Nicotine induces self-renewal of pancreatic cancer stem cells via neurotransmitter-driven activation of sonic hedgehog signalling

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A small subpopulation of pancreatic cancer cells with characteristics of stem cells drive tumour initiation, progression and metastasis. A better understanding of the regulation of cancer stem cells may lead to more effective cancer prevention and therapy. We have shown that the proliferation and migration of pancreatic cancer cell lines is activated by the nicotinic receptor-mediated release of stress neurotransmitters, responses reversed by γ-aminobutyric acid (GABA). However, the observed cancer inhibiting effects of GABA will only succeed clinically if GABA inhibits pancreatic cancer stem cells (PCSCs) in addition to the more differentiated cancer cells that comprise the majority of cancer tissues and cell lines. Using PCSCs isolated from two pancreatic cancer patients by cell sorting and by spheroid formation assay from pancreatic cancer cell line Panc-1, we tested the hypothesis that nicotine induces the self-renewal of PCSCs. Nicotinic acetylcholine receptors (nAChRs) α3, α4, α5 and α7 were expressed and chronic exposure to nicotine increased the protein expression of these receptors. Immunoassays showed that PCSCs produced the stress neurotransmitters epinephrine and norepinephrine and the inhibitory neurotransmitter GABA. Chronic nicotine significantly increased the production of stress neurotransmitters and sonic hedgehog (SHH) while inducing Gli1 protein and decreasing GABA. GABA treatment inhibited the induction of SHH and Gli1. Spheroid formation and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide assays showed significant nicotine-induced increases in self...
1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer deaths in developed countries due to its high mortality within 1 year of diagnosis [4]. Smoking is a documented risk factor for PDAC [19]. However, the mechanisms responsible for this association are poorly understood.

Emerging evidence suggests that a subpopulation of cancer stem cells drives tumour initiation, progression and metastasis of PDAC [17, 23]. A better understanding of the regulation of pancreatic cancer stem cells (PCSCs) may thus lead to the development of more effective PDAC prevention and therapy. However, cancer stem cells only constitute up to 5% of cells in pancreatic cancer tissue and pancreatic cancer cell lines [5, 29]. Data generated in cancer cell lines and their xenografts thus represent mostly the reactions of the more differentiated cancer cells while responses of the small stem cell population may remain obscure.

With the discovery of methods for the isolation of PCSCs from tumour tissue and cell lines [5,29], the sonic hedgehog (SHH) pathway has emerged as a key regulator of PCSCs [12,14,22,27]. Overexpression of SHH and its downstream effector, Gli1, is associated with a poor overall survival of PDAC patients [21] and the SHH pathway is among recently explored therapeutic targets for PDAC [16]. However, a first pilot clinical trial with an SHH inhibitor alone or in combination with gemcitabine failed to improve clinical outcomes in PDAC patients [15]. Similarly, strategies that target signalling pathways overexpressed in more differentiated PDAC cells alone or in combination with conventional cancer therapeutics have disappointed in clinical trials [20]. It hence appears that therapeutic strategies need to simultaneously target regulatory pathways in differentiated cancer cells as well as PCSCs to become more successful.

We have shown that pancreatic duct epithelial cells and PDAC cell lines express an autocrine neurotransmitter loop that is regulated by nicotinic acetylcholine receptors (nAChRs) [2,3]. Nicotine increased the proliferation and migration of these cells by stimulating the synthesis and release of the stress neurotransmitters norepinephrine and epinephrine, which in turn activated multiple signalling cascades downstream of beta-adrenergic receptors [2,3]. In addition, beta-adrenergic receptor agonists increased cell proliferation and migration of PDAC cell lines in vitro in a cyclic adenosine monophosphate (cAMP) dependent manner [13,25,31]. We have shown that nicotine treated mice carrying PDAC xenografts demonstrated increased systemic and tumour levels of norepinephrine, epinephrine, and cAMP accompanied by significant increases in xenograft sizes [1]. These responses were abrogated by treatments in vitro and in the mouse model with the inhibitory neurotransmitter γ-aminobutyric acid (GABA) via G\textsubscript{AB}A-mediated inhibition of cAMP formation [1, 2, 25]. While the reported tumour inhibiting effects of GABA are promising, they would only translate into successful therapeutic applications in PDAC patients if in addition to the more differentiated cells the self-renewal of PCSCs were also inhibited. However, neither the effects of nicotine nor those of stress neurotransmitters or GABA on PCSCs have been studied to date.

