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Tumor-environment biomimetics delay peritoneal metastasis formation by deceiving and redirecting disseminated cancer cells

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A B S T R A C T

Peritoneal metastasis is life threatening and is the result of an extensive communication between disseminated cancer cells, mesothelial cells and cancer-associated fibroblasts (CAF). CAFs secrete extracellular matrix (ECM) proteins creating a receptive environment for peritoneal implantation. Considering cancer as an ecosystem may provide opportunities to exploit CAFs to create biomimetic traps to deceive and redirect cancer cells. We have designed microparticles (MP) containing a CAF-derived ECM-surface that is intended to compete with natural niches. CAFs were encapsulated in alginate/gelatine beads (500–750 μm in diameter) functionalised with a polyelectrolyte coating (MP[CAF]). The encapsulated CAFs remain viable and metabolically active (≥35 days), when permanently encapsulated. CAF-derived ECM proteins are retained by the non-biodegradable coating. Adhesion experiments mimicking the environment of the peritoneal cavity show the selective capture of floating cancer cells from different tumor origins by MP[CAF] compared to control MP. MP[CAF] are distributed throughout the abdominal cavity without attachment to intestinal organs and without signs of inflammatory reaction. Intraperitoneal delivery of MP[CAF] and sequential removal redirects cancer cell adhesion from the surgical wound to the MP[CAF], delays peritoneal metastasis formation and prolongs animal survival. Our experiments suggest the use of a biomimetic trap based on tumor–environment interactions to delay peritoneal metastasis.

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1. Introduction

Peritoneal metastasis develops when primary abdominal and pelvic tumors metastasize to the peritoneal cavity. These metastasis occur, among others, in ovarian and colorectal cancer patients, in respectively 75% and 13% of the cases. Once peritoneal metastasis is present the five-year survival rate is only 20–25% [1,2] These poor survival rates show the need for adequate therapeutic and preventive strategies.

Peritoneal metastasis is a stochastic event requiring adhesive cues from the host tissue. Cancer cells detach from the primary tumor spontaneously or iatrogenically, and are transported by the

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peritoneal fluid [3]. Disseminated cancer cells can be found in the peritoneal fluid before surgery in 18%–25% [3,4] of the colorectal patients. After surgery this is up to 10% higher [2,5], similar results are published for pelvic cancer patients [6]. The presence of disseminated cancer cells indicates a higher recurrence and poorer survival [4–6]. Disseminated cancer cells secrete inflammatory cytokines which compromise the protective, anti-adhesive mesothelial cell layer that lines up the mesothelial wall [3], exposing the submesothelial environment containing extracellular matrix (ECM) and fibroblasts. TGF-β, secreted by cancer cells, will further retract the mesothelial cells, and will activate tissue-resident fibroblasts [7] and mesothelial cells [8] into cancer-associated fibroblasts (CAFs). CAFs secrete a range of ECM proteins (*e.g.* type I collagen, tenascine C) creating a receptive adhesive environment for peritoneal implant formation [9].

Prevention of adhesion of free peritoneal cancer cells to (sub) mesothelial cells is one strategy to target peritoneal metastasis formation [3]. Blocking integrin complexes to prevent adhesion of disseminated cancer cells to the peritoneal wall is a frequently applied preclinical strategy [10] and combined chemotherapy with neutralizing CD44 antibodies improves efficiency [11]. The tumor environment is incessantly considered as a potential target for therapy [12]. Alternatively, viewing tumors as an ecosystem provides the opportunity to consider therapeutics based on ecosystem concepts. The presence of an artificial substrate containing adhesive cues may redirect free peritoneal cancer cells, into an ecological trap. Ecological traps serve as guides to facilitate cancer migration to a new habitat where cancer cells can be eliminated [13]. We designed microparticles (MPs) that encapsulate metabolically active CAF which continuously deposit a pro-adhesive ECM at the surface of the MPs. Free cancer cells in culture or in the peritoneal cavity adhere to the MPs, resulting in a delayed formation of peritoneal implants.

