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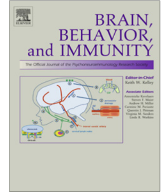

Chronic stress accelerates pancreatic cancer growth and invasion: A critical role for beta-adrenergic signaling in the pancreatic microenvironment

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ABSTRACT

Pancreatic cancer cells intimately interact with a complex microenvironment that influences pancreatic cancer progression. The pancreas is innervated by fibers of the sympathetic nervous system (SNS) and pancreatic cancer cells have receptors for SNS neurotransmitters which suggests that pancreatic cancer may be sensitive to neural signaling. *In vitro* and non-orthotopic *in vivo* studies showed that neural signaling modulates tumour cell behavior. However the effect of SNS signaling on tumor progression within the pancreatic microenvironment has not previously been investigated. To address this, we used *in vivo* optical imaging to non-invasively track growth and dissemination of primary pancreatic cancer using an orthotopic mouse model that replicates the complex interaction between pancreatic tumor cells and their microenvironment. Stress-induced neural activation increased primary tumor growth and tumor cell dissemination to normal adjacent pancreas. These effects were associated with increased expression of invasion genes by tumor cells and pancreatic stromal cells. Pharmacological activation of β-adrenergic signaling induced similar effects to chronic stress, and pharmacological β-blockade reversed the effects of chronic stress on pancreatic cancer progression. These findings indicate that neural β-adrenergic signaling regulates pancreatic cancer progression and suggest β-blockade as a novel strategy to complement existing therapies for pancreatic cancer.

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1. Introduction

Pancreatic cancer is the fourth leading cause of cancer-related death, with approximately 40,000 new cases in the US each year (Siegel et al., 2012). The overall survival rate is less than 5% and effective strategies to improve clinical outcomes are critically needed. Survival from pancreatic cancer depends on successful resection of the primary tumor with 5-year survival rates of 20–30% reported in specialized centers (Gaedcke, 2010). However, even when surgery results in tumor-free resection margins recurrence rates are high and most patients eventually die of local tumor recurrence and metastatic disease (Kleeff, 2007; Hishinuma, 2006; Han, 2006). Additionally, many patients are diagnosed with advanced stage pancreatic cancer, which limits surgical treatment (Kazanjian, 2008; Bilimoria, 2007). Gemcitabine is the standard-of-care chemotherapy regimen for locally advanced and metastatic pancreatic cancer, but the overall prolongation of survival is disappointingly small (5–7 months) (Storniolo, 1999; Herrmann, 2007; Rougier, 2013). Recent trials of combination chemotherapies and targeted therapeutic strategies including FOLFIRINOX, nab-Paclitaxel and the EGFR inhibitor, erlotinib, have shown additional survival benefit of only days to weeks, with increased toxicity that limits their use to otherwise fit patients (Conroy, 2011; Moore,

☆ Please see Brief Commentary by Tim D. Eubank found on page 38 of this issue.

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2007; Von Hoff, 2013). Development of improved therapeutic strategies to treat pancreatic cancer requires a more detailed understanding of the molecular and cellular mediators that influence pancreatic cancer growth and dissemination.

The limited success of existing therapeutic strategies may be due, in part, to our poor understanding of the effect of cross-talk between pancreatic tumor cells and their surrounding stroma on cancer progression (Feig, 2012; Demir et al., 2012; Vonlaufen, 2008). Pancreatic adenocarcinomas interact with a complex stroma that includes nerve fibers, infiltrating immune cells, endothelial cells, and pancreatic stellate cells that secrete abundant extracellular matrix (Demir et al., 2012; Vonlaufen, 2008; Apte et al., 2012; Hamada et al., 2013; Richins, 1945). These stromal cells exert a critical influence on pancreatic cancer progression (Demir et al., 2012; Vonlaufen, 2008; Apte et al., 2012; Hamada et al., 2013; Farrow et al., 2008). However, many therapeutic strategies were developed in cell-based and non-orthotopic disease models that fail to reflect the complexity of *in vivo* interactions (Feig, 2012; Johnson, 2001). Identification of new targets for novel therapies to slow or prevent pancreatic cancer requires studies in disease models that better recapitulate interactions between tumor cells and the pancreatic microenvironment.

The pancreas is innervated by fibers of the sympathetic nervous system (SNS), which regulate pancreatic functions including release of insulin by islet cells and release of digestive enzymes by acinar cells (Richins, 1945; Holmgren and Olsson, 2011). SNS fibers are activated during chronic stress and release catecholaminergic neurotransmitters that act on adrenoceptors to modulate cell behavior. β-adrenoceptors are present on pancreatic tumor cells and *in vitro* studies suggest that tumor cell behavior may be sensitive to β-adrenergic signaling (Guo, 2009; Schuller and Al-Wadei, 2010; Zhang, 2010). However, the effect of stress-induced β-adrenergic signaling on cancer progression within the complex pancreatic microenvironment has not been investigated. To address this we used an orthotopic model of human pancreatic cancer to investigate the effect of chronic stress on primary tumor growth and tumor cell dissemination within the pancreatic microenvironment.

