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A bioengineered 3D ovarian cancer model for the assessment of peptidase–mediated enhancement of spheroid growth and intraperitoneal spread

Daniela Loessner [a,*], Simone C. Rizzi [b], Kathryn S. Stok [c], Tobias Fuehrmann [d], Brett Hollier [a], Viktor Magdolen [e], Dietmar W. Hutmacher [d], Judith A. Clements [a]

[a] Cancer Program, Faculty of Health, Institute of Health and Biomedical Innovation, Queensland University of Technology, 60 Musk Avenue, Kelvin Grove, Brisbane, Queensland 4059, Australia
[b] QGel SA, Innovation Square, École Polytechnique Fédérale de Lausanne (EPFL)-QI Building G, Lausanne, Switzerland
[c] Institute for Biomechanics, Swiss Federal Institute of Technology, ETH Zürich, Zürich, Switzerland
[d] Regenerative Medicine Program, Faculty of Science and Engineering, Institute of Health and Biomedical Innovation, Queensland University of Technology, Brisbane, Australia
[e] Clinical Research Unit, Department of Obstetrics and Gynecology, Technical University of Munich, Munich, Germany

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ABSTRACT

Cancer–associated proteases promote peritoneal dissemination and chemoresistance in malignant progression. In this study, kallikrein–related peptidases 4, 5, 6, and 7 (KLK4–7)–cotransfected OV–MZ–6 ovarian cancer cells were embedded in a bioengineered three–dimensional (3D) microenvironment that contains RGD motifs for integrin engagement to analyze their spheroid growth and survival after chemotreatment. KLK4–7–cotransfected cells formed larger spheroids and proliferated more than controls in 3D, particularly within RGD–functionalized matrices, which was reduced upon integrin inhibition. In contrast, KLK4–7–expressing cell monolayers proliferated less than controls, emphasizing the relevance of the 3D microenvironment and integrin engagement. In a spheroid–based animal model, KLK4–7–overexpression induced tumor growth after 4 weeks and intraperitoneal spread after 8 weeks. Upon paclitaxel administration, KLK4–7–expressing tumors declined in size by 91% (controls: 87%) and showed 90% less metastatic outgrowth (controls: 33%, $P < 0.001$). KLK4–7–expressing spheroids showed 53% survival upon paclitaxel treatment (controls: 51%), accompanied by enhanced chemoresistance–related factors, and their survival was further reduced by combination treatment of paclitaxel with KLK4/5/7 (22%, $P = 0.007$) or MAPK (6%, $P = 0.006$) inhibition. The concomitant presence of KLK4–7 in ovarian cancer cells together with integrin activation drives spheroid formation and proliferation. Combinatorial approaches of paclitaxel and KLK/MAPK inhibition may be more efficient for late–stage disease than chemotherapeutics alone as these inhibitory regimens reduced cancer spheroid growth to a greater extent than paclitaxel alone.

1. Introduction

Cell models that truly reflect the three–dimensional (3D) nature of the microenvironment are favored over two–dimensional monolayer cultures to delineate roles of cancer–associated proteases in malignant progression [1–5]. 3D cultures using collagen matrices or laminin–rich reconstituted basement membranes have been used to elucidate the roles of matrix metalloproteinases, cathepsins, and serine proteases in progression of breast [6,7] and ovarian cancer [8,9]. We established a bioengineered 3D culture model [10] and, in line with others, reported crucial differences in cancer cell growth and survival in 3D compared to traditional monolayer cultures [11,12]. Within this bioengineered 3D microenvironment, ovarian cancer cells form multicellular spheroids that are typical of those accumulated in the tumor fluid (ascites) in late–stage patients. These cancer spheroids exhibit a higher resistance towards the microtubule–stabilizing chemotherapeutic paclitaxel than cell monolayers [10], indicating that the 3D architecture truly replicates the non–responsiveness of patients with progressive disease.

* Corresponding author. Tel.: +61 (0)7 3138 6441; fax: +61 (0)7 3138 6030.
E-mail address: daniela.lossner@qut.edu.au (D. Loessner).

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In this study, we further utilized the modularity of this bioengineered 3D platform, specifically in the light of kallikrein–related (KLK) serine peptidase function, as a spheroid–based therapeutic screening tool and intraperitoneal animal model for ovarian cancer. KLKs have shown potential as diagnostic indicators for ovarian cancer and prognostic biomarkers for malignant progression. To date, 12 of the 15 KLKs are known to be upregulated in ovarian cancer [13]. Of those, KLK4, KLK5, KLK6, and KLK7 (KLK4–7) are associated with an unfavorable prognosis [14]. Elevated serum levels of KLK4–7 are linked to non–responsiveness of patients to paclitaxel [14–16]. KLKs are thought to initiate peritoneal invasion via activation cascades leading to degradation of the extracellular matrix (ECM) and signaling events mediating cell survival and chemoresistance. However, the precise regulatory events are incompletely understood [13–16].

