The effects of analgesics and local anesthetics on gene transcription mediated by NFATc2 and Sp1 in pancreatic carcinoma

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Research article

Keywords: Pancreatic carcinoma, cancer, analgesics, transcription factors, NFATc2, Sp1

Posted Date: September 16th, 2019

DOI: <https://doi.org/10.21203/rs.2.14445/v1>

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Version of Record: A version of this preprint was published at Anticancer Research on September 1st, 2019. See the published version at <https://doi.org/10.21873/anticancer.13654>.

Abstract

Background Pancreatic adenocarcinoma is one of the most lethal cancers worldwide with very poor long-term survival. The treatment of choice next to chemotherapy or radiation treatment is surgical removal of the tumor. However, medication, surgery, and perioperative immunosuppression induce the constitutive activation of important signaling pathways and change the regulation of transcription factors, which may facilitate tumor progression and metastasis. Recent research has identified the transcription factors NFATc2 and Sp1 as key regulators in the carcinogenesis of pancreatic carcinoma. It is still unclear to what extent the transcription factors NFATc2 and Sp1 are influenced by analgesics given via peridural anesthetics or lidocaine infusions administered in perioperative settings or as postoperative pain therapy.

Aims To conduct an in vitro analysis of the impact of clinically achievable dosages of ketamine, s-ketamine, metamizole, and paracetamol as well as of sufentanil, ropivacaine, and lidocaine on pancreatic carcinoma cells in dependency of NFATc2 and Sp1.

Methods Analgesic stimulation and its effects on the expression of NFATc2 and Sp1 were investigated with immunoblot. Cell proliferation was measured with the ELISA BrdU assay. **Results** In PaTu8988t pancreatic carcinoma cells, 48h stimulation with ketamine and s-ketamine significantly inhibited proliferation and contemporaneously decreased endogen expression of NFATc2 in the nucleus. The addition of metamizole and lidocaine to PaTu8988t cells reduced proliferation after 48h.

Conclusions New treatment concepts target the efficient modulation of specific signaling and transcription pathways. The extent to which drugs influence these mechanisms in vulnerable phases of pancreatic carcinoma cells needs to be investigated in future studies. The basis of novel therapeutic approaches to any disease is detailed knowledge of the carcinogenesis and profound molecular and biological understanding of the mechanisms.

Background

Pancreatic adenocarcinoma is the fourth leading cause of cancer-related deaths worldwide [1]. This type of cancer is known for its extremely poor long-term survival rates of 5 years for only about 7% of all affected patients as well as for its almost identical incidence and mortality rates [2]. The treatment of choice next to chemotherapy or radiation treatment is surgical removal of the tumor [3]. However, research carried out in the past few years has shown that the perioperative period is a particularly vulnerable phase in which tumor progression and metastasis are facilitated [4]. Medication, surgery, and perioperative immunosuppression induce the constitutive activation of important signaling pathways and change the regulation of transcription factors [5]. Thus, it is hardly surprising that approximately 80% of affected patients show tumor recurrence even after surgery [2] and that the mean survival rate is reduced to approximately 9 months only [6].

Key elements in the carcinogenesis of pancreatic carcinoma are NFAT transcription factors [7]. NFAT is the abbreviation of 'nuclear factor of activated T cells' that was first described by Shaw et al. in 1988 [8].

The role of NFAT in T lymphocytes in which they control gene expression during cell activation and differentiation has been well documented. In addition, these proteins are also expressed in a wide range of cells and tissue types and regulate genes involved in cell cycle, apoptosis, angiogenesis, and metastasis [9]. As transcription factors, NFAT also control the expression of central genes involved in the control of growth and differentiation in pancreatic carcinoma [10]. Here, NFAT interact with the transcription factor Sp1 in the cells [11].

It is still unclear to what extent the oncogenic transcription factors NFATc2 and Sp1 are influenced by agents given during the perioperative period for anesthesia or pain therapy.

