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Chronic nicotine inhibits the therapeutic effects of gemcitabine on pancreatic cancer in vitro and in mouse xenografts

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Abstract

Aim of Study—Smoking is an established risk factor for pancreatic cancer and nicotine replacement therapy often accompanies chemotherapy. The current study has tested the hypothesis that chronic exposure to low dose nicotine reduces the responsiveness of pancreatic cancer to the leading therapeutic for this cancer, gemcitabine.

Methods—The effects of chronic nicotine (1 $\mu\text{m/L}$) on two pancreatic cancer cell lines in vitro and in a xenograft model were assessed by immunoassays, Western blots and cell proliferation assays.

Results—Exposure in vitro to nicotine for 7 days inhibited the gemcitabine-induced reduction in viable cells, gemcitabine-induced apoptosis as indicated by reduced expression of cleaved caspase-3 while inducing the phosphorylation of signaling proteins ERK, AKT and Src. Nicotine (1 $\mu\text{m/L}$) in the drinking water for 4 weeks significantly reduced the therapeutic response of mouse xenografts to gemcitabine while reducing the induction of cleaved caspase-3 and the inhibition of phosphorylated forms of multiple signaling proteins by gemcitabine in xenograft tissues.

Conclusions—Our experimental data suggest that continued moderate smoking and nicotine replacement therapy may negatively impact therapeutic outcomes of gemcitabine on pancreatic cancer and that clinical studies in cancer patients are now warranted.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer mortality in western countries (1). PDAC is unresponsive to radio- and chemotherapy, resulting in a mortality rate near 100% within 6 months of diagnosis (2). Smoking is a documented risk factor for pancreatic cancer (3). However, the mechanisms of this association are poorly understood. It has been shown that single dose exposures to nicotine in vitro stimulate the proliferation while inhibiting apoptosis of several non pancreatic cancers (4–7). Our

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laboratory has reported that exposure of human PDAC cell lines in vitro to a single dose of nicotine stimulated cell proliferation while inducing the levels of the signaling proteins p-ERK, p-Src, p-AKT and p-CREB (8). These responses were caused by the nicotinic receptor-induced production of norepinephrine and epinephrine, which in turn activated cAMP-dependent intracellular signaling pathways downstream of beta-adrenergic receptors (8). Investigations in mouse xenografts of PDAC (9) revealed significant growth promoting effects on xenografts associated with the induction of beta-adrenergic signaling pathways in mice treated for 4 weeks with nicotine in the drinking water at a dose level ($200\mu\text{g/ml} = 432.4\mu\text{mole/L}$), simulating the nicotine consumption of heavy smokers. It was also shown that the tumor promoting effects of nicotine in this study were completely abolished by the pharmacological inhibition of cAMP-dependent signaling (9). By contrast, studies on the effects of nicotine in animal models of non pancreatic cancers have yielded contradictory results. Nicotine has thus been reported to have stimulating (10), inhibiting (11) or no effects (12, 13) on the development of tumors in laboratory animals, depending on the animal model used, dose, duration or route of nicotine administration chosen. However, the potential effects of chronic low dose nicotine exposure on therapeutic outcomes of pancreatic cancer have not been studied to date.

Smokers diagnosed with PDAC often undergo nicotine replacement therapy (NRT) while numerous patients seek comfort in the continuation of their smoking habits. Cancer therapy is thus often accompanied by chronic exposure to an agonist of nicotinic acetylcholine receptors that may modulate therapeutic efficacy via stimulation of cell proliferation and/or inhibition of drug-induced apoptosis. To test this hypothesis, we have investigated the effects of chronic low dose nicotine on PDAC responsiveness in vitro and in mouse xenografts to the leading chemotherapeutic agent for PDAC, gemcitabine (14). Using numbers of viable PDAC cells, cleaved caspase-3 levels, the activation status of key signaling proteins previously shown by us to be effectors of nicotine-induced adrenaline production(8), and growth of PDAC xenografts in vivo as endpoints, our data show a significant reduction in therapeutic outcomes of gemcitabine by chronic exposure to low doses of nicotine.

