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Effects of ropivacaine, bupivacaine and sufentanil in colon and pancreatic cancer cells *in vitro*

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ABSTRACT

The perioperative period is supposed to be a vulnerable period for cancer progression. Results of clinical studies indicate that the use of regional anesthesia can influence and improve oncological outcome of cancer patients. Uncontrolled cell proliferation and resistance to apoptotic cell death are important characteristics of solid tumors. The aim of this study was to investigate the effects of the clinically used local anesthetics ropivacaine or bupivacaine and the opioid analgesic sufentanil on cell proliferation, cell cycle distribution and apoptosis of colon (HT 29 and SW 480) and pancreatic (PaTu 8988t and PANC 1) cancer cell lines *in vitro*.

Cell proliferation was measured by Cell Proliferation ELISA BrdU Assay. Apoptosis was analyzed by annexin V staining and cell cycle distribution was detected by flow cytometry.

Ropivacaine, bupivacaine and sufentanil did not change apoptosis rate and cell cycle distribution in clinically concentration. Only high concentrations of ropivacaine or bupivacaine revealed antiproliferative potency.

Protective effects of epidural anesthesia observed in clinical studies seem not to be based on direct effects of these drugs on cancer cells.

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1. Introduction

In recent years evidence is growing that, during the perioperative period, the risk of cancer dissemination is increased [1,2]. Injury of tumor vessels and surgical manipulation enables cancer cells to enter the circulation. Enhanced levels of growth factors during the postoperative wound healing process as well as a dysbalance between pro- and antiangiogenetic factors can stimulate the growth of disseminated cancer cells and micro metastases. Furthermore, perioperative immunosuppression compromises anticancer immune surveillance. This fatal combination of released cancer cells, impairment of the immune function and high growth factor levels can enhance the risk for cancer recurrence during the perioperative period.

These findings arise the question if the choice of a special anesthesia technique could influence the risk of cancer recurrence [3,4]. Especially the effect of regional anesthesia on cancer progression was in the focus of clinical investigation over the recent years. Results of retrospective studies showed an association between the perioperative use of regional anesthesia during cancer surgery and better oncological outcome in breast [5], ovarian [6] and prostatic [7] cancer. A metaanalysis including 14 studies demonstrated a benefit of epidural anesthesia compared to general anesthesia alone regarding overall survival. The strongest positive association was found in colorectal cancer [8].

These protective effects of regional anesthesia seem to be based on the suppression of perioperative stress response by neuroaxial blockade [3]. Inhibition of the neuroendocrine stress axis can attenuate both immunosuppression and reduce sympathical activation caused by surgery. Decreased pain levels reduce the requirement of additional analgesic drugs and allow saving anesthetic agents during surgery.

The administration of the long acting local anesthetics ropivacaine or bupivacaine via epidural catheter is a common concept for perioperative pain management for patients undergoing major abdominal surgery. To improve analgesic effects, the opioid analgesic sufentanil usually is added to the local anesthetic agent. Local anesthetics reveal their analgesic effect by inhibition of voltage activated sodium channels. These local anesthetic sensitive sodium channels are expressed in several cancer types including breast,

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colon and prostate cancer and were shown to be associated with cancer metastasis [9,10]. Reduced expression of the voltage gated Nav1.5 channel suppressed cell proliferation and invasiveness and induced apoptosis in astrocytoma cells [11].

We hypothesized that the beneficial effects of regional anesthesia on cancer progression could be based on direct interaction between administered drugs and tumor cells themselves. For this reason, we investigated the effects of clinically achievable concentrations of ropivacaine, bupivacaine and sufentanil on cell proliferation, cell cycle distribution and apoptosis in colon and pancreatic cancer cell lines *in vitro*.

2. Materials and methods

2.1. Reagents

Commercially available ropivacaine (Fagron, Waregem, Belgium), bupivacaine (Fluka, Buchs Switzerland) and sufentanil (Sigma–Aldrich, St. Gallen, Switzerland) were used for this study. Stock solutions of the test reagents were prepared by dissolving the drugs in the ascertained standard growth media for ropivacaine and bupivacaine and standard growth media containing DMSO for sufentanil (as described below). The final concentrations were achieved by diluting the stock solutions with standard growth medium. All solutions were prepared immediately prior to use. For apoptosis and cell cycle analysis concentrations were chosen similar to clinically achievable plasma levels.

2.2. Cell lines

Four cancer cell lines were used in this study: two pancreatic carcinoma cell lines PANC 1 and PaTu 8988t were kindly contributed by Prof. Dr. Ellenrieder, University of Marburg and two colon carcinoma cell lines HT 29 and SW 480 were purchased form the German Collection of Microorganism and Cell Culture (DSMZ). Cells were grown in standard growth media, RPMI 1640 (Pan Biotech, Aidenbach, Germany) for SW 480 and DMEM (Sigma–Aldrich, St. Gallen, Switzerland) for PaTu 8988t, PANC 1 and HT 29. Culture media contained 10% FCS (Sigma–Aldrich) and 2 mM L-glutamine (Sigma–Aldrich) supplemented with 5% penicillin plus streptomycin (Sigma–Aldrich) (HT 29 and SW 480) or Myco Zap (Lonza Verviers SPRL, Verviers, Belgium) (PaTu 8988t and PANC 1). SW 480 culture media additionally contained 2 mM sodium pyruvate (Appli Chem, Darmstadt, Germany). Cells were cultured at 37 °C in a CO_2 atmosphere (5%) and maintained in monolayer culture. Experiments were performed when cells reached ~80% confluence.

