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Oncostatic effects of fluoxetine in experimental colon cancer models

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ABSTRACT

Colon cancer is one of the most common tumors in the human population. Recent studies have shown a reduced risk for colon cancer in patients given the antidepressant fluoxetine (FLX). The exact mechanism by which FLX might protect from colon cancer remains however controversial. Here, FLX reduced the development of different colon tumor xenografts, as well as proliferation in hypoxic tumor areas within them. FLX treatment also decreased microvessel numbers in tumors. Although FLX did not increase serum and tumor glucose levels as much as the colon chemotherapy gold standard Fluorouracil did, lactate levels were significantly augmented within tumors by FLX treatment. The gene expression of the MCT4 lactate transporter was significantly downregulated. Total protein amounts from the third and fifth mitochondrial complexes were significantly decreased by FLX in tumors. Cell culture experiments revealed that FLX reduced the mitochondrial membrane potential significantly and disabled the reactive oxygen species production of the third mitochondrial complex. Furthermore, FLX arrested hypoxic colon tumor cells in the G_0/G_1 phase of the cell-cycle. The expression of key cell-cycle-related checkpoint proteins was enhanced in cell culture and *in vivo* experiments. Therefore, we suggest FLX impairs energy generation, cell cycle progression and proliferation in tumor cells, especially under condition of hypoxia. This then leads to reduced microvessel formation and tumor shrinkage in xenograft models.

1. Introduction

The American Cancer Society estimates the number of new cases and expected deaths for cancer in the United States of America (USA) every year. Last reports have demonstrated colon cancer as the third most common cancer in the country with over 1.2 million diagnosed patients, where numbers *per* year achieved 150 thousand people expecting to be newly diagnosed and 50 thousand deaths [1]. Jemal et al. have projected a 60% increase for colon cancer diagnoses in developing countries until 2030 [2]. This highlights colon tumors as one of the major human malignancies worldwide, and a great challenge for cancer therapy.

Cancer research has shown close interactions among molecular signaling, energy generation machinery, and proliferation in tumors [3,4]. Such mechanisms enable malignant cells to proliferate quickly while high energy generation is maintained and negative feedback regulation from overusing glycolysis avoided [4,5]. Cancer cells thus hyperactivate lactate transporters alkalinizing their intracellular pH (ipH) which allows tumors to use oxidative phosphorylation together with glycolysis, since their mitochondrial membrane potential becomes hyperpolarized [4–8]. Actually, hypoxic tumor cells avoid hypoxia-induced and glycolysis-related acidosis by enhancing the hydration of carbon dioxide (CO_2) to bicarbonate which then promotes the activity of MCT4 lactate transporters in alkalinizing the ipH [5,6,8,9]. Impairing energy generation mechanisms would not only yield less tumor proliferation, but also decreased microvessel density [10].

Recent reports have suggested the antidepressant fluoxetine (FLX) reduces the ATP synthesis in neurons and glial cells [11–13]. Previously, we had shown that FLX, by reducing proliferation in two different cancer models, acts against the development of early colon carcinogenesis steps [14,15]. Our investigation also demonstrated that FLX treatment reduced angiogenesis-related stem cell differentiation processes in preneoplastic lesions by disrupting the stromal cellular proliferation in the colonic microenvironment [14]. Interestingly, other research groups

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reported that the risk for colon cancer was reduced about 45% in patients treated with FLX [16,17].

Considering that FLX clearly has oncostatic effects against colon carcinogenesis [14–19], we sought to investigate the earliest effects by which FLX reduces proliferation in colon tumors. While we had previously observed that FLX has antiproliferative effects in colon preneoplastic lesions from carcinogen-exposed rodents, we now found that FLX treatment reduced colon cancer cell proliferation impairing the tumor energy generation machinery in human xenograft models. Additionally, cell culture experiments were performed to support some of the mechanistic findings from the xenograft studies.

2. Material and methods

2.1. Cell culture conditions

HT29 and Caco-2 human colorectal adenocarcinoma cell lines were obtained from the American Type Culture Collection (ATCC; USA) and grown under standard conditions in DMEM (4.5 g/l glucose, 10% FBS, 1% L-glutamine, penicillin [100 units/ml] and streptomycin [0.1 mg/ml]) and Eagle's MEM medium (20% FSB, 1% L-glutamine, 1% Na-Pyruvate, 1% nonessential amino acids, penicillin and streptomycin). Cell cultures underwent normoxia (5% CO_2, 95% air, 90% humidity), and hypoxia (1% O_2 and 5% CO_2; incubator C42, Labotect GmbH, Germany) at 37 °C (further description is given in Supplementary mat. 1 to 3).

2.2. Xenograft transplantation studies

Nonobese diabetic, severe combined immunodeficient mice (NOD/SCID; Charles River, Germany; 5 weeks, 20 ± 2 g) were subcutaneously transplanted with Caco-2 or HT29 cells (1.5×10^6 cells *per* mice) in agreement with the protocol approved by the Internal Ethical Committee (no 121/2012). All mice were acclimated for 1 week before starting the experiment, and maintained under specific pathogen-free conditions.