PCSCs have the unique ability to self-renew and form differentiated progeny [17,23,29]. The maintenance of cancer cells in serum free medium selects for the self-renewal of cancer stem cells as three dimensional floating aggregates (spheroids), a method widely used to generate cell populations enriched in cancer stem cells from cancer cell lines [6,7,9]. Spheroid formation assays and cell sorting by stem cell markers are both commonly used to isolate cancer stem cells from tumour tissues and cell lines [8,23,29]. Using PCSCs isolated by cell sorting and PCSCs enriched by spheroid formation assays, the current study has tested the hypothesis that nicotine induces the self-renewal of PCSCs by modulating the autocrine production of regulatory neurotransmitters and that this response can be reversed by treatment with GABA.

2. Materials and methods

2.1. Cell culture

The human PDAC cell line Panc-1 was purchased from the American Type Culture Collection (Manassas, VA, USA) and was authenticated at the end of the experiments by species-specific polymerase chain reaction (PCR) in March 2015 (IDEXX BioResearch, Columbia, MO, USA). Two batches of PCSCs isolated from different donors by cell sorting were purchased from Celprogen (San Pedro, CA, USA). The purchased PCSCs expressed the stem cell markers CD133, CD44, SSEA3/4, Oct4, alkaline phosphatase, aldehyde dehydrogenase, telomerase and nestin. Cancer stem cells isolated from
cell line Panc-1 by spheroid formation assay express the stem cell markers CD133, CD44, Oct4, nestin and CD24 [30,32]. Monolayers of Panc-1 cells comprised of mostly differentiated cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) medium supplemented with 10 % foetal bovine serum (FBS). Monolayers of the purchased PCSCs comprised of mixed populations of differentiated and cancer stem cells were maintained in complete (containing FBS) cancer stem cell growth medium provided by the vendor (Celprogen). Enrichment of stem cells from cell line Panc-1 and the sorted PCSCs was accomplished by maintenance of the cells in serum-free spheroid formation media (Celprogen) consisting of 1:1 proportion of 1X DMEM/F12 and cell specific basal media (without FBS) supplemented with B-27, N-2, glutamax and beta-mercaptoethanol (Invitrogen Life Technologies, Grand Island, NY, USA). All cells were maintained without antibiotics in an atmosphere of 5 % CO2, 99 % relative humidity, and 37 °C.

2.2. Spheroid formation assays

Single cell suspensions (1000 cells/well) were plated in ultra-low adherent 6-well plates (Corning Inc., Corning, NY, USA) in serum-free spheroid formation media to assess PCSC self-renewal. All treatments were started 24 h after plating of the cells and continued daily for 15 d with subculture of spheroids after 7 d (nicotine: 1 µM/L (-)-nicotine hydrogen tartrate, Sigma–Aldrich, St. Louis, MO, USA; GABA: 30 mM/L, Sigma–Aldrich). This concentration of nicotine is within the range of plasma nicotine levels in smokers [24]. The generated spheroids were counted and photographed using a Nexcelom cellometer. The proportion of spheroid-generating cells for each treatment group was calculated by dividing the counted number of spheroids by the number of cells seeded (1000 cells).

2.3. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide assays

Following instructions of the vendor, the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide) colorimetric assay (Sigma–Aldrich) was used to assess the number of viable cells as an indicator of cell proliferation in spheroids. The spheres were either left untreated or treated with 1 µM/L nicotine, 30 µM/L GABA or nicotine + GABA for 7 d. Absorbance values of the collected spheres from all treatment groups were read using an uQuant Bio-Tek Instrument enzyme-linked immunosorbent assay (ELISA) reader at 570 nm primary and 650 nm reference wavelengths.