2. Materials and methods

2.1. Cell lines

Human CAF were isolated from a colorectal adenocarcinoma resection specimen obtained in accordance with the local ethics committee (Ghent University Hospital) described by De Boeck et al. [9,14]. In sort, tissue fragments were cut in small pieces and transferred into a 6-well late fetal FBS. Cultures were incubated at 37 °C with 10% CO_2 in air for 24 h. After 24 h, DMEM (Invitrogen) containing 10% FBS was added ell outgrowth was observed after 3–6 days. CAFs are positive for vimentin and α-SMA (Suppl. Fig. 1). Isolated CAFs were infected with a pBABE retroviral vector expressing the hTERT open reading frame. The replicative life span of hTERT transduced pool was examined and compared with that of mock-transduced pool. Growth in control CAF populations typically plateaued by population doubling 15, whereas hTERT populations continued to divide far beyond the senescence point of control cells. CAF-eGFP are hTERT-immortalized CAF lentivirally transduced by eGFP expressing pLVTH plasmid. HCT8/E11 is a human colon cancer cell line (ATCC number: CCL-244). SK-OV-3 is a human ovarian cancer cell line (ATCC number: HTB-77). HT29hCG-Luc is a human colon cancer cell line (ATCC number: HTB-38) kindly provided by Dr. C. Hackl [15]. MSTO-211h is a human mesothelioma cancer cell line (ATCC number: CRL-2081). HCA2-hTERT are normal foreskin fibroblasts immortalized by hTERT [16]. LP-9 is a normal human mesothelial cell line (corriell ID: AG07086). Luciferase (Luc) positive HCT8/E11, SK-OV-3 and MSTO-211H cells were prepared by pFL4.76 plasmid transfection and selection (Promega). SK-OV-3-Luc cells were inoculated intraperitoneally of immunodeficient female mice to isolate populations that form peritoneal implants. After tumor dissociation and expansion in culture, the resulting cell populations (SK-OV-3-Luc-IP1) were subjected to a second round of *in vivo* selection, yielding SK-OV-3-Luc-IP2 cell populations that showed a significant increase in peritoneal metastatic activity. All cell lines, except LP-9 were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% FBS and antibiotics (penicillin/streptomycin), and incubated at 37 °C with 10% CO_2 in air. LP-9 is cultured on a 1% type A gelatin coating (Sigma–Aldrich) in a mixture of HAM's F12/Medium 199, 1:1 mixture of culture medium (Invitrogen) supplemented with 10 ng/ml EGF, 0.4 μg/ml, 10% FBS and antibiotics (penicillin/streptomycin, incubated at 37 °C with 5% CO_2 in air. Cancer cells were red fluorescent labeled with DiI Vybrant ™ cell-labelling solutions (Molecular Probes). Authenticity of ATCC cell lines was confirmed by short tandem repeat profiling in the last 6 months before use.

2.2. Cell-encapsulation

CAFs were encapsulated in alginate (Sigma–Aldrich) and gelatin type B (Sigma–Aldrich). 2% Na-alginate (MW: 12 000–80 000, M/G 1.56) and 2% gelatin type B (produced by alkaline hydrolysis, thus having an IEP below 7.4) were separately dissolved in PBS^{D-}. Na-alginate was sterilized by filtration through a 0.22 μm filter (Nalgene), gelatin by boiling. Na-alginate and gelatin were mixed in a concentration of 1.5% Na-alginate and 0.5% gelatin and vortexed. This mixture was left to settle overnight, to discard air bubbles. A 80% confluent culture of CAF(-eGFP) cells was trypsinised to a single cell suspension, counted and centrifuged (200 g). Supernatant was removed and the cell pellet was suspended in the Na-alginate/gelatin mixture in a 2×10^6 cells/ml concentration. The mixture was dropped in a 1.3% $CaCl_2$, pH7.4 bath through a needle (Hamilton N Needle (25/51/3) 6 pK) with an inner diameter of 260 μm. The tip of the needle was 8 cm removed from the surface of the $CaCl_2$ solution. Droplets were made with a speed of 40 ml/h while the tip of the needle was surrounded by a sterile air-flow. When the small droplets came in contact with $CaCl_2$, Na^+ was replaced by Ca^{2+} gelous forming Ca-alginate (Suppl. Fig. 2A). The CAF(-eGFP) became imbedded in the matrix. As a control, empty microparticles (MP[E]) were made by using the Na-alginate/gelatin mixture without adding CAFs or by adding formol fixed CAFs [17].