2.2.1. First experiment

NOD/SCID mice bearing Caco-2 tumor xenografts were treated daily for 6 days with intraperitoneal (i.p.) injections of vehicle (saline, 100 μl; CTRL) or FLX (30 mg/kg/day; Sigma).

2.2.2. Second experiment

NOD/SCID mice bearing HT29 tumor xenografts were treated daily for 3 days with vehicle or FLX.

2.2.3. Third experiment

Mice bearing HT29 tumor xenografts were treated daily for 3 days with i.p. injections of vehicle, or FLX, or Fluorouracil (5-FU; 50 mg/kg/day, provided by the Department of Surgery I, Molecular Oncology and Immunology, University of Wuerzburg, Germany).

Tumor volume was monitored daily by measuring tumors with a caliper (formula, volume = πLWT/days; L, length; W, width; T, thickness). Relative tumor growth was determined as the ratio between initial (1st day treatment) and final (before sacrifice) tumor volumes *per* number of days. Mice were sacrificed under general anesthesia (1.5% Forane in 98.5% oxygen; 2 l/min) by cardiac puncture, and tumors were weighted. Serum was collected by centrifuging blood samples (835 *g*, 10 min, 4 °C). Tissue samples were snap-frozen in liquid nitrogen or within RNAlater RNA stabilization buffer (Qiagen, Germany) and TissueTek (Sakura, Germany), and kept at −80 °C. Tissue samples were also fixed in formalin (4%; 48 h) for histopathological and immunohistochemical analyses.

2.3. Histopathological analysis

Tumor slices were stained with H&E according to standard procedures [14,15] for pathological exam.

2.4. Immunohistochemistry analysis

According to previous description [14,15], IHC staining was performed on tissue sections (4 μm) incubated with anti-Ki67 (clone MM1 at 1:100) and anti-CD44 (clone NCL-CD44-2 at 1:100) primary antibodies overnight (Novocastra, USA). The brown color was displayed by incubating sections with Picture-MAX Polymer Kit (Invitrogen). In positive reactions a brown precipitate at the nucleus was detectable for Ki67, and in the cell membrane for CD44. Positive Ki67 and CD44 positive cells were counted manually throughout five randomly chosen microscopic fields in each sample. Calculations for either Ki67(+) or CD44(+) cells were done by determining a ratio between stained nuclei and counted fields (area = 3.15 mm^2). Necrotic areas of tumors were excluded from counting.

2.5. Computer-assisted three-dimensional (3D) reconstruction

Formalin-fixed tumors were embedded in paraffin, cut at 12.5 μm and stained according to standard H&E procedure. About 400 slices were serially obtained from half tumors, which were then photographed using a stereo microscope (Olympus SZH, Tokyo, Japan; conventional binocular lens [Olympus BH2, Tokyo, Japan]; Olympus®Zuiko automacro 50 mm 1:3.5 macro-lenses [Tokyo, Japan]; and, Canon® EOS 5D Mark II 21.1 Megapixel DSLR camera [Tokyo, Japan]). The obtained pictures were analyzed with the Amira® 5.4.1 software package (Visage Imaging GmbH, Germany) for 3D reconstruction.

2.6. Western blotting (WB)

A TissueLyser system was used to extract protein from tumor samples, according to the manufacturer's guideline (Qiagen, Germany). Protein extraction from cell culture experiments was carried out with direct lyses in 1× RIPA buffer. WB analysis was performed as described in NuPAGE Technical Guide (Invitrogen) for NuPAGE 4–12% Bis-Tris Mini Gels (Invitrogen) and iBlot Dry Blotting System (Invitrogen). Briefly, membranes were incubated at 4 °C overnight with antibodies from Cell Cycle/Checkpoint Antibody Sampler Kit (9917, Cell Signaling) and Cell Cycle Regulation Antibody Sampler Kit II (9870, Cell Signaling). Anti-β-actin antibody was used as an endogenous control (13E5, Cell Signaling). Data analysis was performed with ImageJ software (National Institutes of Health, NIH, USA).

2.7. Histopathological analysis for microvessel density (MVD)

Tumor microvessels were detected with double staining for cytochrome C oxidase (COX) and anti-CD31 antibody. COX reaction was performed as previously described by Sotgia et al. [20]. Briefly, cryostat sections (10 μm) were washed with 25 mM sodium phosphate buffer (5 min), and then incubated with the COX incubation mixture (2 h). Sodium phosphate buffer was used to stop reactions (5 min incubation). Afterwards, sections were fixed with 10% formalin, and washed with PBS. Blocking steps were made with biotin blocking kit (Dako, Germany) and 10% goat serum in PBS (1 h incubation for each one). Anti-CD31 antibody (clone 1A10 at 1:100; Novocastra, US) was applied overnight at 4 °C. Positive reactions were detected with the Vector Red Alkaline Phosphatase Substrate Kit I (Vector, US). Double-positive reactions were seen as brown cytoplasmic dots for COX, and light-red cytoplasmic staining for CD31. Double-stained microvessels were traced (CD31 and COX double stained cells aligned along of a microvessel wall) and enumerated within up to 10 different fields (area = 3.15 mm^2) for each tumor. Microvessel density (MVD) was determined as the ratio between microvessel numbers and counted areas.