2.4. Western blotting

The protein expression of nAChR subunits z3, z4, z5 and z7 and of the transcription factor Gli1 was determined by Western blots as previously described [2]. Protein samples were collected using lysis buffer (RIPA buffer 1X; Halt Protease Inhibitor Single-Use Cocktail, Ethylenediaminetetraacetic acid (EDTA)-Free (100X); 10 µl of 100 mM phenylmethylsulfonylfluoride/ml RIPA; 10 µl of 100 mM Na3VO4/ml RIPA; 10 µl of 1 M NaF/ml RIPA; Thermo Scientific, Rockford, IL, USA). After heat denaturation, protein samples were electrophoresed using 12% SDS gels (Invitrogen) and were then blotted onto membranes. The membranes were blocked (5% nonfat dry milk solution in 1X Tris-buffered Saline Tween (TBST)) for 1 h at room temperature, incubated overnight at 4 °C with the following primary antibodies: anti-nAChR subunits z3 (57 kDa, Abcam, Cambridge, MA, USA), z4 (55 kDa, Millipore, Billerica, MA, USA), z5 (53 kDa, Abcam) and z7 (56 kDa, Abcam) or anti-Gli1 (118 kDa, Santa Cruz Biotechnologies, Dallas, TX, USA). The primary antibody β-actin (42 kDa, Abcam) was used as a loading control. All membranes were then washed (0.5 % Tween 20/TBS) and incubated with secondary antibodies for 2 h. Protein bands were visualised with enhanced chemiluminescence reagent (Pierce ECL Western Blotting Detection Substrate, Thermo Scientific). Following background subtraction, mean densities of three rectangular areas of standard size per band from three independent westerns were determined using NIH ImageJ software and mean values and standard deviation (n = 9) of protein expression were calculated.

2.5. Real-time PCR

Having previously identified the expression of nAChRs with subunits z3, z4, z5 and z7 in PDAC cell lines and in immortalised pancreatic duct epithelial cells [3], the mRNA levels of these receptors were assessed in untreated and nicotine treated (1 µM/L for 7 d) spheroids by real-time PCR as previously described [26] using a Cepheid SmartCycler. RNA samples from each group were isolated using an Absolutely RNA Miniprep kit (Agilent Technologies, Santa Clara, CA, USA). The collected RNA samples along with the QuantitTect Primer assays (Qiagen, Germantown, MS, USA) for genes CHRNA3 (NM_000743), CHRNA4 (NM_000744), CHRNA5 (NM_000745) and CHRNA7 (NM_000746) were used in the Quantifast SYBR Green PCR kit to quantify mRNA expression levels of nAChR subunits z3, z4, z5 and z7. Detection reagent for 18S rRNA (Eurogentec, San Diego, CA, USA) served for normalisation. Data were analysed using the 2-ΔΔT method [18].

2.6. Determination of neurotransmitters and SHH

Spheroids were treated for 7 d (nicotine: 1 µM/L; GABA: 30 µM/L). Untreated control and treated spheroids were then harvested into 1.5 ml Eppendorf tubes (5 samples per group) after one time wash with warm 1X phosphate buffered solution. Norepinephrine, GABA for 7 d. Absorbance values of the collected spheres from all treatment groups were determined by Western blots as previously described [2]. Protein samples were collected using lysis buffer (RIPA buffer 1X; Halt Protease Inhibitor Single-Use Cocktail, Ethylenediaminetetraacetic acid (EDTA)-Free (100X); 10 µl of 100 mM phenylmethylsulfonylfluoride/ml RIPA; 10 µl of 100 mM Na3VO4/ml RIPA; 10 µl of 1 M NaF/ml RIPA; Thermo Scientific, Rockford, IL, USA). After heat denaturation, protein samples were electrophoresed using 12% SDS gels (Invitrogen) and were then blotted onto membranes. The membranes were blocked (5% nonfat dry milk solution in 1X Tris-buffered Saline Tween (TBST)) for 1 h at room temperature, incubated overnight at 4 °C with the following primary antibodies: anti-nAChR subunits z3 (57 kDa, Abcam, Cambridge, MA, USA), z4 (55 kDa, Millipore, Billerica, MA, USA), z5 (53 kDa, Abcam) and z7 (56 kDa, Abcam) or anti-Gli1 (118 kDa, Santa Cruz Biotechnologies, Dallas, TX, USA). The primary antibody β-actin (42 kDa, Abcam) was used as a loading control. All membranes were then washed (0.5 % Tween 20/TBS) and incubated with secondary antibodies for 2 h. Protein bands were visualised with enhanced chemiluminescence reagent (Pierce ECL Western Blotting Detection Substrate, Thermo Scientific). Following background subtraction, mean densities of three rectangular areas of standard size per band from three independent westerns were determined using NIH ImageJ software and mean values and standard deviation (n = 9) of protein expression were calculated.

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epinephrine (Kat ELISA kits, Rocky Mountain Diagnostics, Colorado Springs, CO, USA), GABA (GABA Research ELISA kit, Rocky Mountain Diagnostics Inc.) or SHH (SHH human ELISA kit, Abcam) were determined by ELISA assays per instructions by the vendors. Absorbance of samples was read using an uQuant Bio-Tek Instrument ELISA reader at 450 nm primary wavelength with a 630 nm reference wavelength.