Besides proteases, integrins are critical for spheroid formation, adhesion of ovarian cancer cells onto secondary sites, thereby affecting metastatic outgrowth and chemoresistance [17–19]. Importantly, integrins mediate tumor–ECM interactions, via the ligand–binding motif RGD, having growth–promoting effects that lead to chemoresistance [20]. In particular, αvβ3 and α5β1 integrins are upregulated in late–stage disease [15,21–23]. Hence, a concomitant KLK4–7 expression together with integrin engagement might contribute to disease progression and a lack of therapy response given that KLKs degrade ECM proteins, and therefore, influence the ECM–integrin binding dynamics.

KLK4–7 are individually implicated in metastatic outgrowth and chemoresistance of ovarian cancer cells [14–16,24,25], and KLK4–7–cotransfection in OV–MZ–6 ovarian cancer cells is correlated with increased invasion *in vitro* and enhanced tumor burden *in vivo* [26]. Thus, we hypothesized that the cellular effects of concomitant expression of these four KLKs, facilitating the malignant behavior of cancer spheroids, will be evident in a spheroid–based model with an impact on proliferation, interaction with integrins, survival responses to paclitaxel treatment and the combination treatment of paclitaxel with KLK, integrin or MAPK inhibition as well as tumor growth and intraperitoneal dissemination in mice.

2. Materials and methods

2.1. DNA constructs, generation of stable transfectants and 3D cell culture

Human KLK4, KLK5, KLK6, and KLK7 full–length cDNA was derived from ovarian cancer tissue, and stable KLK4–7–expressing OV–MZ–6 cells generated using G418–selection as reported [26]. OV–MZ–6 cells transfected with the vector only ('OV–Vector') and non–transfected parental OV–MZ–6 cells behaved identically in all assays. Cells were cultivated at 37 °C/5% CO_2 until reaching confluency of 60–80% and harvested with EDTA (0.48 mmol/L). For 3D cultures, cells (3.5×10^5 cells/mL) were encapsulated within biomimetic PEG–based hydrogels and grown over 14 days in 500 μL media, replaced every three days [10]. For animal experiments, OV–Vector/OV–KLK4–7 cells were transfected with a lentiviral luciferase expression system (pLenti6/V5–D–TOPO; Invitrogen) using blasticidin–selection [27] and encapsulated within hydrogels (RDG–functionalized, proteolytic degradable; QGel).

2.2. Cell proliferation post paclitaxel treatment and integrin/KLK/MAPK inhibition

Preparation of samples for proliferation assays, CyQuant® (Invitrogen) to measure the DNA content and AlamarBlue® (Invitrogen) to detect metabolic activities, was described earlier [10]. CyQuant® fluorescent signals (excitation 485 nm, emission 520 nm) were detected using a plate reader (BMG PolarStar). A λDNA standard (0.01–2 μg/mL) was applied to calculate the fold change of DNA content per culture condition normalized to the starting time point. AlamarBlue® reagent (monolayer/spheroid – 10/4%) was added to the media and fluorescent signals (excitation 544 nm, emission 590 nm) detected as above. For chemosensitivity assays, spheroids were treated with paclitaxel (10 nmol/L; Sigma–Aldrich; DMSO as control) on day 7 of 3D culture for 7 days [10]. Cell survival using CyQuant®/AlamarBlue® was detected as above, and DNA content/metabolic activities calculated as percentage of fluorescence in non–treated controls. Treatments with functional blocking β1 integrin (10 μg/mL; Chemicon, #P2D5), KLK4 (10 μg/mL; R&D Systems, #325712), KLK5 (10 μg/mL; R&D Systems, #193318), KLK7 (10 μg/mL; R&D Systems, #333931) antibodies and a MEK1/2 (U0126) inhibitor (50 μmol/L; Cell Signaling Technology) were either performed alone or in combination with each other or paclitaxel on day 7 of 3D culture for 7 days. Each condition was performed three times in triplicate.