2.3. Cell proliferation

Cell proliferation was analyzed using cell proliferation ELISA BrdU (Roche Applied Science, Mannheim, Germany), a proliferation test based on the detection of BrdU incorporation during DNA synthesis. The test was performed according the manufactures protocol. In brief, cells ($3–5 \times 10^3$) were seeded in 96-well plates (Costar, Bodenheim, Germany) and allowed to attach overnight. Cells were incubated with 100 μL of the test substances for 48 h (0.1–1000 μM ropivacaine and bupivacaine, 0.1–30 nM sufentanil). After 32 h incubation time cells were additionally treated with BrdU labeling solution for the remaining 16 h. After an overall treatment time of 48 h, culture medium was removed, cells were fixed and DNA was denatured. Cells were incubated with Anti-BrdU-POD solution for 90 min and antibody conjugates were removed by washing three times. After incubation with a TMB substrate for 15 min, absorbance at 405 and 490 nm was measured to detect immune complexes. All tests were performed in duplicates, eight wells per treatment group were used and tests were repeated at least twice.

2.4. Cell cycle analysis

Cell cycle distribution was measured by flow cytometry. Cancer cells were incubated with test reagents (10 μM ropivacaine, 5 μM bupivacaine, 1 nM sufentanil or the combination of 1 nM sufentanil with 1 or 10 μM ropivacaine) or vehicle for 24 h. 5 mM ASS was used for positive control and standard culture medium was used for negative control. Cells were detached by standard trypsination, counted (10^6 cells) and washed twice with cold PBS/5 mM EDTA. After fixing with 100% ethanol for 30 min at room temperature, cells were washed again with PBS/5 mM EDTA and treated with 1 mg/mL RNase A. After 30 min incubation time, cells were stained with 100 μg/mL propidium iodide (Sigma–Aldrich) and analyzed using a FACS Calibur (BD Bioscience, Heidelberg, Germany) and Cellquest™ Pro software (BD Bioscience). 10^4 cells were counted for each sample and all tests were performed in duplicates and repeated twice.

2.5. Apoptosis analysis

Cells ($3.5–5 \times 10^5$) were seeded in T 12.5 cm^2 cell culture flasks (BD Falcon, Heidelberg, Germany) and allowed to attach overnight. Cells were incubated with test reagents (10 μM ropivacaine, 5 μM bupivacaine, 1 nM sufentanil or the combination of 1 nM sufentanil with 1 or 10 μM ropivacaine). Standard growth medium was used for negative control and 1 μM staurosporine (Sigma–Aldrich) was used for positive control. After 0, 3, 6, 16 or 24 h incubation time, supernatant was decanted from the cells to preserve floating cells. Adherent cells were rinsed with warm (37 °C) PBS (Sigma–Aldrich) and harvested by standard trypsination. FITC Annexin V Apoptosis detection Kit (BD Pharming) was performed according to the manufacturer's protocol. In brief, the combined harvested and floating cells were mixed, washed twice with cold PBS and resuspended in binding buffer at a final concentration of 10^6 cells/mL. 5 μL of FITC Annexin and 5 μL of propidium iodide were added to 100 μL of the cell suspension containing 10^5 cells. The cell suspension was mixed by gently vortexing and then incubated for 15 min at room temperature in the dark. 400 μL of binding buffer were added and cells were analyzed by flow cytometry using FACS Calibur (BD Bioscience) and Cellquest™ Pro software (BD Bioscience). All tests were performed in duplicates and repeated twice. Cells staining positive for both annexin V and PI were defined to be apoptotic, necrotic cells were detected by positive staining for PI only and vital cells did dot stain.

2.6. Statistical analysis

Results are expressed as mean ± SD. For comparison between mean values the non-parametric Mann–Whitney-*U*-test was used. Differences were considered statistically significant at $p < 0.05$. IBM SPSS Statistics (V 20; IBM, New York, USA) and Excel 2010 (Microsoft, Redmond, USA) were employed for statistical analysis.

3. Results

3.1. Cell proliferation

In all cell lines, 1000 μM bupivacaine significantly inhibited cell growth (Fig. 1a). In PaTu 8988t 0.1–100 μM bupivacaine induced a slight but significant increase of cell growth.

1000 μM ropivacaine reduced cell growth in HT 29, SW 480 and PANC 1 cell lines (Fig. 1b). Lower concentrations of ropivacaine

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