2.7. Statistical analysis

GraphPad Instat 3 and GraphPad Prism 6 software (GraphPad Instat Biostatistics, San Diego, CA, USA) was used for the statistical evaluation of data. Statistical tests used included non-parametric one way analysis of variance, non-parametric Mann-Whitney test, two-tailed t-test and F-test for comparison of dose-response curves. Densitometry data of Western blots are expressed as mean values and standard deviations of three density determinations per band from three independent westerns per antibody (n = 9). Dose–response curves of GABA on SHH production in the presence and absence of nicotine (1 μM) are expressed as mean values and standard errors normalised to untreated controls of triplicate samples that were fitted to non-linear regression curves. All other data are expressed as mean values and standard deviations of five samples per treatment group.

3. Results

Assessment of cancer stem cell self-renewal by spheroid formation assays showed significant (p < 0.0001) increases in the number of spheroids formed during 14 d of daily exposure to nicotine (Fig. 1a,b). Simultaneous treatment with GABA completely (p < 0.0001) blocked this response to nicotine (Fig. 1a,b). As exemplified in Fig. 1a, the increases in spheroid numbers were associated with increases in spheroid sizes while spheroids were smaller when their numbers decreased. In accord with this observation, exposure of spheroids to nicotine for 7 d significantly (p < 0.001) increased the number of viable cells suggesting enhanced cell proliferation in spheroids (Fig. 2a). GABA significantly (p < 0.001) inhibited the nicotine-induced increases in viable cells.
and additionally significantly (p < 0.001) reduced the number of viable cells below that observed in control spheroids when administered as a single agent (Fig. 2a).

As exemplified in Fig. 2b, PCSCs enriched in spheroids produced the neurotransmitters norepinephrine, epinephrine and GABA. Nicotine significantly (p = 0.0079) increased the production of both cancer stimulating stress neurotransmitters significantly (p < 0.0079) when administered as a single dose for 30 min (Fig. 2b) and these responses were enhanced further when identical doses of nicotine were administered daily for 7 d (Fig. 2b). By contrast, the production of inhibitory GABA was significantly reduced by chronic nicotine (p = 0.0079), whereas a single dose of nicotine did not significantly change GABA levels (Fig. 2b).

Real-time PCR revealed the expression of mRNA for nAChR subunits α3, α4, α5, and α7 in spheroids (Fig. 3a). In accord with the behaviour of these receptors in the brain [10] and in the predominantly differentiated cells represented in monolayers of pancreatic cancer cell lines [2], exposure to nicotine did not significantly change the expression levels of their mRNA (Fig. 3d). However, as shown in Fig. 3b–d, the protein expression of α3, α4 and α7 nAChRs was significantly (p < 0.0001) increased by chronic nicotine in sorted cells and spheroids.

PCSCs in spheroids synthesised and released SHH as detected by ELISA assays. Total SHH (intracellular plus secreted) was significantly (p = 0.0286) increased in spheroids from both sources (Fig. 4a) by chronic nicotine. This response was significantly (p = 0.0286) inhibited by simultaneous treatment with GABA (Fig. 4a). In addition, exposure to GABA alone significantly (p = 0.0286) decreased SHH production below the levels observed in untreated control spheroids (Fig. 4a). As shown in Fig. 4b, the inhibition of SHH by GABA was concentration-dependent. Analysis of the data by nonlinear regression revealed that the IC50 of GABA in the presence of nicotine was almost 40 times higher than in the absence of nicotine (Fig. 4b).

In accord with the documented function of the Gli1 transcription factor as an effector of SHH signalling [14], Western blots revealed significant (p < 0.001) increases in Gli1 protein in spheroids treated for 7 d with nicotine (Fig. 5a–c). This response was completely abrogated (p < 0.001) by simultaneous exposure to GABA while GABA treatment alone significantly (p < 0.001) reduced Gli1 protein below the levels observed in control spheroids (Fig. 5a–c).

4. Discussion

Our data show, for the first time, that nicotine induces the self-renewal of PCSCs derived from PDACs via increased SHH production caused by a simultaneous increase in stress neurotransmitter production and suppression of GABA (Fig. 6). These findings add novel mechanistic insights to the recently reported nicotine-induced PCSC activation and acinar dedifferentiation via down-regulation of the GATAt6 gene in a Kras mutated mouse model during initiating events in the development of PDAC [11]. Exogenous administration of GABA prevented these effects of nicotine in a
concentration-dependent manner, emphasizing the antagonistic effects of this inhibitory neurotransmitter on the adverse effects of nicotine on PCSCs.

