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Original article

Chronic stress increases experimental pancreatic cancer growth, reduces survival and can be antagonised by beta-adrenergic receptor blockade

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ABSTRACT

Background/objectives: Chronic stress could promote tumour growth and reduce survival of pancreatic cancer patients via beta-adrenergic receptors of tumour cells. We have tested the impact of chronic acoustic and restraint stress on tumour development in an orthotopic syngeneic murine model of pancreatic cancer.

Methods and results: Tumour-bearing C57BL/6 mice exposed to chronic stress had 45% ($p = 0.0138$) higher circulating steroid and 111% ($p = 0.0052$) higher adrenal tyrosine hydroxylase levels. Their immune response was significantly suppressed: The *in vitro* LPS response of splenocytes was significantly reduced regarding Th1- and Th2-cytokines including IFN-gamma, IL-6, IL-10 and MCP-1 ($0.0011 < p < 0.043$). Also, tumours of stressed mice showed a tendency towards fewer total CD4 cells, more regulatory T cells (Treg), less T cell/tumour cell contacts and a reduction of CTLA-4 in CD4 cells ($p > 0.05$). TGF-beta *in vitro* was increased by 23.4% using catecholamines ($p < 0.012$) and *in vivo* employing chronic stress ($p < 0.001$). After 5 weeks tumour volumes were 130% ($p = 0.0061$) larger and median survival reduced by 13.5% ($p = 0.0058$). Tumours expressed more VEGF ($p = 0.0334$), had greater microvessel densities ($p = 0.047$), and an increased MMP-9 expression ($p = 0.0456$). Beta-catecholamines increased proliferation in tumour cells by 18% ($p < 0.0001$) and migration by 78% ($p = 0.0348$) whereas the beta-blocker propranolol reduced these effects by 25% ($p < 0.0001$) and 53% ($p = 0.045$), respectively. When stressed tumour-bearing animals were treated with propranolol tumour volumes were reduced by 69% ($p = 0.0088$) and survival improved by 14% ($p < 0.0058$).

Conclusions: The potential treatment with beta-blockers of patients with pancreatic cancer or other malignancies should be further evaluated as an adjuvant anti-neoplastic agent in clinical trials.

Introduction

Cancer is the second leading cause of death in Europe and the United States with pancreatic carcinoma representing a particularly malignant variety. The latter one is responsible for more than 33,000 fatalities in the United States and more than 16,000 in Germany [1–3]. Despite intensive research on improved surgical and non-surgical treatment modalities the five year survival rate

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has remained at an unfavourable 5%. Many patients are diagnosed with late stage disease showing little long term effects of most therapeutic agents [4].

In addition to this present dilemma there is a large body of evidence that chronic stress can further impair the prognosis of malignancies [5–8]. Stress may lead to immunosuppression [9] and thereby influence the prognosis of cancer patients. Furthermore, stress hormones of the sympathetic nerve system may themselves stimulate tumour growth and invasiveness of tumour cells. This may be mediated via beta-adrenergic receptors present on tumour cells themselves [6,10]. Thereby, chronic stress could further worsen the prognosis of malignancies in general and pancreatic cancer in particular leaving the beta-adrenergic system as a possible target for therapies [6,8]. In the present study we have tested the impact of chronic stress on tumour development in an orthotopic syngeneic murine tumour model of pancreatic cancer [11–13].

Methods

Ethics statement

This study was carried out in strict accordance with the recommendations of and approved by the Ethics Committee for Animal Care of Mecklenburg-Vorpommern, Germany (permit number LALLF M-V TSD/7221.3-1.1-056/06). All surgery was performed under body weight adapted anaesthesia using intraperitoneally injected ketamine hydrochloride and xylometazoline hydrochloride. For all MRI studies anaesthesia was carried out using isoflurane. All efforts were made to minimise suffering as well as numbers of research animals. For survival studies humane endpoints were used according to the Ethics Committee for Animal Care of Mecklenburg-Vorpommern, i.e. animals with any signs of suffering were euthanized/sacrificed using intraperitoneal anaesthesia and finally cervical dislocation. Signs of suffering included but were not limited to obvious changes in behaviour including increasing lethargy; notable changes of fur/ruffled fur; limitation of movements/moving; obvious tumour growth leading to suffering and/or the above signs. A detailed list as supporting information.

