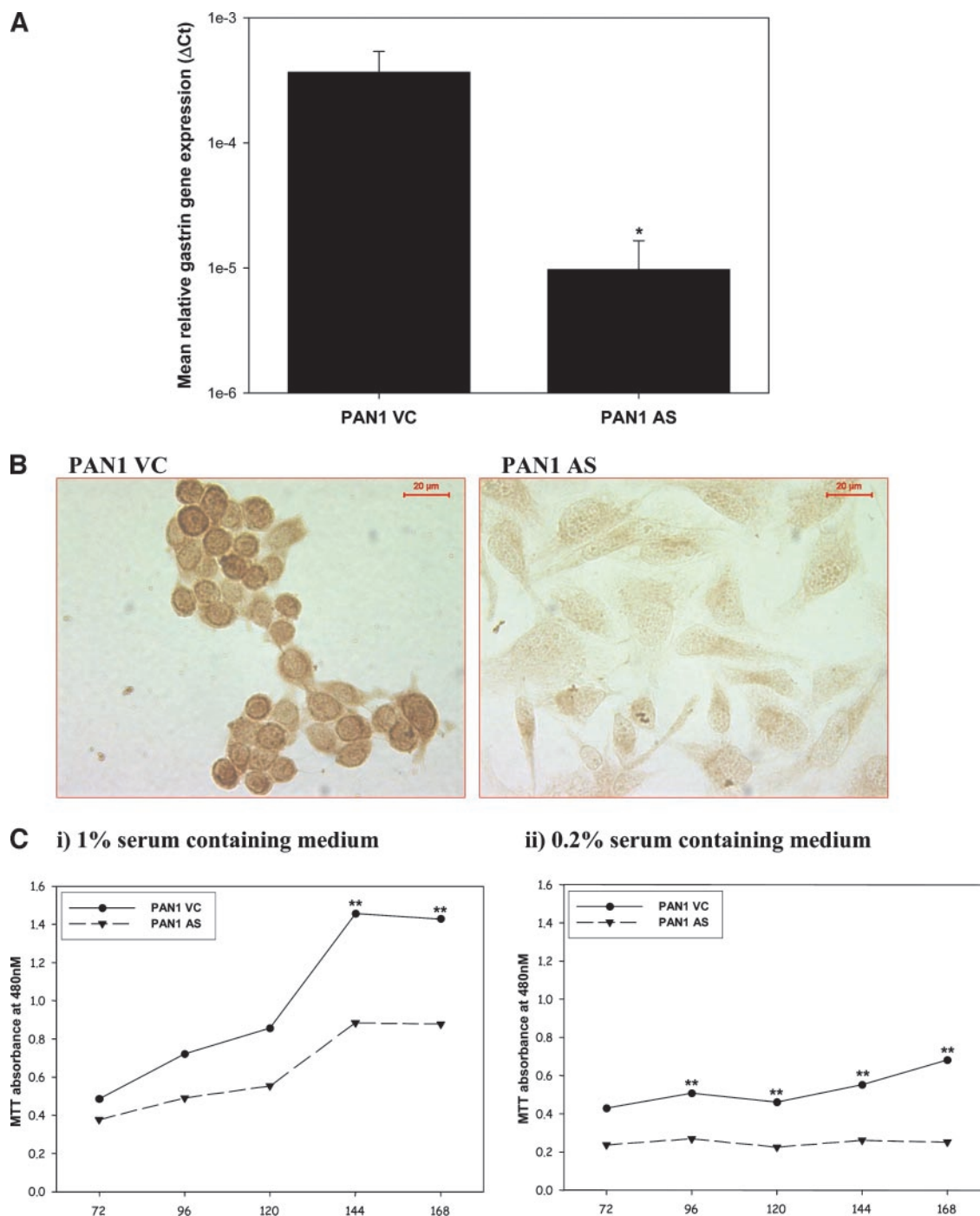


Fig. 2. Gastrin expression of PAN1 vector control and PAN1 gastrin antisense cell lines. **A**, gastrin gene expression determined by real-time PCR. *, $P < 0.001$, Mann Whitney U, $n = 3$ replicates of two separate cDNA samples. **B**, immunocytochemical staining using progastrin antiserum raised against the NH_2 terminus. **C**, clonogenicity assay with PAN1 VC and PAN1 AS cell lines in: (i) 1% serum-containing medium; and (ii) 0.2% serum-containing medium. Ten replicates/time point and results are representative of a single assay that was repeated three times. **, $P < 0.01$, Student's t test. (MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