2. Methods

2.1. Orthotopic pancreatic cancer model

The human pancreatic ductal adenocarcinoma cell lines Panc-1, HPAF-II and Capan-1 were obtained from the American Type Culture Collection, and maintained at 37 °C, 5% CO_2. These cell lines were chosen because they have mutated *TP53* and *KRAS*, which are common driver mutations in pancreatic cancer and because they range from well differentiated (Capan-1) to moderately and highly undifferentiated (HPAF-II and Panc-1, respectively) (Yachida, 2012). Panc-1 cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Bovogen Biologicals) and 1% penicillin–streptomycin (Sigma–Aldrich). HPAF-II cells were cultured in RPMI (Invitrogen) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin. Capan-1 cells were cultured in DMEM supplemented with 20% fetal bovine serum and 1% penicillin–streptomycin. To model human pancreatic cancer, 4×10^5 tumor cells in Matrigel (BD Bioscience) were injected into the tail of the pancreas of six week old female BALB/c-Foxn1nu nude athymic mice (The University of Adelaide, Australia) by laparotomy as previously described (Chai, 2013). Panc-1 was chosen for *in vivo* studies as tumors are poorly differentiated which is characteristic of patients who are diagnosed with advanced stage and grade (Chai, 2013; Hotz, 2003). To track tumor progression *in vivo*, cell lines were transduced with FUhLucW construct that expresses firefly luciferase under control of the ubiquitin C promoter (Morizono, 2005). Tumor progression was monitored longitudinally over 42 days by *in vivo* and *ex vivo* optical bioluminescent imaging using an IVIS Lumina ll system (Perkin Elmer) as described previously (Chai, 2013; Sloan, 2010). The presence of tumor cell dissemination beyond the tumor margins and into adjacent normal pancreas and metastasis to distant organs was measured by *ex vivo* optical imaging using long exposure times (>60 s), and confirmed by hematoxylin and eosin staining. Findings were validated in 2–4 independent experiments. All procedures were conducted in accordance with protocols approved by Institutional Animal Care and Use Committee of Monash University.

2.2. Chronic stress

Mice were randomly assigned to home cage control conditions (control) or 2 h per day restraint (stress) for 28 days commencing 7 days before tumor cell injection. Mice were restrained in a confined space that prevented them from moving freely but did not press on them (Thaker, 2006). This paradigm has been shown to induce chronic stress as shown by neuroendocrine activation (Thaker, 2006; Manni, 2008), weight loss (Smagin, 1999) (Supplementary Fig. 1), and anxiety-like behaviors (Hermann, 1994) but does not cause pain or wounding (Sheridan, 2004).

2.3. Pharmacological studies

For β-adrenergic antagonist studies 10 mg/kg/day (R/S)-propranolol (treatment) or water vehicle (placebo) was delivered to mice subcutaneously by osmotic minipump (Model 1004, Alzet). Propranolol was delivered for the duration of the experiment commencing seven days prior to tumor cell injection, with pumps replaced 24 days after implantation. Drug plasma concentration was assessed 20 days after pump implantation by UPLC-MS using a Micromass Quattro Premier coupled to an Acquity UPLC (Waters). For β-adrenergic agonist studies, 5 mg/kg/day (S/S)-isoproterenol (treatment) or 1 mM HCl vehicle (placebo) was delivered subcutaneously to mice via osmotic minipump (Model 1002, Alzet). Isoproterenol was delivered for 28 days, commencing seven days prior to tumor cell injection, with pumps replaced 14 days after implantation. Mice were maintained in their home cage for the duration of experiments that included isoproterenol treatment.

2.4. Invasion assay

2.5×10^5 pancreatic cancer cells in serum free culture medium were seeded into the top well of a transwell chamber with 8.0 μm pores (BD Falcon) that was coated with 15 mg/mL Matrigel. Cells were allowed to migrate towards medium containing 20% serum for 22 h and then stained with DAPI. Cells that had migrated to the underside of the membrane were counted.

2.5. Proliferation assay

The influence of isoproterenol on proliferation was assessed using the CellTiter 96® AQueous One Proliferation Assay (Promega). 8×10^3 cells were seeded into a 96-well plate and assayed over 120 h, according to manufacturer's instructions.

2.6. Gene expression studies

RNA was isolated from cell lines or primary pancreatic tumors using RNeasy Mini Kit (Qiagen). Transcript levels were quantified by RT-PCR using iScript One-Step RT-PCR kit (Biorad) and species-specific Taqman probes (Applied Biosystems) to identify

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