Influence of Paclitaxel and Heparin on Vitality, Proliferation and Cytokine Production of Endometrial Cancer Cells

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Key words
endometrial cancer, paclitaxel, heparins, cytokines, apoptosis, proliferation

ABSTRACT
Background Cancer patients have a higher risk for thromboembolic events compared to healthy individuals and are often treated with heparins. A beneficial effect of heparins on tumor patients above and beyond the classic anticoagulation effect has been reported, leading to an increased focus on the use of heparins in anticancer treatment. In recent years, it has become apparent that microenvironments greatly affect tumor development and can be a major source of tumor-promoting factors. Cytokines play an important role in tumor microenvironments, inducing carcinogenesis and influencing tumor progression by promoting angiogenesis, metastatic potential and immunosuppression. The possible interaction of heparins and cytokines could also have an effect on cancer cells.

Methods This study investigated the effect of paclitaxel (PTX) combined with heparins on the vitality of endometrial cancer cells using viability and cytotoxicity assays. The study also examined whether treatment with paclitaxel and heparin influences cytokine secretion or expression.

Results Heparin treatment did not influence cell viability, and no influence of heparins in combination with paclitaxel was seen for the evaluated cancer cell lines HEC-1-A, KLE, RL 95-2 and AN3-CA compared to untreated cells. Secretion of the cytokines CCL5, CCL2 and IL-6 increased after paclitaxel treatment in several endometrial cancer cell lines, but no general effect on cytokine secretion was detected after heparin treatment. A significant decrease in CCL5 expression was only detected in KLE cells following treatment with heparin and paclitaxel, and an increase in the expression of CCL5 in RL 95-2 cells.

Conclusion Further in-depth studies are needed to investigate the functions of cytokines CCL2, CCL5 and IL-6 in endometrial cancer cells treated with paclitaxel. Although no general effect on cytokine secretion was detected following heparin treatment, a selective modulatory impact could exist.
Introduction

Endometrial cancer is the most common gynecologic malignancy with a peak incidence between the ages of 55 and 65 years [1]. Around 142 000 women are affected by endometrial cancer worldwide every year [1]. Endometrial cancer is frequently diagnosed at an early stage as affected women often present with abnormal vaginal bleeding. In its early stages endometrial cancer can be treated surgically with intent to cure [2]. Adjuvant radiation therapy or adjuvant chemotherapy have been shown to improve the outcome of patients with high-risk endometrial cancer [3].

Around 13% of all patients with endometrial cancer develop recurrent disease [2]. Different therapeutic modalities including radiotherapy and surgery and systemic therapies such as chemotherapy and hormone therapy are used to treat recurrent endometrial cancer [3]. Clinical trials evaluating chemotherapy regimens in patients with endometrial cancer include combinations of doxorubicin and cisplatin, cyclophosphamide or paclitaxel and carboplatin, most of them administered in a palliative setting [3]. These systemic treatment options are often accompanied by high toxicity.

Cancer patients have a generally higher risk for thromboembolic events compared to healthy individuals and are often treated with heparins [4]. Different studies have reported a synergistic effect of heparins above and beyond their standard anticoagulation effect in tumor patients; this has led to an increased focus on heparins in anticancer treatment. Lapiere et al. reported that treatment with heparin inhibited tumor growth of pancreatic adenocarcinoma in a mouse model [5]. Other studies found no antiproliferative effect but did report an inhibition of metastatic potential [6, 7].

To develop new strategies for further therapeutic interventions, a detailed understanding of the molecular mechanisms in endometrial cancer is necessary. In recent years, it has become apparent that microenvironments greatly affect tumor development, because they are a major source of tumor-promoting factors for a huge range of tumors [8]. It is known that cytokines play an important role in tumor microenvironments: TNF-alpha and IL-6, for example, are proinflammatory cytokines which also induce carcinogenesis [9]. High serum levels of TNF-alpha, IL-6 and IL-8 are often detected in cancer patients, and very high IL-6 levels seem to be associated with a poor prognosis [10]. The CC-chemokine ligands CCL2 and CCL5 influence tumor progression by promoting angiogenesis, metastatic potential and immunosuppression [11, 12].

