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Original Research

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

Mohammed H. Al-Wadei, Jheelam Banerjee, Hussein A.N. Al-Wadei, Hildegard M. Schuller*

Experimental Oncology Laboratory, Department of Biomedical & Diagnostic Sciences, College of Veterinary Medicine, University of Tennessee, Knoxville, TN, USA

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Abstract 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

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* *Corresponding author*: Experimental Oncology Laboratory, Department of Biomedical & Diagnostic Sciences, College of Veterinary Medicine, University of Tennessee, 2407 River Drive Knoxville, TN 37996-4543, USA. Tel.: +1 865 974 8217; fax: +1 865 974 5616.
E-mail address: hmsch@utk.edu (H.M. Schuller).

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renewal and cell proliferation, responses blocked by GABA. Our data suggest that nicotine increases the SHH-mediated malignant potential of PCSCs and that GABA prevents these effects.

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 $G\alpha_i$-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