The purpose of this study was to conduct an in vitro analysis of the impact of clinically achievable dosages of the analgesics ketamine, sketamine, metamizole, and paracetamol as well as of sufentanil, ropivacaine, and lidocaine on the pancreatic carcinoma cell lines PaTu8988t in dependency of NFATc2 and Sp1.

Materials And Methods

Cell lines

The human pancreatic cancer cell lines PaTu8988t were obtained from Professor Ellenrieder (Philipps University of Marburg, Germany). PaTu8988t cells were maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% fetal calf serum (FCS) (Sigma-Aldrich) and 5% Myco Zap (Lonza Verviers SPRL). Cells were cultured in humidified CO₂ atmosphere (5%) at 37°C and maintained in monolayer culture. Experiments were done with cells at ~70–80% confluence.

Reagents

Commercially available ropivacaine (Fagron), sufentanil (Sigma-Aldrich), and lidocaine (Sigma-Aldrich) were used for this study. Ketamine and s-ketamine were purchased from Sigma-Aldrich, metamizole from Fluka, and paracetamol from Merck. Final concentrations were obtained by diluting drugs in standard growth media. All solutions were freshly prepared prior to use.

Subcellular fractionation and immunoblotting

For subcellular fraction in nuclear and cytoplasmic cell lysate, cells were washed twice with cold DPBS and re-suspended in Puffer A (200µl 1M Hepes KOH, 100µl 1M KCL, 100µl 1mM EDTA, 10µl 1mM DTT, proteinase inhibitors, and 9.5ml aqua dest) for 15min and centrifuged at 3.600rpm for 12min. The supernatant (cytoplasmic part) was transferred into Eppendorf cups, and the pellet (nuclear part) was dissolved in RIPA buffer (5mL Triton X100, 190mg EDTA, 0.5g SDS, 2.5g Deoxycolid Acid, 500mL DPBS, proteinase inhibitors) for 15min and centrifuged at 13.000rpm for 30min. Supernatants were transferred to new cups and incubated on ice. For Western blotting, 30µg protein extracts were analyzed by SDS-

PAGE and blotted onto nitrocellulose. Upon protein extraction and gel transfer, membranes were washed in TBS washing buffer and incubated with peroxidase-conjugated secondary antibodies. Immunoreactive proteins were visualized by means of an enhanced chemiluminescence detection system (Western Blotting Detection Reagent, GE Healthcare). For immunoblotting, membranes were probed with antibodies against NFATc2 (Cell Signaling), Sp1 (Cell Signaling), Lamin B (Santa cruz), and β -actin (Sigma-Aldrich).

Cell proliferation

Quantification of cell proliferation was based on the measurement of BrdU incorporation during DNA synthesis. The test was done according to the manufacturer's protocol (Cell proliferation ELISA BrdU, Roche applied science). In brief, cells were incubated with 100 μ L of the test compounds for 0h, 24h, 48h, and 72h. After 8h, 32h, or 56h of incubation, cells were additionally treated with BrdU labeling solution for the last 16h. The culture medium was removed, cells were fixed, and DNA was denatured. Afterwards, cells were incubated with Anti-BrdU-POD solution for 90min, and antibody conjugates were removed with three washing cycles. Immune complexes were detected by means of TMB substrate for 15min and quantified by measuring the absorbance at 405nm and 490nm. All tests were done in duplicates with eight wells per treatment group and were performed as three independent experiments.

Statistical analysis

Data are presented as mean $\pm$ SD. The non-parametric Mann Whitney U-test was used for the statistical evaluation of the data. P-values of <0.05 were considered significant. IBM SPSS Statistics (Vs. 22; IBM New York, US) and Excel Vs. 2013 (Microsoft, Redmond, USA) packages were employed for statistical analysis.

Results

Effects of ketamine and s-ketamine

PaTu8988t pancreatic cancer cells were stimulated with 5 μ M ketamine or 5 μ M s-ketamine for 0h, 24h, 48h, and 72h. Cell proliferation was significantly decreased after 48h stimulation with ketamine and s-ketamine (*Figure 1a*).