2.3. Confocal laser scanning microscopy (CLSM) and quantitative analysis

3D cultures were processed as described earlier [10]. F–actin filaments were stained with rhodamine415–conjugated phalloidin (0.3 U/mL; Invitrogen) and nuclei with DAPI (2.5 mg/mL; Invitrogen) in 1% bovine serum albumin (BSA; Sigma–Aldrich)/PBS. Immunofluorescence was visualized and photographed using a confocal microscope (TCS SP5 II, Leica) with a 20/40× oil objective at three–five different positions per sample. Z–stacks were acquired with constant thickness of 2 μm reconstructing a cross–section profile of 100–150 equidistant XY–scans using the Leica Microsystems LAS AF software (v.1.8.2 build 1465) to generate maximal projections. Quantitative analyses of CLSM images, using an image processing and evaluation protocol, were employed to categorize the distribution pattern clusters, cluster numbers and volumes using both rhodamine415–conjugated phalloidin and DAPI channels [10].

2.4. Transmission electron microscopy (TEM)

To image the morphology of 3D cultures upon paclitaxel administration, samples were processed and photographed with a 1200EX TEM (JEOL) operating at 80 kV as reported earlier [10].

2.5. Real–time reverse transcription quantitative PCR (RT–qPCR)

Equal amounts (1 μg) of total RNA from 3D cultures (extracted using an RNeasy micro kit, Qiagen) were used for cDNA synthesis. RT–qPCR was performed in triplicate with SYBR® Green chemistry (AB Applied Biosystems) on an ABI7300 thermal cycler (AB Applied Biosystems). Reaction setup and normalization applying the standard curve method ($R^2 = 0.99–0.96$) were conducted as reported [10]. Gene specific primers used are listed in Supplementary Table 1. Each condition was performed three times in duplicate.

2.6. Western blotting

Cell lysates from 3D cultures were collected in lysis buffer (according to RNeasy micro kit, Qiagen), protein concentrations determined using protein detection reagents (bicinchoninic acid; Sigma–Aldrich) and 40 μg electrophoresed on 10% SDS–PAGE, transferred onto nitrocellulose membranes and treated with Odyssey® blocking buffer (LI–COR Biosciences). Membranes were incubated with primary [α5 integrin (1:500; Chemicon); β1 integrin (1:500; Chemicon); caspase8 (1:2000; BD Bioscience); GAPDH (1:10,000; Abcam)] and secondary [IRDye 680/800–conjugated rabbit/mouse IgG (1:5000; LI–COR Biosciences)] antibodies overnight at 4 °C and 1 h at room temperature respectively. Images were obtained using the Odyssey® system (LI–COR Biosciences).

2.7. Immunohistochemistry, H/E and fluorescent staining

3D cultures were washed with PBS, fixed with 4% paraformaldehyde (PFA)/PBS for 20 min, embedded in TissueTek (ProSciTech), and stored at −80 °C. Samples were cryo–sectioned (5–7 μm), nuclei stained with hematoxylin (H) and cytoplasm with eosin (E) and imaged using a widefield microscope (LaborLux, Leitz; DXM1200C digital camera, Nikon; ACT–1C software v.1.01) with a 40× air objective at three–five different positions per sample. For immunofluorescence, sections were permeabilised with 0.2% triton X–100/PBS for 15 min, washed with PBS and blocked with 1% BSA/PBS for 2 h. Primary [α5 integrin (1:50; Chemicon); β1 integrin (1:200; Chemicon, #P5D2); caspase8 (1:50; Abcam); KLK4 [1:100; [28]; KLK5 (1:100; R&D Systems); KLK7 (1:20; generated in Prof Magdolen's group, Technical University of Munich, Germany)] and secondary [Alexa488–conjugated anti–mouse/rabbit IgG (1:1000/2000; Invitrogen)] antibodies in 1% BSA/PBS were incubated 1 h each. Secondary antibody only or mouse IgG (Sigma–Aldrich) served as negative controls. F–actin filaments and nuclei were stained as per CLSM. Sections were mounted with ProLong Gold® anti–fade reagent (Invitrogen) and glass coverslips (ProSciTech) and immunofluorescence visualized and imaged using a confocal microscope with a 40× oil objective at three–five different positions per sample. Z–stacks were acquired with constant thickness of 0.5 μm reconstructing 5 μm cross–section profiles shown as maximal projection.

2.8. Intraperitoneal ovarian cancer mouse model

Animal experiments were performed in compliance with the *Australian Code of Practise for the Care and Use of Animals for Scientific Purposes* and approved by the University Animal Ethics Committee. Female NOD/SCID mice (mean body weight of 16.5 g; Animal Resource Centre, WA) were housed in individually ventilated cages