($P < 0.005$, ANOVA; Table 2). No significant synergy was observed with antagastrin mAbs in combination with gemcitabine.

With PAN1, the IC_{50} with taxotere was achieved at a concentration of 15.0 $\mu g/ml$. The inhibitory effect of antagastrin mAbs increased from 12.7% to 70.2% after correction for the effect of cytotoxics alone ($P < 0.05$; Table 2). PAN1 was highly resistant to the antiproliferative effects of gemcitabine in cell culture, and an IC_{50} was not achieved. Synergy was examined using a concentration of 250 $\mu g/ml$, which induced a maximum inhibition of 18.0%, and significant synergy was achieved with antagastrin mAbs with inhibition increasing from 12.0% to 28.6% ($P < 0.01$; Table 2).

Characterization of PAN1 Cells Transfected with an Antisense Gastrin Gene Construct

Fig. 2A shows the gastrin gene expression in PAN1 vector control (PAN1 VC) transfected cells in comparison with a PAN1 clone transfected with a gastrin antisense plasmid (PAN1 AS). There was a significant log-fold reduction of gastrin gene expression in the antisense cell line compared with the vector control ($P < 0.01$, Mann Whitney). Gastrin secretion was measured by assessing progastrin levels in supernatant concentrated from cells. Supernatant from the vector control cells secreted 58 pmols of

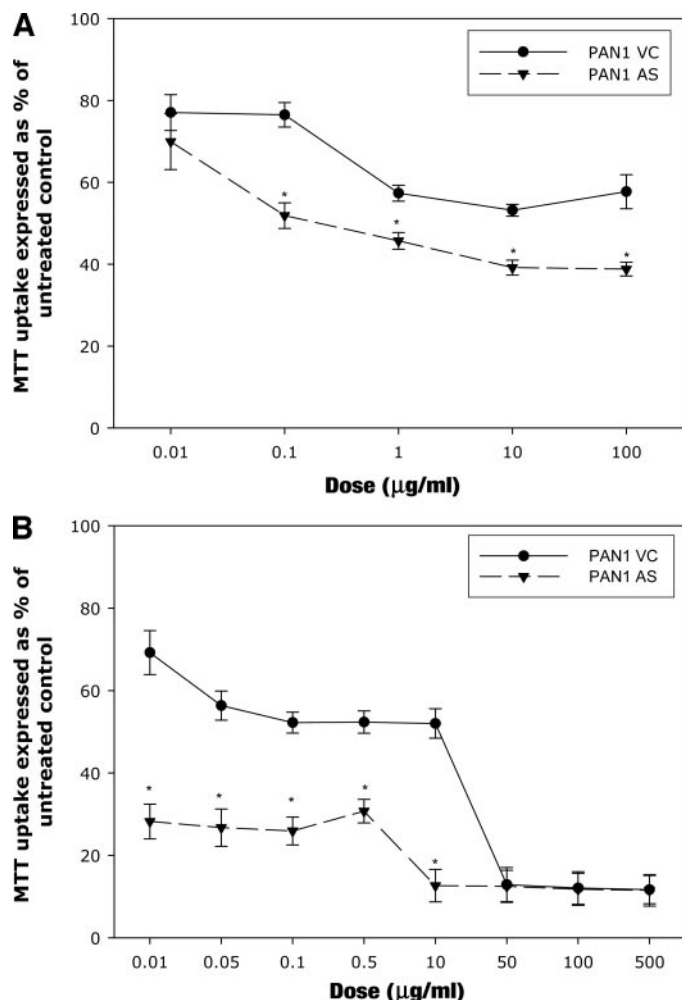


Fig. 3. Typical dose-response curve for gemcitabine and taxotere in the PAN1 vector control and PAN1 gastrin antisense cell lines as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide uptake. A, gemcitabine; B, taxotere. *, $P < 0.0001$ as assessed by one-way ANOVA comparing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide uptake in PAN1 VC versus PAN1 AS cells; bars, \pm SD.

progastrin per 8×10^6 cells compared with nondetectable levels in the gastrin antisense cell line.