Materials and Methods

Cell culture

The human PDAC cell lines Panc-1 and BXPC-3 were purchased from the American Type Culture Collection (Manassas, VA) and maintained in an atmosphere of 5% CO₂ and 37°C in the culture medium recommended by the vendor supplemented with 10% FBS and without antibiotics. The cell lines were authenticated at the beginning of the current study by RADIL (Research Animal Diagnostic Laboratory, Columbia, MO, USA) by species-specific PCR evaluation.

Establishment of dose-response curves for gemcitabine in unpretreated and nicotine pretreated cells in vitro

Cells were pretreated for 7 days with (–)-nicotine hydrogen tartrate ($1\mu\text{m/L}$, Sigma, St Louis, MO). During this time period, the culture medium containing nicotine was changed every 24 hours. Control cells were maintained under identical conditions with daily changes of media not containing nicotine. The nicotine pretreated and unpretreated Panc-1 cells and BXPC-3 cells were then seeded into 6-well plates at a density of 50,000 cells per well and grown to 30%–40% confluence. Triplicate samples were then incubated for 72h with gemcitabine (Gemzar hydrochloride; Eli Lilly (Indianapolis, IN, USA) dissolved in sterile water at concentrations ranging from 1 nm/L to $1\mu\text{m/L}$. Following trypan blue dye exclusion staining, the numbers of viable cells were measured by an automated cell counter

(Cellometer, Nexcelom, Bioscience, Lawrence, MA, USA) and verified by manual cell counts by haemocytometer. The data were analyzed by nonlinear regression for the establishment of sigmoidal dose response curves and calculation of EC₅₀ values, using Graphpad Prism software (Graphpad Software, Inc., La Jolla, CA, USA).

Visualization of signaling proteins by Western Blotting

Unpretreated and 7 day nicotine pretreated cells were grown in 100 mm plates in their complete media until they reached 30–40% confluence. Cells were then switched to their respective basal media without any supplements for 24 hours starvation. Fresh basal media was then added to the cells and treated with nicotine (1 μ M/L), gemcitabine (1 nm/L for BXPC-3 cells; 12 nm/L for Panc-1 cells) or the combination of both drugs for 72 hours. The concentrations of gemcitabine were based on the EC₅₀ values of this drug in the two cell lines. Cells were lysed in lysis buffer (50mmol/L Tris-HCl, 1% NP-40, 150mmol/L NaCl, 1mmol/L phenylmethylsulfonylfluoride, 1 mmol/L Na₃VO₄, 1mmol/L NaF and 1 μ g/ml of aprotinin, leupeptin and pepstatin) and the supernatants were obtained. Similarly, snap frozen xenografts harvested from the animal experiment (see below) were thawed and lysed using lysis buffer. Protein concentrations were determined using the BCA Protein Assay (Pierce, Rockford, IL). After heat denaturation, protein samples (20 μ g) were electrophoresed using 12% and 14% SDS gels (Invitrogen, Carlsbad, CA, USA) and blotted onto membranes. The membranes were blocked with 5% nonfat dry milk for 1 hour at room temperature. The antibodies for signaling proteins and Caspases were from Cell Signaling Technology (Danvers, MA, USA). The blots were probed with phospho-AKT (Ser473), AKT (pan), phospho-ERK 1/2 (Thr202/Tyr204), ERK 1/2 (p44/42), phospho-SRC (Tyr416), SRC (Tyr416), Caspase-3 (full length Caspase-3) and cleaved Caspase-3 (Asp175) or beta-Actin (mouse monoclonal AC-15 to beta-actin ab6276; Abcam, Cambridge, MA, USA) at 4°C overnight. The membranes were then washed (0.5% Tween 20/TBS) and incubated with their respective fluorescent secondary antibodies for 1 hour. Protein bands were then visualized with enhanced chemiluminescence reagent (Pierce ECL plus Western Blotting Detection Substrate, Thermo Scientific, Rockford, IL, USA).