The protein expression of NFATc2 and Sp1 in PaTu8988t pancreatic carcinoma cells could be proven by means of Western blotting. Cells treated with FCS showed presence of NFATc2 in the nucleus. 24h and 48h stimulation with 5 μ M ketamine or 5 μ M s-ketamine reduced the expression levels of NFATc2 in the nucleus of pancreatic carcinoma cells. Concurrently, stimulation with ketamine increased the expression of NFATc2 in cytoplasm. After 72h stimulation, expression of NFATc2 in the cell nucleus was significantly increased again. The expression level of Sp1 remained unchanged, and Lamin B and β -actin served as a loading control (*Figure 1b*).

Effect of metamizole and paracetamol

PaTu8988t pancreatic cancer cells were stimulated with 75µM metamizole, 100µM paracetamol, or a combination of 75µM metamizole and 100µM paracetamol for 0h, 24h, 48h, and 72h each (*Figure 2a, 2b, 2c*). Proliferation was significantly inhibited after stimulation with metamizole for 48h. Paracetamol and the combination of 75µM metamizole and 100µM paracetamol did not significantly reduce cell growth (*Figure 2a*).

Western Blot analysis of samples incubated in metamizole, paracetamol, or the combination of metamizole and paracetamol showed no change in the expression of NFATc2 or Sp1. Column 3 depicts the endogenous expression of Lamin B and column 4 the expression of βactin that served as a loading control (*Figure 2b + 2c*).

Effect of ropivacaine, lidocaine and sufentanil

PaTu8988t pancreatic cancer cells were stimulated with 5µM ropivacaine, 1.5nM sufentanil, 5µM ropivacaine, and 1.5nM sufentanil or 10µM lidocaine for 0h, 24h, 48h, and 72h each (*Figure 3a + 3b + 3c*). In PaTu 8988t cell lines, stimulation with 10µM lidocaine for 48h resulted in a slight but statistically significant decrease in cell proliferation (*Figure 3a*). Stimulation for 0h, 24h, or 72h or use of the other test substances did not change the proliferation rate in comparison to untreated control (*Figure 3a*).

Western blot analysis did not yield any effects at the protein levels of NFATc2 and Sp1 after stimulation for 0h, 24h, 48h, or 72h. Furthermore, translocation of NFATc2 in the cell remained unchanged. Columns 3 and 4 depict the loading control (*Figure 3b + 3c*).

Discussion

Because of the different effects of medications given in the context of anesthesia and postoperative pain therapy of pancreatic carcinoma, such as ketamine, sketamine, metamizole, and paracetamol but also sufentanil, ropicavaine, and lidocaine, the administration of such medications requires careful consideration. The purpose of this study was to use drug concentrations in clinically achievable dosages to facilitate the transfer of experimental data to clinical practice.

As an NMDA receptor antagonist, ketamine in narcotic dosages causes profound analgesia; thus, this drug is not only successfully used for the management of cancer pain and opioid-refractory pain but also in preventive pain therapy [12,13]. Plasma concentrations achievable in clinical settings range between 0.1 and 0.42µg/ml ($\approx 2.3\text{--}9.5\mu\text{M}$) [14]. The main impact of ketamine and s-ketamine is based on the non-competitive blockade of the NMDA receptor complex. In the process, ketamine as well as its race mate s-ketamine bind to the binding site of phencyclidine (PCP) inside the NMDA channel, thus inhibiting the effect of NMDA antagonists [15]. This process results in the inhibition of the intracellular calcium concentration and in the inactivation of Ca²⁺-dependent cytosolic guanylate cyclase [16]. Several studies

have described the influx of calcium into the cell as a key trigger factor or a regulator of cellular processes relevant to tumor progression including proliferation and apoptosis [17]. Interestingly, 24h and 48h stimulation with ketamine and s-ketamine initially reduced NFAT transcription factors in the nucleus of pancreatic carcinoma cells PaTu8988t; at the same time, stimulation with ketamine increased NFATc2 in cytoplasm.