An antibody raised against the NH₂ terminus of progastrin was used to stain the cells to indicate gastrin immunoreactivity, and these results are shown in Fig. 2B. Mean percentage of staining in PAN1 VC was 52.12 and 49.66 (2 separate assays) compared with 4.91 and 2.19 in PAN1 AS ($P < 0.0001$, Student's *t* test).

Clonogenic assays were performed with PAN1 VC and AS in both 1% and 0.2% serum-containing growth medium (Fig. 2C). In 1% serum, PAN1 VC had significantly greater growth at the 144- and 168-h time points ($P < 0.01$, Student's *t* test). In the 0.2% serum concentration, PAN1 VC cells grew modestly, whereas PAN1 AS cells failed to grow with significantly lower 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide absorbance compared with PAN1 VC at all of the time points after 72 h ($P < 0.01$).

Sensitivity of PAN1 Vector Control and Antisense Cell Lines to Cytotoxic Agents.

In Vitro. The effect of abrogation of the gastrin gene on the sensitivity of PAN1 VC and AS cells to taxotere and gemcitabine was assessed by *in vitro* proliferation and typical dose-response curves (from a series of two to three repeats per cell line) are shown in Fig. 3, A and B.

The PAN1 AS cell line was more sensitive to the antiproliferative effects of gemcitabine with the IC₅₀ achieved at $\sim 0.1 \mu\text{g/ml}$ compared with $>100 \mu\text{g/ml}$ with PAN1 VC (IC₅₀ at $\sim 250 \mu\text{g/ml}$ but was difficult to achieve due to a flat dose-response curve at gemcitabine doses $>100 \mu\text{g/ml}$). At gemcitabine concentrations between 0.1 and $100 \mu\text{g/ml}$ there was significantly more inhibition ($P < 0.000$, ANOVA) observed with PAN1 AS when compared with PAN1 VC cells (Fig. 3A).

With taxotere the IC₅₀ was achieved at a concentration of $\sim 20 \mu\text{g/ml}$ in PAN1 VC compared with $<0.01 \mu\text{g/ml}$ in PAN1 AS. There was a significant difference in inhibitory effects between the two cell lines at taxotere concentrations between 0.01 and $10 \mu\text{g/ml}$ (Fig. 3B).

In Vivo. PAN1 VC and AS cells were grown as xenografts orthotopically in the pancreas of nude mice and treated with therapeutic doses of the cytotoxic agents taxotere and gemcitabine. The final pancreatic tumor cross-sectional areas and weights are shown in Fig. 4, A and B, respectively.