Quantitative assessment of phosphorylated signaling proteins and cleaved caspase-3 by ELISA assays

Unpretreated and 7 day nicotine pretreated PANC-1 and BXPC-3 cells were grown in complete media and were seeded in 100mm plates and grown to 30–40% confluence. Cells were then treated with nicotine, gemzar or the combination of both (5 samples per treatment group) as outlined under Western blots. The cells were then washed twice with ice cold PBS, lysed with lysis Buffer and harvested into 1.5ml Eppendorf tubes. Similarly, snap frozen xenograft tissue samples harvested from the animal experiment (see below) were thawed and lysed using lysis buffer. Quantitative analyses of phosphorylated signaling proteins were conducted using c-SRC (MBL International Corporation, Woburn, MA, USA), AKT and ERK 1/2 kits (Invitrogen, Carlsbad, CA, USA) following the vendors' recommendations. Absorbance of samples was measured with an Epoch microplate spectrophotometer (Bio-Tek, Winooski, VT, USA) at 450nm primary wavelength with a 630nm reference wavelength. Quantitative assessment of cleaved caspase-3 levels was performed using an Enzchek Caspase-3 assay kit (Invitrogen). Absorbance of samples was measured using the Epoch microplate spectrophotometer at a primary wavelength of 496nm and a reference wavelength of 520nm.

Animal Experiment

The animal experiment was approved by the Institutional Animal Care and Use Committee. Male, 6-week-old athymic nude mice (Harlan Sprague Dawley Inc.) were housed (10 mice per cage) in our laboratory animal facility under standard laboratory conditions with free

access to food (autoclaved Purina rodent chow) and autoclaved water. All mice were subcutaneously inoculated in the flank region with BXPC-3 cells (3×10^6 in 0.2 mL of PBS, viability > 95%). The mice were randomly assigned to four groups (n=10). The control group remained untreated while the remaining groups were treated either with 50mg/kg Gemzar (Eli Lilly) twice a week by intra-peritoneal injections, 1 μ m/L nicotine in the drinking water (with changes of water bottles twice a week) or both nicotine in the drinking water and Gemzar intra-peritoneally. All treatments started 1 day after subcutaneous inoculation of the tumor cells and the animals were observed for 30 days. Body weights were recorded weekly. Liquid consumption was monitored by measuring the tap water with and without the nicotine in and out every day. Two perpendicular diameters (length and width) of each xenograft were measured weekly, and tumor volumes were calculated as follows: $(\text{length}/2) \times (\text{width}^2)$. At the end of the 30-day observation period, the animals were euthanized by CO₂ inhalation. The tumors were excised and snap frozen in liquid nitrogen for additional analyses by Western blots and Elisa assays as detailed above.

Statistical Analysis of Data

GraphPad Instat 3 software (GraphPad Instant biostatistics) was used to test significant differences between different treatment groups. Statistical tests used for the analysis of Elisa assays (n = 5) included nonparametric one-way ANOVA and Tukey-Kramer multiple comparison tests. Statistical significance of differences in EC₅₀ values of Gemzar in the concentration-response curves from unpretreated and nicotine pretreated cells was determined by unpaired t-tests with Welch correction. Statistical analysis of tumor volumes from all treatment groups (n = 10) was conducted by one-way analysis of variance. Statistically significant differences between xenograft volumes of selected treatment groups (n = 10) were additionally determined by unpaired, 2-tailed t-tests, following verification of a Gaussian distribution of data by the method of Kolmogorov and Smirnov.

Results

Our data show that chronic exposure of PDAC to nicotine significantly reduces the therapeutic efficacy of gemcitabine in vitro and in vivo. As shown in Figs. 1, A and B, treatment of cells in vitro for 7 days with nicotine (1 μ m/L) significantly ($p < 0.01$) increased the concentration of gemcitabine required to yield a 50% reduction in the number of viable cells in both cell lines, yielding increases in EC₅₀ values for gemcitabine from 790 pm/L in unpretreated BXPC-3 cells to 2.03 nm/L after 7 days of nicotine and from 9.6 nm/L in unpretreated Panc-1 cells to 340 nm/L in the nicotine pretreated cells. In accord with previous reports that cell line Panc-1 is relatively resistant to gemcitabine (15), the EC₅₀ for gemcitabine in unpretreated BXPC-3 cells was 12 times lower than that for Panc-1 cells. Gemzar concentrations of 1 nm/L (BXPC-3 cells) and 12 nm/L (Panc-1 cells) were therefore used for the in vitro assays shown in Figs. 2–4. Assessment by Western blots and Elisa assays of the levels of phosphorylated signaling proteins AKT, Src and ERK, all of which are frequently overexpressed in pancreatic cancer tissues (16–18), provided evidence that this reduction in sensitivity to gemcitabine was mediated in part by significant ($p < 0.001$) inductions in the levels of these phosphorylated proteins by chronic nicotine (Figs. 2A, B and 3A, B). At the same time, the induction of cleaved caspase -3 by gemcitabine was significantly ($p < 0.001$) reduced by pre-exposure of both cell lines to chronic nicotine (Figs. 4A–D), indicating that a nicotine-induced reduction in apoptosis contributed to the observed loss of sensitivity of either cell line to gemcitabine.