In dormant inactive cells, NFATc2 proteins are present in the cytoplasm in a phosphorylated form. The proteins have only a low affinity for DNA [18] and are activated by stimulation with FCS. The resulting intracellular increase in calcium activates the protein serine/threonine phosphatase calcineurin, which can thus bind to the PxxIT motifs located at the N-terminal of NFAT proteins. This way, NFAT proteins are dephosphorylated on 13 serine residues [19]. Due to the resulting conformational change, NFAT proteins are translocated into the nucleus and bind to specific DNA-binding sequences (GGAAA) [20]. NFAT proteins eventually interact with other transcription factors and exert their carcinogenic effect [21]. Both 24h and 48h stimulation with ketamine or s-ketamine seem to be able to inhibit this protein activation cascade. NFAT translocate into cytoplasm in a dormant stage, and cell proliferation decreases after stimulation for 48h.

The pyrazolone derivative metamizole (dipyrone) and the aminophenol derivative paracetamol (acetaminophen) are non-acidic, non-opioid analgesics [22,23]. The administration of these drugs is a key element of the WHO's cancer pain ladder [24] and an important part of postoperative analgesia [25]. Oral administration of 1g metamizole results in a maximum plasma concentration of $17.3 \pm 7.5 \text{ mg/l}$ ($\approx 50\text{--}75 \mu\text{M}$ metamizole) and the intravenous injection of 1g paracetamol in a plasma concentration of $95 \pm 36 \mu\text{M}$ [26,27]. According to the literature, metamizole and paracetamol primarily inhibit cyclooxygenase activity (COX), thus influencing prostaglandin synthesis as the central regulator of inflammation and inhibiting the transformation of arachidonic acid into endoperoxide, the preliminary stage of prostaglandin, thromboxane A₂, and prostacyclin [28,29].

In this study, cell proliferation in PaTu8988t pancreatic carcinoma cells was inhibited by the administration of metamizole. Paracetamol had already shown a slight but significant anti-proliferative effect also in PaTu8988t and Panc-1 pancreatic carcinoma cells in an earlier study [30]. Stimulation with metamizole and paracetamol did not change the level of expression of NFATc2 or Sp1.

Recent study results have shown that COX-2 inhibitors increase Sp1 protein degradation [31]. Tolfenaminic acid is a non-steroidal anti-inflammatory drug (NSAID) that additionally activates the degradation of Sp1, Sp3, and Sp4 and decreases the expression of several Sp-regulated growth-promoting, angiogenic, survival, and inflammatory gene products [32]. These characteristics are of particular significance given the long existing assumption that Sp1 expression is a key factor in tumor development, growth, and metastasis. In some types of cancers, Sp1 overexpression is associated with poor survival [33].

Ropivacaine and lidocaine are amide local anesthetics [34] that block the voltage-gated sodium channels of neuronal axons. Here, the local anesthetic binds to the inside of the inactivated sodium channel, thus impeding the fast sodium influx into the cell that is important for depolarization [35]. The conduction of stimuli in the nerve is inhibited, thus stopping the transmission of pain [36]. Several studies have shown that plasma concentrations of 0.61–4 µg/ml ($\approx$ 1.6–10.9 µM) are achievable with ropivacaine and 1–5 µg/ml ($\approx$ 2.3–11.5 µM) with lidocaine. [37,38]. In peridural anesthesia, ropivacaine is often combined with the opioid sufentanil [39]. As a pure agonist, sufentanil binds to the opioid receptors of the nervous system [40] and has been proven to improve the quality of analgesia. In peridural anesthesia, the addition of opioids to local anesthetics results in a faster onset of effects and reduces the dosage of the individual drugs [41]. Plasma concentrations achievable with sufentanil are 0.40+/-0.14 ng/ml ($\approx$ 1.5 nM) [42]. This phenomenon can also be observed in the case of intravenous injections of lidocaine in large abdominal surgical interventions in contrast to singular general anesthesia. The reduction in peri- and postoperative pain significantly decreases the requirement of anesthetics and opioid analgesics [43]. In our study, the administration of lidocaine decreased proliferation after 48h, but ropivacaine, sufentanil, and lidocaine had no effect on the expression of the transcription factors NFATc2 and Sp1 in pancreatic carcinoma cells.