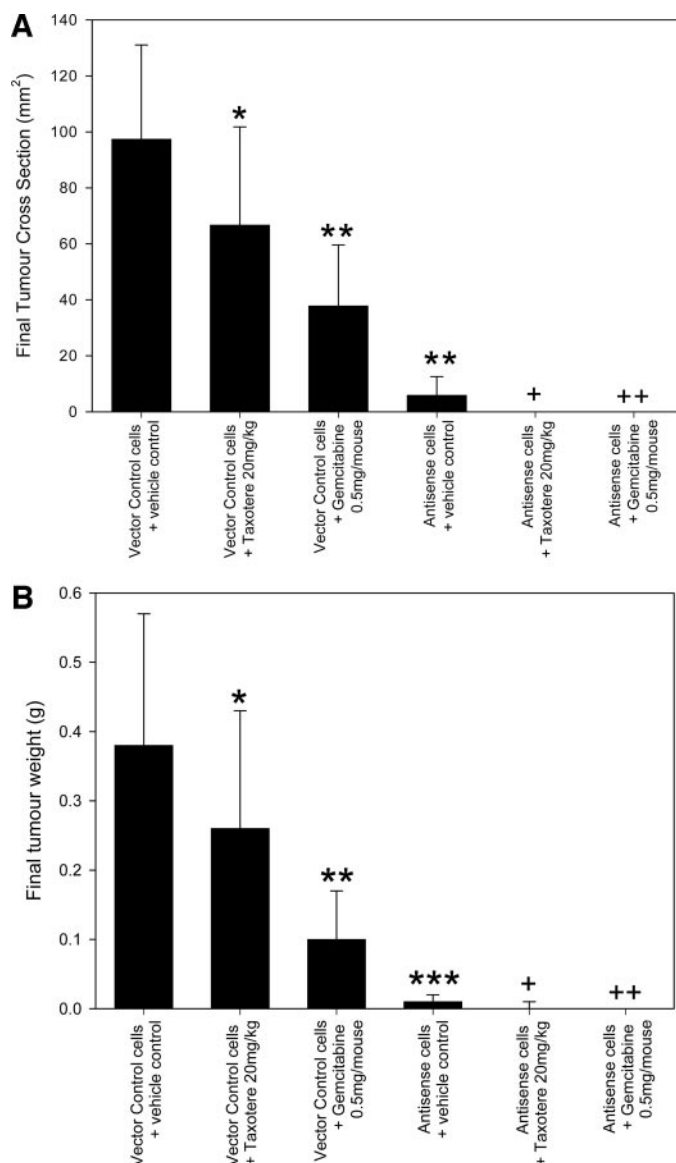


Fig. 4. Effect of taxotere and gemcitabine on the orthotopic growth of PAN1 VC and PAN1 AS cells in the pancreas. A, final tumor cross-sectional area (mm²). *, $P = 0.04$ and **, $P = 0.000$ when compared with PAN1 VC; +, $P = 0.013$ and ++, $P = 0.009$ when compared with PAN1 AS as assessed by ANOVA. B, final tumor weight. *, $P = 0.000$; **, $P = 0.115$; and ***, $P = 0.000$ when compared with PAN1 VC. +, $P = 0.03$ and ++, $P = 0.006$ when compared with PAN1 AS as assessed by ANOVA; bars, \pm SD.

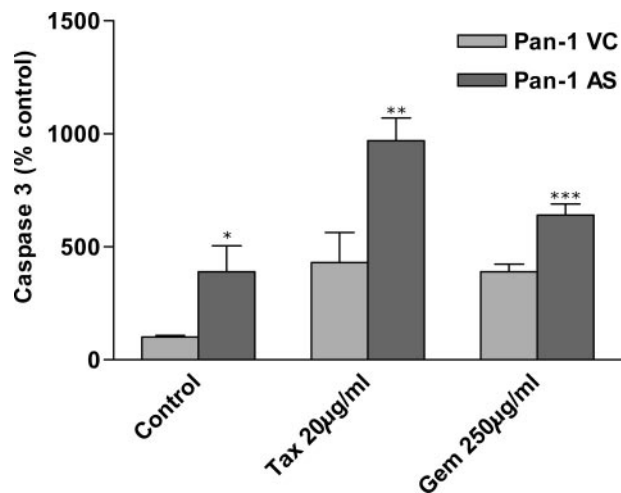


Fig. 5. A comparison of caspase-3 levels in PAN1 VC and PAN1 AS cell lines after treatment with gemcitabine or taxotere. Cells were incubated for 4 h with 20 µg/ml taxotere or 250 µg/ml gemcitabine. Cumulative results of two separate assays performed in duplicate. *, NS, **, $P = 0.032$, and ***, $P = 0.013$ compared with PAN1 VC cells treated with the same conditions, one-way ANOVA; bars, \pm SD.