Spratte et al. showed that heparins inhibited TNF-alpha signaling in endometrial stromal cells [13]. The possible interaction between heparins and cytokines could also affect cancer cells.

Doster and co-workers reported a selective modulatory impact of unfractionated heparin on the expression of cytokines (CXCL8, CCL2 and CCL5) in endometrial cancer cells [14].

Based on these findings, this study aimed to investigate the effects of treatment with paclitaxel and heparins on the vitality, procliferation and cytokine secretion of endometrial cancer cells.

Material and Methods

Endometrial cancer cell lines

Endometrial cancer cells were obtained from ATCC (Manassas, VA, USA). HEC-1-A were cultivated in McCoy’s medium (Biochrom AG, Berlin, Germany). KLE and RL 95-2 were cultivated in DMEM/F12 medium (Invitrogen, Karlsruhe, Germany) and AN3-CA were cultivated in MEM Earle’s medium (Invitrogen). All media contained 10% fetal bovine serum and 50 µg/mL gentamycin (Invitrogen) and cells were cultivated at 37 °C in a 5% CO2 humidified atmosphere.

Cell lines differ in their grading and pattern of metastatic spread; the cells of HEC-1 A and RL 95-2 are moderately differentiated and originate from the epithelial layer of the endometrium. KLE and AN3-CA are poorly differentiated cell lines of endometrial adenocarcinoma, with AN3-CA originating from lymph node metastasis [15].
Cell lines were cultured 48 h prior to the different experiments. Different numbers of cells were used for all experiments as follows: 50,000 RL 95-2 cells, 30,000 HEC-1A cells, 15,000 KLE cells and 40,000 AN3CA cells.

**Viability assay**

Endometrial cancer cells of all four cell lines were treated for up to 144 h with 0.1 nM or 0.1 µM paclitaxel and/or 0.1 U/mL, 1 U/mL or 10 U/mL unfractionated heparin (UFH) or low-molecular-weight heparin (LMWH) (dalteparin/enoxaparin/reviparin/fondaparinux). All experiments were performed under normoxic (21% O₂) and hypoxic (1–5% O₂) conditions.

The relative number of viable endometrial cancer cells influenced by the indicated agents (paclitaxel and/or heparin) was measured at the end of each respective experiment (every 24 h between 0 h and 144 h) using the CellTiter-Blue assay (Promega, Madison, WI, USA) in accordance with the manufacturer’s instructions. Fluorescence was recorded using the FLUOstar OPTIMA system (BMG Labtech, Offenburg, Germany). Untreated cells were used as controls. Excitation was measured at 560 nm and emission at 590 nm.

**Cytotoxicity assay**

Endometrial cancer cells of the four cell lines were treated for up to 144 h with 0.1 nM or 0.1 µM paclitaxel and/or 0.1 U/mL, 1 U/mL or 10 U/mL UFH. All experiments were performed under normoxic and hypoxic conditions. The number of viable endometrial cancer cells influenced by the indicated agents (paclitaxel and/or heparin) was measured at the end of each respective experiment using the CytoTox-ONE homogeneous membrane integrity assay (Promega) in accordance with the manufacturer’s instructions. The test was used to detect cytotoxicity when cytokine secretion was evaluated to differentiate between the active cytokine secretion of vital cells and passive cytokine release through cell death.

**Enzyme-linked immunosorbent assay**

Endometrial cancer cells were treated with different doses of paclitaxel (0.01 µM, 0.1 M or 1 µM) for 24 h and 72 h. At the end of each time period, enzyme-linked immunosorbent assays (ELISAs) were performed to detect cytokine secretion, using commercial ELISA kits to detect human IL-1β, IL-6, CCL2, CCL5 (R & D Systems, Wiesbaden, Germany) and TNF-alpha (eBioscience, Frankfurt, Germany) in accordance with the manufacturer’s instructions. Absorbance values were measured with the Fluostar Optima system and normalized to the relative number of viable endometrial cancer cells.