The cell line with the higher sensitivity to gemcitabine (BXPC-3) was used to assess the modulating effects of chronic low concentrations of nicotine (1 μ mole/L in the drinking water) on the therapeutic efficacy of gemcitabine in mouse xenografts. All mice inoculated with BXPC-3 cells survived until the end of the 30-day observation period. The mice

weighed between 25g and 30 g throughout the experiment, with no statistically significant differences among individual treatment groups. The animals receiving nicotine treatments consumed $3.44\mu\text{g} \pm 0.74\mu\text{g}$ of nicotine/day per mouse. Contrary to our previous observations (9) with high doses of nicotine ($432.4\mu\text{mole/L}$ in the drinking water), xenograft growth in the group receiving nicotine alone of the current study was not significantly increased (Fig. 5A). Treatment of mice with gemcitabine alone significantly ($p < 0.001$) reduced xenograft volumes in weeks 2–4 (Fig. 5A) with 2 out of the 10 animals showing no detectable tumors. Nicotine treatment significantly ($p < 0.001$) reduced this therapeutic effect of gemcitabine in weeks 2–4 (Fig. 5A).

In accord with the documented ability of gemcitabine to induce apoptosis (15), BXPC-3 xenografts of the animals treated with gemcitabine alone showed induced protein levels of cleaved Caspase-3 in Western blots (Fig 5B) accompanied by a significant ($P < 0.001$) increase in activated caspase-3 levels in the quantitative Elisa assay (Fig. 5C). Nicotine treatment alone did not significantly change the low levels of cleaved caspase-3 detected in xenograft tissues of untreated mice (Figs. 5C, D). By contrast, the induction of cleaved caspase in response to gemcitabine was completely abolished by nicotine ($p < 0.001$; Fig. 5C, D).

Western blots also revealed reductions in the levels of the phosphorylated signaling proteins AKT, SRC and ERK1/2 by gemcitabine (Fig. 6A), a finding verified by a significant ($p < 0.001$) reduction of their levels in quantitative Elisa assays (Fig. 6B). These responses were reversed above the levels of untreated control xenografts ($p < 0.001$) by treatment of the mice with nicotine while nicotine exposure alone significantly ($p < 0.001$) induced all three phosphorylated proteins (Figs 6A, B).

Discussion

Our data show, for the first time, that low levels of chronic nicotine of relevance to nicotine exposure in moderate smokers (19) or patients undergoing NRT (20) significantly reduce the therapeutic efficacy of gemcitabine in PDAC in vitro and in vivo. The significantly higher concentrations of gemcitabine required to yield a 50% reduction in viable cells from both PDAC cell lines exposed for 7 days to nicotine and the significant decrease of gemcitabine-induced inhibition of xenograft development in nicotine treated mice identify the low nicotine concentrations used in the current experiments as highly effective in reducing the anti-tumorigenic effects of gemcitabine in PDAC. These effects appear to have been primarily caused by inhibition of gemcitabine-induced apoptosis and not by nicotine-induced stimulation of cell proliferation. This interpretation is supported by the lack of significant increases in xenograft volumes of the animals treated with nicotine alone and is in accord with the known apoptosis inducing properties of gemcitabine (15). In response to cytotoxic stimulation by gemcitabine, a set of initiator caspases are activated (caspase-2, -8, -9, and -10). In turn, these initiator caspases cleave effector caspases (caspase-3, -6, and -7) from an inactive or procaspase structure to an active form, leading to apoptosis (21). The observed significant induction of cleaved caspase-3 by gemcitabine in vitro and in the xenografts and inhibition of these responses by nicotine thus further supports inhibition of drug-induced apoptosis by nicotine as the predominant mechanism in the current experiments. These findings are in accord with earlier reports in lung cancer cells in vitro that showed inhibition of drug-induced apoptosis by a single low dose ($100\text{--}200\text{ nm/L}$) of nicotine (5) whereas a higher dose of nicotine ($1\mu\text{m/L}$) induced cell proliferation (4).

