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Combined expression of KLK4, KLK5, KLK6, and KLK7 by ovarian cancer cells leads to decreased adhesion and paclitaxel-induced chemoresistance

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HIGHLIGHTS

- We have elaborated on functional roles of ovarian cancer-specific kallikreins due to regulation of integrin expression and cell adhesion.
- Paclitaxel-insensitivity of KLK-expressing cancer cells was accompanied by differential expression of drug-resistance-genes, indicative of disease progression.
- These key findings provide new mechanistic insight into peritoneal spread and chemoresistance of advanced ovarian cancer.

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ABSTRACT

Objective. Chemoresistance is a critical feature of advanced ovarian cancer with only 30% of patients surviving longer than 5 years. We have previously shown that four kallikrein-related (KLK) peptidases, KLK4, KLK5, KLK6 and KLK7 (KLK4–7), are implicated in peritoneal invasion and tumour growth, but underlying mechanisms were not identified. We also reported that KLK7 overexpression confers chemoresistance to paclitaxel, and cell survival *via* integrins. In this study, we further explored the functional consequenses of overexpression of all four KLKs (KLK4–7) simultaneously in the ovarian cancer cell line, OV-MZ-6, and its impact on integrin expression and signalling, cell adhesion and survival as contributors to chemoresistance and metastatic progression.

Methods. Quantitative gene and protein expression analyses, confocal microscopy, cell adhesion and chemosensitivity assays were performed.

Results. Expression of α5β1/αvβ3 integrins was downregulated upon combined stable KLK4–7 overexpression in OV-MZ-6 cells. Accordingly, the adhesion of these cells to vitronectin and fibronectin, the extracellular matrix binding proteins of α5β1/αvβ3 integrins and two predominant proteins of the peritoneal matrix, was decreased. KLK4–7-transfected cells were more resistant to paclitaxel (10–100 nmol/L: 38–54%), but not to carboplatin, which was associated with decreased apoptotic stimuli. However, the KLK4–7-induced paclitaxel resistance was not blocked by the MEK1/2 inhibitor, U0126.

Conclusions. This study demonstrates that combined KLK4–7 expression by ovarian cancer cells promotes reduced integrin expression with consequently less cell–matrix attachment, and insensitivity to paclitaxel mediated by complex integrin and MAPK independent interactions, indicative of a malignant phenotype and disease progression suggesting a role for these KLKs in this process.

Introduction

Ovarian cancer is the most common malignancy among gynaecological cancers and is the major cause of all female cancer deaths [1,2]. The predominant cancer type is epithelial ovarian carcinoma (EOC). More than 75% of ovarian cancer patients are diagnosed at a late-stage with evidence of metastatic spread within the peritoneal cavity and pelvic region. Cytoreductive surgery and chemotherapy is the standard treatment for advanced ovarian cancer. Although platinum–taxane combination therapies have improved, the 5 year survival rate of patients is only 30%, with the overall mortality due to relapse and chemoresistance is unchanged for decades. The main reasons for unsuccessful therapies are a limited understanding of

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the heterogeneous pathobiology of this disease, the mechanisms underlying drug resistance and a limited number of prognostic and therapeutic biomarkers [2–4].

Currently, the serum biomarker CA125 is clinically used to indicate the presence of ovarian cancer, although not all patients have elevated CA125 levels and its detection can result in false positives. Due to the low specificity and sensitivity, its use for monitoring treatment responses and predicting prognosis after treatment is limited. Therefore, novel ovarian cancer-specific serum biomarkers are needed to detect this disease and to follow up patient outcomes [5]. Cancer-related proteases, such as kallikrein-related peptidases (KLK; proteins encoded by *KLK* genes), are under investigation as potential diagnostic indicators for ovarian cancer and prognostic biomarkers for disease progression [6–9]. Among the fifteen human KLKs, twelve, including *KLK4*, *KLK5*, *KLK6*, and *KLK7* (*KLK4–7*), are upregulated in cancerous ovarian tissues, serum and ascitic fluid from patients, and cell lines [6,10,11]. KLK5 serves as a biomarker for early detection of ovarian cancer and its levels in serum or ascitic fluid did not correlate with the established cancer biomarker CA125, thus it provides independent information from CA125 in this disease [12]. *KLK4–7* overexpression is associated with late-stage and high-grade disease, shorter disease-free and overall survival rates [12]. Elevated serum levels of KLK4–7 are associated with non-responsiveness of ovarian cancer patients to paclitaxel [9,12–14]. Both *KLK*/KLK4 and *KLK*/KLK7 are also associated with multicellular aggregation and paclitaxel resistance in ovarian cancer *in vitro* [13,14]. KLKs likely play a crucial role throughout the progression of ovarian cancer by contributing to invasion, metastasis and modification of the tumour microenvironment *via* degradation and/or activation of extracellular matrix (ECM) and other proteins leading to altered cell–cell/cell–ECM interactions, ECM remodelling, cell survival and differentiation [15]. However, the precise signalling pathways involved are not yet fully elucidated.