**Real-time reverse transcription polymerase chain reaction**

To detect whether paclitaxel treatment alone influences the secretion of cytokines or cytokine expression, real-time reverse transcription polymerase chain reaction was performed. Endometrial cancer cells lines RL 95-2 and HEC-1A were treated with 0.01 µM, 0.1 µM or 1 µM paclitaxel for 72 hours. Untreated cells were used as controls.

Total ribonucleic acid (RNA) was isolated from endometrial cancer cells using peqGOLD Trifast (Peqlab) and reverse-transcribed using the high capacity cDNA archive kit (Applied Biosystems, Foster City, CA, USA).

Semi-quantitative real-time polymerase chain reaction (PCR) was performed to quantify the mRNA levels of CCL5 in relation to the housekeeping gene β-actin. cDNA samples were amplified with the Power Sybr Green PCR Master Mix (Applied Biosystems) and the respective forward and reverse primers. The primers (Invitrogen) were designed using Primer Express primer design software, version 2.0 (Applied Biosystems), with the resulting amplimers having an intron-overlapping sequence. The sequences of the primers used are summarized in **Table 1**.

PCR amplification was performed in duplicate in an ABI Prism 7300 sequence detector (Applied Biosystems) using a standard cycling program [16]. PCR products were analyzed by thermal dissociation to verify that a single specific PCR product had been amplified. Relative expression levels of CCL5 in relation to β-actin were determined using the mathematical model: ratio: ¼ 2DDCT [17].

**Statistical analysis**

Statistical analysis was done by one-way ANOVA, followed by Dunnett’s and Bonferroni multiple comparison tests or unpaired Mann-Whitney t-tests, using GraphPad PRISM version 5.01 software (GraphPad Software Inc., La Jolla, CA, USA). P < 0.05 was considered to indicate a statistically significant difference.

**Results**

**Viability assay**

After treatment with UFH or LMWH no influence on the cell viability of the four different endometrial cancer cell lines could be demonstrated compared to untreated cells. As expected, the toxic PTX dose showed a decreasing signal in the CellTiter-Blue assay, which could not be influenced by combining PTX with heparins. Hypoxic conditions did not change the results demonstrated under normoxic conditions (data not shown).

**Viability and cytotoxicity after treatment with paclitaxel**

All four evaluated endometrial cancer cell lines showed a decrease in vital cells after treatment with paclitaxel compared to untreated cells. No significant change in membrane integrity was measured in the CytoTox-ONE assay after treatment with paclitaxel (data shown in **Fig. 1**).
Basal cytokine secretion in untreated endometrial cancer cells

Basal secretion of IL-1β and TNF-alpha could not be detected in the four different cell lines due to values below the detection limit of the commercial ELISA kits used.

CCL5 secretion was shown in all four cell lines; the highest secretion was detected in HEC-1A cells (61 pg/mL/10^4 cells). IL-6 was secreted by KLE, RL-95 and HEC-1A; the highest secretion was detected in KLE cells (18.6 pg/mL/10^4 cells). Only in AN3-CA cells was the secretion level too low to be detected.

CCL2 secretion was measured in RL-95, HEC-1A and KLE cells; in AN3-CA cells the secretion levels were again too low to be detected (data shown in ▶ Table 2).

Cytokine secretion after treatment with paclitaxel

CCL5 secretion increased 72 h after paclitaxel treatment in three of the examined cell lines. In HEC-1A and RL 95-2 cells, secretion changed significantly for all three doses of paclitaxel (0.01 M; 0.1 µM; 1 µM). In KLE cells secretion was reduced under paclitaxel treatment, but the results were only significant for 1 µM paclitaxel.

Secretion of IL-6 increased significantly after paclitaxel treatment in RL 95-2 cells. Treatment with 0.1 µM paclitaxel led to a 52-fold increase in secretion.

In HEC-1A cells the increase in secretion was only significant for 0.01 µM and 0.1 µM paclitaxel. In KLE cells, the secretion of IL-6 did not increase significantly and the secretion levels of IL-6 in AN3-CA cells were below the detection limit.

CCL2 secretion after paclitaxel treatment only showed a significant increase for HEC-1A cells (data shown in ▶ Table 3).