For investigating human pancreatic cancer specimens written consent as well as Institutional Review Board ("Ethics Committee of the University Medicine Greifswald", formerly University Hospital Greifswald) approval (IRB study number/permit number III UV 84/03, March 3rd 2003 and amendment December 12th 2005) was obtained.

Laboratory animals

Six to eight weeks old male C57BL/6 mice with a body weight of 20–23 g were obtained from Charles River Laboratories (Bad Sulzfeld, Germany) and allowed to adapt to the new surrounding for seven to fourteen days. They were maintained in an open pathogen-free environment receiving food (ssniV Spezialdiäten GmbH, Soest, Germany) and water ad libitum. Stress levels were kept to a minimum before starting experiments. Animal rooms had a twelve to 12 h light–dark/day–night cycle and were maintained at constant temperature and humidity [11–13].

Cell lines and culture

The murine and human pancreatic adenocarcinoma cell lines 6606PDA [14], Panc02 [15] and Colo-357 [16] have been used previously [11–13]. BxPC-3, MIA PaCa-2, PANC-1 and PaTuS are human pancreatic cancer cell lines obtained from ATCC (ATCC, Manassas, Virginia, USA) or DSMZ (PaTuS: DSMZ, Braunschweig, Germany). TD1 and TD2 were kind gifts from Christoph Weber, Ulm University (TD1 and TD2 are murine pancreatic cancer cell lines institutionally established at Ulm University, Ulm, Germany). All cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin. Tissue culture reagents were obtained from Gibco (Invitrogen, Carlsbad, California, USA). Cell cultures were kept pathogen-free in a humidified incubator at 37 °C with 5% CO_2. Cell cultures were regularly tested for Mycoplasma species. They were consistently negative for mycoplasma contamination.

Transplantation of tumours into the head of the pancreas by orthotopic injection

All mice used during the experiments (i.e. control mice as well as stressed mice) had been orthotopically injected into the head of the pancreas with 6606PDA cells. This technique had already been described before [11–13].

Chronic stress model

To mimic patients' chronic stress in a murine setting we adapted Kiank's model [9] to our purposes. Primary stress experiments were carried out without tumour implantation; in tumour experiments stress sessions started three days after tumour implantation: Mice were exposed to combined acoustic and restraint stress. Two stress sessions were carried out per day, with 2 h of stress each session: morning sessions from 8 a.m. to 10 a.m. and afternoon sessions from 4 p.m. to 6 p.m. Restraint stress was induced by immobilising mice in 50 ml conical centrifuge tubes. These tubes contained multiple holes for ventilation. Special care was taken to avoid penning of tails pulling the tail through a whole of the tube's cap. All tubes containing mice were horizontally placed into a styrofoam rack exhibiting appropriate wholes for the tubes. The rack was positioned in front of the speaker of the ultrasound device. This device (ASC-100, ISOTRONIC Mezger KG, Horb/Neckar, Germany) induced acoustic stress by randomly emitting ultrasound waves using frequencies between 19 and 25 kHz. The volume of waves was also randomly changed between 0 and 35 dB. Ultrasound waves were emitted in random intervals and time periods to prevent adaptation of mice to acoustic stress. Between stress sessions mice were kept within their original cages. They had free access to food and tap water. Control mice (i.e. mice only injected with tumour cells but not receiving any form of stress) were kept isolated from stressed animals to avoid any impact of stressed on control mice and vice versa.

Stress severity score (SSS)

The observational Stress Severity Score (SSS) was originally established by Kiank et al. [9] and could be adapted for C57BL/6 mice. The parameters urination, normal defecation, diarrhoea, and muscle tone were evaluated as described. After placing the animal back into its home cage the condition of fur was recorded (0–2 points): normal fur (0), dorsal fur ruffled (1), whole animal's fur strongly ruffled (2). Lethargy (locomotor activity, explorative activity, grooming, 0–6 points), and muscle tone (0–3 points, see above) were also estimated: normal activity (0 points), slightly increased activity (1), increased exploration activity with short grooming intervals (2), more than 10 intervals of grooming in the first 2 min (3), only short breaks of grooming in the first 2 min (4), reduced activity and rare grooming intervals (5), lethargy and no activity (6). The SSS for each mouse finally consisted of points rated by the investigator after each stress cycle theoretically ranging from 0 to 29 points of maximum performance.

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