Both taxotere and gemcitabine significantly inhibited the cross-sectional area of PAN1 VC xenografts by 31.6% ($P = 0.040$, ANOVA) and 61.2% ($P = 0.000$), respectively (Fig. 4A). The mean cross-sectional area of PAN1 AS xenografts was reduced by 94% ($P = 0.000$) when compared with PAN1 VC with tumors detectable in 8 of 15 mice. When PAN1 AS xenografts were treated with cytotoxics, 1 of 10 tumors grew in the taxotere-treated group ($P = 0.013$ compared with control-treated PAN1 AS) and 0 of 10 in the gemcitabine-treated group ($P = 0.009$).

When assessing final tumor weights (Fig. 4B), similar trends were observed: gemcitabine inhibited the weights of PAN1 VC xenografts ($P = 0.000$, ANOVA), whereas the effect of taxotere failed to reach statistical significance ($P = 0.115$). PAN1 AS xenografts were significantly smaller than PAN1 VC xenografts ($P = 0.000$), and taxotere- and gemcitabine-treated PAN1 AS tumors had significantly lower mean tumor weights when compared with PAN1 AS control-treated xenografts ($P = 0.03$ and $P = 0.006$, respectively).

Apoptotic Activity of Taxotere and Gemcitabine in PAN1 VC- and PAN1 AS-Transfected Cell Lines

Apoptosis was detected by measurement of caspase-3 after short-term (4 h) treatment with taxotere and gemcitabine. When comparing the level of apoptosis in untreated PAN1 VC versus PAN1 AS cells, the levels were not significantly different ($P = 0.37$, ANOVA; Fig. 5). However, the levels of caspase-3 after treatment with taxotere and

gemcitabine were significantly higher in PAN1 AS cells when compared with PAN1 VC ($P = 0.032$ and 0.013 , respectively; Fig. 5).

Protein Kinase B/Akt Expression/Phosphorylation in PAN1 VC and PAN1 AS Cell Lines after Treatment with Taxotere and Gemcitabine

The basal protein kinase B/Akt expression and phosphorylation status of the PAN1 vector control and gastrin antisense cell lines are shown in Fig. 6A.

The PAN1 vector control cell line showed higher basal levels of phosphorylated protein kinase B/Akt than the control cell line (AR42J). The basal levels of phosphorylated protein kinase B/Akt were reduced in the gastrin antisense cell line, and both cell lines were shown to respond modestly to gastrin stimulation in serum-free medium as determined by increased phosphorylation, which peaked at 30 min in PAN1 VC and 60 min in PAN1 AS cell extracts (Fig. 6A).

To determine the effects of cytotoxic treatment on protein kinase B/Akt phosphorylation levels, cells were grown in 1% serum-containing medium and treated with either taxotere or gemcitabine for 1 h. In the gastrin antisense cell line, phosphorylated protein kinase B/Akt levels were reduced after taxotere treatment, unlike the PAN1 VC cells (Fig. 6B).

Trypan blue viability assays were carried out on the PAN1 cells after 1 h of treatment with the cytotoxics to ensure that changes in protein kinase B/Akt phosphorylation were not due to a reduction in cell number. There was no viability loss in either cell line (data not shown).

Sensitivity of a Panel of Gastrin Antisense-Transfected Gastrointestinal Cell Lines to a Series of Cytotoxic Agents

Stable gastrin antisense transfectants of HCT116 and ST16 were derived, and together with PAN1, *in vitro* sensitivity to a wider panel of cytotoxics was determined (Table 3). Significantly increased sensitivity to taxotere was observed in all three of the gastrin antisense cell lines when compared with their corresponding VC lines ($P \geq 0.007$, ANOVA; Table 3). Increased sensitivity was also seen with camptothecin for all of the gastrin AS-transfected lines. However, only PAN1 AS retained increased sensitivity to cisplatin with similar sensitivity shown between the vector control and gastrin antisense in the gastric and colorectal cell lines (Table 3).