Influence of paclitaxel on cytokine expression of endometrial cancer cells

To determine whether paclitaxel treatment only affects the secretion of cytokines or whether it also affects cytokine expression, real-time reverse transcription polymerase chain reaction was performed.

In RL 95-2 cells, CCL5 mRNA expression increased 5.3-fold following treatment with 0.01 µM paclitaxel. The other paclitaxel doses resulted in no significant changes in the mRNA expression of CCL5. In HEC-1A cells, the increase in CCL5 mRNA expression was even higher; treatment with 0.1 µM paclitaxel resulted in

▶ Fig. 1 Cytotoxicity assay for all four evaluated cell lines. No significant difference in membrane integrity was measured after treatment with paclitaxel (PTX). a AN3-CA, b HEC-1A, c KLE, d RL 95-2.
a 58-fold increase in expression, and treatment with 0.1 µM paclitaxel increased expression 42-fold (data shown in ▶ Fig. 2).

**Influence of paclitaxel combined with heparin on cytokine expression of endometrial cancer cells**

In KLE cells, a statistically significant decrease in CCL5 expression was detected after treatment with paclitaxel and UFH.

In RL 95-2 cells, CCL5 expression increased after treatment with paclitaxel and UFH.

No significant differences were noted for the other examined cell lines (data not shown).

**Discussion**

Endometrial cancer is often detected in its early stages and can be treated and cured. But for patients with advanced or recurrent disease the therapeutic options are poor, and new strategies will have to be evaluated. Antitumor research has begun to focus on heparins because different studies have shown that cancer patients treated with heparins to prevent thromboembolic events lived longer than cancer patients who did not receive anticoagulation treatment. In-vitro models and animal models showed an inhibition of tumor growth or metastatic potential following heparin treatment [5 – 7].

This study aimed to evaluate the effect of paclitaxel combined with heparins on the vitality of endometrial cancer cells. The study also investigated whether paclitaxel and heparin treatment affects cytokine secretion or expression. Cytokines play a pivotal role in tumor microenvironments and influence the activation and migration of immune cells [8]. The chemokines CCL2 and CCL5 influence tumor progression by promoting angiogenesis, metastatic potential and immunosuppression [11, 12]. Higher serum levels of IL-6 have been found in breast cancer patients compared to healthy individuals, and higher serum levels correlated strongly with tumor stage and lymph node metastasis [18]. Ma et al. reasoned that serum levels might help to identify patients with a poor prognosis [18]. Another study showed that CCL2 and CCL5 are highly expressed by breast cancer cells compared to regular breast epithelial cells, suggesting that both cytokines play a role in the development and progression of breast cancer. It could
be demonstrated that both chemokines promote a pro-malignant phenotype of cancer cells. These findings suggest that CCL2 and CCL5 could be potential therapeutic targets in breast cancer treatment [19, 20].

In our study, treatment with paclitaxel was found to affect the expression and secretion of CCL2, CCL5 and IL-6. An increase in the secretion of CCL2, CCL5 and IL-6 was detected; this could promote angiogenesis and metastatic potential but could also be a sign of the migration of immune cells into the tumor microenvironment. But the results and changes in secretion or expression varied considerably between the different cell lines, and further evaluations are needed to analyze the impact of heparins on the molecular size and negative charge. This could be an interesting starting point for further studies of heparins in the setting of endometrial cancer.

No antiproliferative effect of heparins was detected in our study and there was no increase in the antimitotic effect of paclitaxel when it was combined with heparin. Heparins do not appear to influence cytotoxic treatment negatively either.

Further studies are needed to investigate the function of cytokines CCL2, CCL5 and IL-6 in endometrial cancer cells treated with paclitaxel. Studies into mechanistic processes and regulations will be necessary to determine the purposes of the different cytokines in the setting of endometrial cancer.

Although no general effect of heparin treatment on cytokine secretion was detected, a selective modulatory impact could exist and further evaluations are needed to analyze the impact of heparins on tumor microenvironments.

Conflict of Interest

The authors declare that they have no conflict of interest.

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