The phosphorylated proteins ERK, AKT and Src are frequently overexpressed in PDAC tissue and contribute to the regulation of cell proliferation and apoptosis (17, 18, 22) and are current targets for PDAC therapy (2). We have shown that the in vitro exposure of immortalized pancreatic duct epithelial cells, Panc-1 cells, or BXPC-3 cells to a single dose

of nicotine causes the synthesis and release of the noradrenaline and adrenaline (8). This induced phosphorylation of multiple proteins (8). The observed reduction in p-ERK, AKT, and Src by gemcitabine in vitro and in xenografts and partial reversal of these effects by chronic nicotine suggest that modulation of all three proteins by nicotine contributed to the observed reduced sensitivity of PDAC to gemcitabine. Our laboratory has previously shown that chronic treatment of mice with a high dose of nicotine (200 µg/ml = 432.4 µmole/L in the drinking water for 4 weeks) in the range of the daily nicotine consumption by heavy smokers significantly increased the growth of Panc-1 xenografts in mice not treated by an anti-cancer agent (9). The lack of significant increases in xenograft volumes in the mice treated with the current low dose (1 µm/L in the drinking water for 4 weeks) of nicotine alone thus suggest that relatively high nicotine doses are required for the induction of PDAC proliferation whereas much lower concentrations of nicotine still effectively inhibit drug-induced apoptosis. These findings have immediate clinical implications as the nicotine concentrations used in the current experiments are within the range of the daily nicotine exposure of light smokers and patients undergoing NRT. Clinical investigations in PDAC patients are now warranted to further investigate this important issue.

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Figure 1.

Concentration-response curves for gemcitabine (Gemzar) in unpretreated and 7-day nicotine (1 μ M/L) pretreated BXPC-3 (**A**) and Panc-1 (**B**) cells in vitro. Data points are mean values and standard deviations of triplicate samples per Gemzar concentration expressed as normalized numbers of viable cells. The curves and EC_{50} values were established by nonlinear regression analysis of data fitted to sigmoidal dose-response curves with variable slope.

Figure 2.

Results of Western blots (A) and Elisa assays (B) for the detection of phosphorylated signaling proteins AKT, Src and ERK1/2 in BXPC-3 cells in vitro. Nicotine (1 μ M/L) pretreatment was for 7 days. Gemcitabine (Gemzar) was used at a concentration of 1 nM/L. Columns in the graph are mean values and standard deviations of 5 samples per treatment group expressed as fold change over controls.

Figure 3. Results of Western blots (**A**) and Elisa assays (**B**) for the detection of phosphorylated signaling proteins AKT, Src and ERK1/2 in Panc-1 cells in vitro. Nicotine (1 μ M/L) pretreatment was for 7 days. Gemcitabine (Gemzar) was used at a concentration of 12 nM/L. Columns in the graph are mean values and standard deviations of 5 samples per treatment group expressed as fold change over controls.

Figure 4.

Expression of cleaved caspase-3 in vitro in BXPC-3 cells by Western analysis (A) and Elisa assay (B) and in Panc-1 cells by Western blot (C) and Elisa assay (D). Columns in the graph are mean values and standard deviations of 5 samples per treatment group expressed as fold change over controls.

Figure 5.

Volumes of xenografts from BXPC-3 cells (A) over time in control mice and in mice treated with nicotine (1 μ M/L in the drinking water), Gemzar (50 mg/kg twice a week i. p.) or with the combination of both drugs. Data points in the graph are mean values and standard errors (n = 10) of tumor volumes. Levels of cleaved caspase-3 in BXPC-3 xenograft tissues by Western blot (B) and Elisa assay (C). Columns in the graph are mean values and standard deviations of 5 samples per treatment group expressed as fold change over controls.

Figure 6. Results of Western blots (A) and Elisa assays (B) for the detection of phosphorylated signaling proteins AKT, Src and ERK1/2 in tissues from BXPC-3 xenografts. Treatments with nicotine and Gemzar are as detailed in Figure 5. Columns in the graph are mean values and standard deviations of 5 samples per treatment group expressed as fold change over controls.