DISCUSSION

The therapeutic options for pancreatic cancer are limited, and new approaches are needed to either work in concert with existing therapeutic modalities such as gemcitabine and/or to improve treatment for

Fig. 6. Protein kinase B (P)/Akt expression of PAN1 vector control and PAN1 gastrin antisense cell lines after treatment with gastrin, taxotere, or gemcitabine. A, P/Akt phosphorylation in PAN1 VC, PAN1 AS, and AR42J (positive control) cells in the absence and presence of gastrin-17 (10 nM assessed up to 60-min incubation). B, PKB/Akt phosphorylation in PAN1 VC and PAN1 AS after treatment with the cytotoxic agents, gemcitabine (250 µg/ml) and taxotere (20 µg/ml), after 1-h treatment. Representative blots repeated twice.

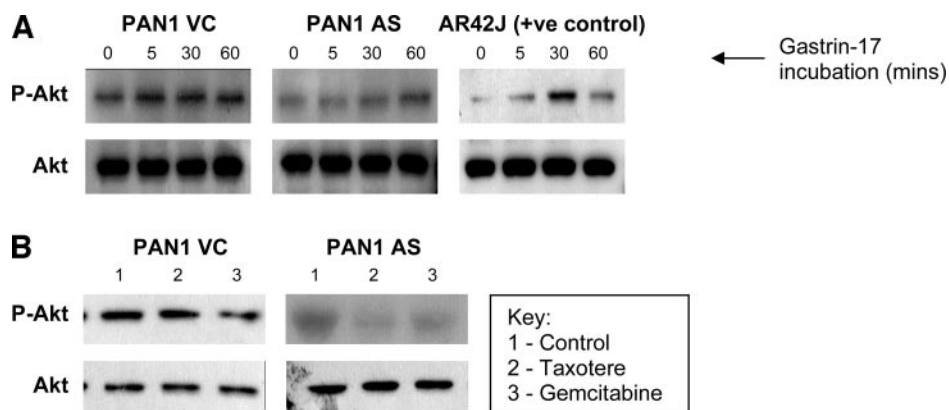


Table 3 Cytotoxic concentrations inducing IC₅₀ in a vector control and gastrin antisense cell line panel

Cell line		IC ₅₀ (μg/ml) for each cytotoxic		
		Taxotere	Cisplatin	Camptothecin
PAN1	VC	20.0	3.0	60.0
	AS	0.01*	0.30*	3.0†
HCT116	VC	0.020	2.0	30.0
	AS	0.003‡	2.5§	9.0¶
ST16	VC	10.0*	4.0	80.0
	AS	0.006*	3.0§	20.0*

NOTE. Results for a single representative experiment, five replicates per assay.

* Statistical significance from VC cell line determined by ANOVA at concentration relating to IC₅₀, $P = 0.000$.

† Statistical significance from VC cell line determined by ANOVA at concentration relating to IC₅₀, $P = 0.003$.

‡ Statistical significance from VC cell line determined by ANOVA at concentration relating to IC₅₀, $P = 0.007$.

§ Statistical significance from VC cell line determined by ANOVA at concentration relating to IC₅₀, not significant.

¶ Statistical significance from VC cell line determined by ANOVA at concentration relating to IC₅₀, $P = 0.006$.

patients with advanced disease for which there are currently no viable options.

Gastrin has now been confirmed as a central growth factor for malignancies of the gastrointestinal tract having proliferative and antiapoptotic effects possibly indirectly through increasing transcription of ligands of the epidermal growth factor receptor (22, 23), the REG protein (24), and cyclooxygenase 2 (25). Treatments exist, which are directed at interacting with serum gastrin including CCK-2 receptor antagonists and the gastrin vaccine G17DT, and have been proven to be effective clinically (26–28).

The current study has attempted to define the effects associated with gastrin gene expression by pancreatic adenocarcinomas on apoptotic potential and, thus, chemotherapeutic sensitivity. The pancreatic cell lines used (PAN1 and BXPC3) were shown to be representative of tissue obtained from resectable human pancreatic tumor specimens. Their relevance to unresectable disease is not known due to lack of experimental tissue. However, when evaluating serum gastrin levels, in particular progastrin, as a marker of activation of the gastrin gene in tumor tissue (29, 30), serum levels were detectable and significantly higher than those in patients with resectable disease.