Conclusions

Pancreatic adenocarcinoma is one of the most aggressive cancers worldwide. Its oncogenic potential is mainly marked by extremely fast growth triggered by the activation of important signaling cascades during vulnerable phases. Thus, new therapeutic concepts also target the efficient modulation of specific signaling and transcription pathways. A wide variety of inhibitors is being investigated in the context of preclinical studies or is currently being established in clinical practice [34,35]. One possible novel therapeutic concept for pancreatic carcinoma cells is the inhibition of the interaction between NFATc2 and Sp1. The extent to which medication influences mechanisms in vulnerable phases of pancreatic carcinoma needs to be investigated in future studies. The basis of novel therapeutic approaches to any disease is detailed knowledge of the carcinogenesis and profound molecular and biological understanding of the mechanisms.

Abbreviations

Ca²⁺calcium

COXcyclooxygenase

DNAdeoxyribonucleic acid

FCSfetal calf serum

NFAT nuclear factors of activated T-cells

NMDAN-methyl-D-aspartate

NSAIDnon-steroidal anti-inflammatory drug

PCPphencyclidin

Sp1specificity protein 1

WHOworld health organization

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The data set supporting the conclusions of this article is included in the article.

Competing interests

The authors declare that they have no competing interests.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Author contributions

All authors have made substantial contributions to the conception, design, analysis, and the interpretation of this research article. They have been involved in the critical revision of the manuscript with regard to important intellectual content. All authors have given their final approval for the version to be published and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Acknowledgements

We thank Sigrid Bamberger, Regina Lindner, Gabriele Bollwein, Marion Schindler, Ruth Spaeth and Michael Gruber for technical assistance. We thank Monika Schoell for linguistic support.

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Figures

Figure 1

The effect of ketamine and s-ketamine on the proliferation (a) and endogenic expression of NFATc2, Sp1, Lamin B, and β-actin (b) in PaTu8988t pancreatic cancer cell lines after incubation for 0h, 24h, 48h, and 72h. The proliferation rate was determined by means of proliferation BrdU assays. 100% correspond to untreated control. (*) p <0.05 in comparison to untreated control.

Figure 2

a The effect of metamizole, paracetamol, and the combination of metamizole and paracetamol on PaTu8988t pancreatic carcinoma cell proliferation after stimulation for 0h, 24h, 48h, and 72h. The

proliferation rate was detected by means of BrdU uptake. (*) indicate statistical significance at $p < 0.05$ compared to untreated control. b + c: Immunoblotting and proof of the endogenous expression of NFATc2, Sp1, Lamin B, and β -actin after stimulation with metamizole, paracetamol, (b) and the combination of metamizole and paracetamol (c) in PaTu8988t pancreatic cancer cells for 0h, 24h, 48h, and 72h.

Figure 3

a: The effects of ropivacaine, sufentanil, the combination of ropivacaine and sufentanil, and lidocaine on cell proliferation in PaTu 8988t pancreatic carcinoma cell lines in vitro. Cell proliferation was quantified by measuring BrdU incorporation. (*) indicate statistical significance at $p < 0.05$ compared to untreated control. b + c: Western blot analysis and proof of the endogenous expression of NFATc2, Sp1, Lamin B, and β -actin after stimulation with ropivacaine, sufentanil (b), the combination of ropivacaine, sufentanil, and lidocaine (c) in PaTu 8988t pancreatic cancer cells for 0h, 24h, 48h, and 72h.