Cell–cell/cell–ECM adhesion molecules, such as integrin cell adhesion and signalling receptors are also critical factors in tumour progression. Integrin signalling is involved in multiple functions of tumour cells, such as adhesion, invasion, proliferation, survival and apoptosis, thereby affecting tumour growth and metastasis [16]. Integrins are non-covalently associated heterodimeric cell surface receptors promoting bidirectional signal transduction *via* preferential binding to distinct ECM proteins, such as fibronectin, vitronectin and collagens. Although transmembrane integrins lack kinase activity, by clustering they recruit and activate kinases, such as focal adhesion kinase (FAK) and src family kinases (SFK), to form focal adhesions and crosstalk with other cell surface receptors, thereby activating the MAPK signalling cascade [17]. Of particular interest, it has been reported for various cancer types including ovarian cancer, that integrins mediate tumour–ECM interactions, like matrix adhesion and alteration of apoptotic mediators, leading to drug resistance [18]. Different integrins contribute to tumour progression and chemoresistance of ovarian cancer. For example, $\alpha v\beta 3$, $\alpha 5\beta 1$ and $\alpha 2\beta 1$ integrins are upregulated in late-stage ovarian cancer [19–21].

The objective of this study was to determine the functional consequences on cell adhesion, cell survival, integrin expression and related signalling responses towards the commonly used chemo-agents paclitaxel and carboplatin upon stable KLK4–7 overexpression in the EOC cell line OV-MZ-6 [22,23]. In this previously generated cell line, combined KLK4–7 transfection promoted increased invasion *in vitro* and enhanced tumour growth in an EOC mouse model compared to vector controls [22], although the underlying mechanisms involved were not studied. An advantage of this model is that simultaneous expression of KLK4, KLK5, KLK6, and KLK7 in EOC cells allows their proposed activation cascades which have been described *in vitro* by pro-KLK fusion protein digestions on cleavage of the pro-region [24] to proceed. For instance, KLK4 and KLK5 autoactivate; active KLK4 activates KLK5 and KLK6; active KLK5 activates KLK6 and KLK7; and KLK6 partially activates KLK5 [24]. Although one should be circumspect in extrapolation of this data to an *in vivo* environment, especially considering many additional potential interactions of (pro)KLKs within the extracellular proteolytic web, several tissue-specific KLK cascades have been described in detail, for instance, within the central nervous system, semen or skin [24–27]. In fact, we only saw the marked tumourigenic effect when the four KLKs (KLK4–7), and not the individual KLKs or other combinations, were overexpressed in OV-MZ-6 cells [22]. Therefore, it is not unreasonable to assume that an activation cascade, dependent on combined expression of certain KLKs, akin to that described by Yoon and colleagues [24], is operational in these cells.

We demonstrate that stable overexpression of KLK4–7 by EOC cells promoted decreased cell adhesion to ECM proteins and integrin expression as well as paclitaxel-induced chemoresistance accompanied by differential expression of drug-resistance associated genes representative of a malignant phenotype and disease progression. Our results also showed that integrin and MAPK independent pathways may mediate the cellular responses of KLK4–7-expressing EOC cells that contribute to their chemoresistance.

Materials and methods

DNA constructs, generation of stable cell lines and cell culture

Human KLK4, KLK5, KLK6, and KLK7 cDNAs including pre–pro-domains were isolated from EOC tissue, stable KLK-expressing OV-MZ-6 cells generated using G418-selection (1 g/mL; Invitrogen, Mulgrave, VIC, Australia) and grown in DMEM (Invitrogen) containing 10% (v/v) FCS as described previously [22]. The human EOC cell line OVCAR-3 was purchased from the American Type Culture Collection (ATCC). Cells were cultured at 37 °C/5% CO_2 until reaching confluency of 60–80% and harvested with EDTA (0.48 mmol/L; Invitrogen).

Cell adhesion assays

Attachment of cells onto the ECM proteins (10 μg/mL) fibronectin (FN) (BD Biosciences, Eight Mile Plains, QLD, Australia), vitronectin (VN) (Promega, Alexandria, NSW, Australia), collagen (Col) type I (Sigma-Aldrich, Castle Hill, NSW, Australia) and type IV (Calbiochem, South Granville, NSW, Australia) and the effect of blocking β1 (#P2D5)/αv (#AV1) integrin antibodies (Chemicon, North Ryde, NSW, Australia) or mouse IgG (10 μg/mL; Sigma-Aldrich) was measured as reported previously [13]. For each condition, three different biological assays were conducted in triplicate and normalised to BSA-coated plates and non-blocked controls.

Cell survival post paclitaxel/carboplatin/U0126 treatment

Chemosensitivity assays using anti-cancer agents (Sigma-Aldrich) were performed as reported previously [28]. Briefly, 1 day after seeding (3.0×10^4 cells) onto 96-well plates (Perkin Elmer, Melbourne, VIC, Australia), cells were treated with paclitaxel (0, 0.01, 0.1, 1, 2.5, 5, 10, 50, and 100 nmol/L), carboplatin (0, 1, 5, 10, 50, and 100 μmol/L) for 3 days, and in 1 day intervals using paclitaxel (100 nmol/L), carboplatin (50 μmol/L) or in combination, control agents (DMSO (Merck, Kilsyth, VIC, Australia) or H_2O). For inhibition, 1 day before seeding, cells were serum-starved with media containing 1% (v/v) FSC cells, and then pre-treated with the MEK1/2 inhibitor (U0126; 50 μmol/L; Cell Signaling Technology, Arundel, QLD, Australia) or control agent (DMSO) for 2 h followed by paclitaxel (100 nmol/L) over 3 days. Cell survival post anti-cancer agents or inhibition was detected using CyQuant®/AlamarBlue® assays (Invitrogen) and DNA content/metabolic activity calculated as percentage of fluorescence in non-treated control samples. The percentage of OV-Vector cells was set to 100% for each dose and the proportion of