It is well established that nAChRs are expressed in embryonic stem cells and adult cancer stem cells [33]. However, their function in these cells is poorly understood. By contrast, an important regulatory role of the SHH pathway for self renewal of embryonic stem cells and adult cancer stem cells, including PCSCs, has been well documented [14]. Interestingly, a potential functional link between neurotransmitter receptors and the SHH pathway in cancer stem cells has not been investigated to date. Our findings that chronic exposure to nicotine significantly increases SHH signalling, self-renewal and proliferation of PCSCs by augmenting SHH production has important clinical implications and are in accord with the reported strong growth stimulation of PDAC xenografts by chronic nicotine, which increased the systemic levels of stress neurotransmitters while reducing GABA [1]. In light of the current data and the observation that PCSCs drive the progression of pancreatic cancer [5], these effects of chronic nicotine on xenograft progression was not only caused by the reported activation of multiple signalling pathways in

Fig. 3. (a) Real-time PCR showed that the mRNA levels of nAChR receptor subunits α3, α4, α5 and α7 were expressed in spheroids but did not significantly change in response to treatment for 7 d with nicotine (1 μM/L), suggesting that the upregulations in receptor protein determined by Western blots (b) in these spheroids were caused by post-translational mechanisms. Densitometry values of Western blots (c and d) revealed that protein upregulation of nAChR subunits α3, 4 and 7 were significant (p < 0.0001). Columns in graphs c and d represent the mean values and standard deviations of three densitometric readings per band from three independent Western blots. PCR = polymerase chain reaction; nAChR = nicotinic acetylcholine receptor.
differentiated cancer cells but also by the activation of the SHH pathway in PCSCs. In vitro studies have additionally shown that pancreatic cancer cell lines and immortalised pancreatic duct epithelial cells have the ability to synthesise and release their own norepinephrine, epinephrine and GABA, with the stress neurotransmitters stimulating cell proliferation and migration whereas GABA inhibited [2, 3]. As our current data show, the regulatory functions of these neurotransmitters are not limited to the more differentiated PDAC cell populations but are also fully functional in PCSCs.

Exposure to nicotine by smoking or chronic abuse of nicotine replacement products often persists after cancer diagnosis. In these scenarios, PCSCs and differentiated cancer cells are continuously stimulated by increases in stress neurotransmitters and simultaneous decrease in GABA. Our current data in conjunction with our published in vivo findings [1] indicate that reversal of the nicotine-induced GABA deficiency by exogenous GABA supplementation yields high anti-tumorigenic efficiency in pancreatic cancer by inhibiting differentiated cancer cells and PCSCs simultaneously. GABA has been safely used as a nutritional supplement for many years without detectable adverse effects [28].

Fig. 4. (a) Results of ELISA assays for the detection of total (intracellular plus secreted) sonic hedgehog (SHH) in spheroids from Panc-1 and sorted PCSCs. Exposure of spheroids for 7 d to nicotine (1 μM/L) significantly (p < 0.003) increased SHH levels. This response was completely blocked by simultaneous treatment for 7 d with GABA (30 μM/L) while GABA alone significantly (p < 0.003) reduced SHH below control levels. Data are mean values and standard deviations of five samples per treatment group. (b) Dose-response curves for GABA-induced SHH inhibition in Panc-1 spheroids in the presence and absence of 7-d nicotine (1 μM). The IC50 in the presence of nicotine was 23.5 times higher than in the absence of nicotine, emphasizing the antagonistic effects of nicotine and GABA on SHH production. Data points are mean values and standard deviations of triplicate samples normalised to controls with untreated controls set as 100% and maximum inhibition of non-nicotine exposed samples set as 0%. The curves and IC50 values were determined by nonlinear regression analysis. ELISA = enzyme-linked immunosorbent assay; GABA = γ-aminobutyric acid.

Fig. 5. Western blots showing protein expression of Gli1 in spheroids from Panc-1 (a) and sorted PCSCs (b). Nicotine (1 μM/L) for 7 d significantly (p < 0.001) induced Gli1 protein, a response completely blocked by simultaneous treatment with GABA (30 μM/L) GABA alone significantly (p < 0.001) reduced Gli1 protein below control levels. Columns in the graph (c) are mean values and standard deviations of three densitometric readings per band from three independent Western blots. PCSCs = pancreatic cancer stem cells; GABA = γ-aminobutyric acid.
Accordingly, nutritional GABA supplementation may significantly improve clinical outcomes in pancreatic cancer patients.

Conflict of interest statement

The authors declare no conflicts of interest.

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References