2.3. Coating of the microparticles

The microparticles (MPs) were coated by layer-by-layer [18] technique with each two alternating layers of PSS (poly-styrene sulfonate, MW~70000, Sigma–Aldrich) and PAH (poly-allyamine, MW: ~65000, Sigma–Aldrich). $CaCl_2$ was replaced with PAH solution (2 mg/ml in 0.9% $CaCl_2$ pH 7.4), for each ml MPs 1 ml PSS was added. This was placed on an orbital shaker at 350 rpm. After 10 min the MPs were rinsed 3 times with 0.9% $CaCl_2$, pH7.4. This was repeated with the PSS solution (2 mg/ml in 0.9% $CaCl_2$ pH 7.4). To ensure stability, four layers were used, each individual layer added two times. Coated MPs were washed three times with 0.9% $CaCl_2$ pH7.4 followed by three washes by culture medium (DMEM 10% FCS, PSF) to avoid calcium-phosphate crystal formation. When ironoxide nanoparticles (FeOxNP, PlasmaChem) were used, 3 mg FeOxNP/ml 0.9% $CaCl_2$ solution was added between the first two alternating layers (Suppl. Fig. 2B).

2.4. Microscopy

Following microscopes were used: light microscope Leica DM750, phase contrast microscope Leica DMI3000B and stereo fluorescent microscope Leica M205FA with the LAS4.1 software packet, fluorescent microscope Zeiss Axiovert 200M with the software package Axiovision 4.1, and a Leica DMI6000 coupled to an Andor D8D2 spinning disc system for confocal images processed in the Imanis software package (see Suppl. data).

2.5. Functional assays

The viability and metabolic activity of encapsulated cells was analyzed once a week during 5 weeks, and performed with the LIVE/DEAD viability/cytotoxicity kit for mammalian cells (Invitrogen) and the MTS CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Promega) respectively (see Suppl. data). Collagen invasion assay was performed according to De Wever et al. [19]. with 10^5 HCT8/E11 cells (see Suppl. data). For protein analyses Western blot was performed with a 8% SDS-PAGE gel and immunostained with anti-TNC (clone BC8; provided by L. Zardi [20]) and anti-β1-integrin (BD: 610468) antibodies (see Suppl. data). Conditioned medium (CM) was prepared according to De Wever et al. [14] (see Suppl. data).

2.6. Gelatin decay

Dequenched (DQ) gelatin becomes fluorescent when gelatin is cleaved. DQ gelatin (Invitrogen) was added to the gelatin (1/20) before encapsulation and coating (PAH/PSS/PAH/PSS). The cleavage of gelatin was monitored by calculating the total surface of fluorescent signal during 8 days by digital image analysis (Axiovision 4.1).

2.7. Polymer and protein labeling

PAH and proteins present in the CM of CAF were labeled red fluorescent with rhodamine isothiocyanate (RITC) (Sigma–Aldrich). PAH (6 mg) or CM (2 ml) was mixed with 8 ml borate buffer (1M boric acid with 1MNaOH to pH9). 1 mg RITC was added and incubated during 24 h. Unbound RITC was removed by dialysis using a cellulose ester membrane for 72 h.

2.8. eQCM: electrochemical quartz crystal microbalance

Monitoring of the adsorption of proteins by the PSS/PAH coating was performed using a Gamry eQCM 10M quartz crystal microbalance equipped with an ALS flow cell. Gold coated quartz chips with a nominal resonance frequency of 10 MHz (International Crystal Manufacturing) were first precoated by 15 min immersion in an aqueous solution of mercaptosuccinic acid (2 mg/ml) followed by extensive water rinsing. Next, the quartz chip was immersed into an aqueous poly(ethylenimine) 22 kDa PEI solution (2 mg/ml) for 15 min and again extensively washed. In addition the quartz chip was immersed into alternating PSS and PAH solutions (2 mg/ml in

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