Treatment with a neutralizing antiserum directed against both amidated and glycine-extended gastrin significantly reduced the *in vitro* basal growth of both PAN1 and BXPC3 and induced synergistic inhibitory effects with the chemotherapeutic agents gemcitabine and taxotere.

Reduction of gastrin gene expression in PAN1 cells significantly reduced growth in 1% serum by ~40% and completely suppressed growth in 0.2% serum-containing medium. *In vivo* PAN1 AS cells were poorly tumorigenic when transplanted orthotopically with a 93% reduction in final tumor size and weight and almost complete elimination of tumor growth after treatment with either taxotere or gemcitabine. This relates to previous studies by Smith *et al.* (31) where it was shown that BXPC3 cells transfected with gastrin antisense oligonucleotides had a > 30% reduction in final tumor weight when transplanted orthotopically, although in this latter study there was no combination with cytotoxics.

Sensitivity to cytotoxic agents was increased when comparing PAN1 VC with PAN1 AS, because IC₅₀s achieved with taxotere, gemcitabine, camptothecin, and cisplatin were significantly lowered in PAN1 AS compared with PAN1 VC, with the greatest sensitivity being to taxotere. This effect was not limited to the PAN1 cell line, because a colorectal and gastric cell line also had increased sensitivity to taxotere and camptothecin but not cisplatin after stable transfection with gastrin antisense. The combination of antigastrin mAbs with

taxotere was also shown to be synergistic with both pancreatic cell lines. Gastrin increases cell migration and activates focal adhesion kinase (32, 33), and combined blockade of these pathways by either antigastrin mAbs or gastrin AS may, therefore, enhance efficacy of taxotere to inhibit spindle formation. The effects were of lower magnitude with gemcitabine and cisplatin, both inhibitors of DNA replication (2, 3, 34), which may affect pathways that overlap with the effect of gastrin neutralization. Increased sensitivity to taxotere was also been seen when combined with the c-erbB2 mAb preparation, Herceptin (35).

It is known that gastrin can increase the level of antiapoptotic proteins such as Bcl-2 (36, 37) and has been shown to enhance phosphorylation of protein kinase B/Akt in response to serum withdrawal in the pancreatic cell line AR42J (16). Once phosphorylated, protein kinase B/Akt can go on to inactivate a range of proapoptotic factors including caspase-9, Bad and fork-head/winged-helix transcription factors important in the transcription of the cell death ligand, *fas*, as well as activating the antiapoptotic inhibitor of nuclear factor κB/nuclear factor κB cascade (38).

The role of tumor-associated gastrin on constitutive protein kinase B/Akt phosphorylation has not been investigated. In the present study, basal levels of phosphorylated protein kinase B/Akt were detected in PAN1 cells, which were reduced after transfection of gastrin antisense. A previous study has shown that in the rat pancreatic cell line, AR42J, exogenous gastrin can protect cells from the apoptosis-inducing effects of serum withdrawal *in vitro* through up-regulation of protein kinase B/Akt (16). The findings of the present study suggest that protein kinase B/Akt may be autonomously phosphorylated by autocrine gastrin in the PAN1 cell line.

To assess whether apoptosis was induced in response to chemotherapeutic agents in the cell lines with low expression of the gastrin gene, caspase-3 was measured. Basal levels of caspase-3 were increased in PAN1 AS cells and were significantly greater after treatment with taxotere and gemcitabine. Furthermore, protein kinase B/Akt phosphorylation was reduced in the PAN1 AS cell line after treatment with taxotere and gemcitabine, which correlates with increased levels of caspase-3 activity. These studies suggest that gastrin peptides secreted by the tumor cells themselves interact with antiapoptotic pathways resulting in increased resistance to cytotoxic agents.

In conclusion, gastrin gene neutralization may provide an adjunct to conventional chemotherapy for the treatment of pancreatic adenocarcinoma and may provide a therapeutic option for patients with advanced disease.

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