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Research paper

Targeting the extracellular matrix of ovarian cancer using functionalized, drug loaded lyophilisomes

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ABSTRACT

Epithelial ovarian cancer is characterized by a high mortality rate and is in need for novel therapeutic avenues to improve patient outcome. The tumor's extracellular matrix ("stroma") offers new possibilities for targeted drug-delivery. Recently we identified highly sulfated chondroitin sulfate (CS-E) as a component abundantly present in the ovarian cancer extracellular matrix, and as a novel target for anti-cancer therapy. Here, we report on the functionalization of drug-loaded lyophilisomes (albumin-based biocapsules) to specifically target the stroma of ovarian carcinomas with the potential to eliminate cancer cells. To achieve specific targeting, we conjugated single chain antibodies reactive with CS-E to lyophilisomes using a two-step approach comprising sortase-mediated ligation and bioorthogonal click chemistry. Antibody-functionalized lyophilisomes specifically targeted the ovarian cancer stroma through CS-E. In a CS-E rich micro-environment *in vitro* lyophilisomes induced cell death by extracellular release of doxorubicin which localized to the nucleus. Immunohistochemistry identified CS-E rich stroma in a variety of solid tumors other than ovarian cancer, including breast, lung and colon cancer indicating the potential versatility of matrix therapy and the use of highly sulfated chondroitin sulfates in cancer stroma as a micro-environmental hook for targeted drug delivery.

1. Introduction

Epithelial ovarian cancer is the fifth leading cause of cancer-related death in women worldwide [1]. Most patients are diagnosed with an advanced stage of disease (Fédération Internationale de Gynécologie et d'Obstétrique (FIGO) stage III–IV) and suffer from extensive abdominal metastases [2,3]. Aggressive surgical cytoreduction and chemotherapy are used as primary treatment, but nevertheless up to 70% of these patients will develop recurrent disease and eventually succumb. Long term survival is poor with a 5-year survival of less than 35% [2,3]. Overall survival statistics have not significantly improved over the last decades and new avenues for better treatment are clearly warranted [4].

Conventional chemotherapeutics affect proliferating cancer cells as well as normal cells, resulting in systemic adverse events that greatly affect quality of life. As a consequence, the dose administered has to be limited resulting in a suboptimal treatment that negatively affects prognosis of cancer patients. The use of drug delivery systems may be helpful to overcome these problems by improving biodistribution, resulting in high local drug concentrations at the tumor site while minimizing exposure to healthy cells [5]. Beneficial effects of drug delivery systems such as liposomal doxorubicin (Caelyx/Doxil) and albumin bound paclitaxel (nab-paclitaxel), have been reported in several (pre)-clinical studies [6–9]. Previously we described a novel class of drug delivery vehicles, lyophilisomes, which are spherical nano- to microsized biocapsules that can be prepared from various proteins (e.g. albumin, collagen, and elastin) [10,11]. Albumin-based lyophilisomes can be efficiently loaded with doxorubicin and are able to eliminate ovarian cancer cells *in vitro* [10]. In addition, the albumin wall of lyophilisomes offers opportunities for functional modification, e.g. by the incorporation or conjugation of components in and/or on the wall. Antibody-conjugated lyophilisomes have been shown to specifically bind to cancer cells expressing the corresponding antigen, thus enabling active cancer-targeting [12].

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Although it is hypothesized that active targeting of cancer cells by drug delivery systems using specific antibodies or ligands has the potential to broaden the therapeutic index of anti-cancer drugs, the favorable effect of tumor-cell targeting over non-targeting systems was reported to be disappointing [5,6,13]. As most of these studies have focused on targeting cancer cells, other approaches such as targeting the cancer extracellular matrix (ECM) may offer valuable alternatives [14].

The ECM represents a network of proteins and proteoglycans that is abundantly remodeled during cancer development and actively contributes to cancer progression [15,16]. A large amount of intratumoral matrix correlates with poor prognosis in cancer, including ovarian cancer [17]. Major components of the ECM are collagen, laminin and proteoglycans. Proteoglycans function to a large extent through their glycosaminoglycan side chains; linear negatively charged polysaccharides built from repeating disaccharides [18]. Highly 4,6-sulfated chondroitin sulfate (CS-E), a specific class of glycosaminoglycans, is found to be abundantly expressed in the ovarian cancer stroma while being absent or present in only very small amounts in healthy stroma, thus representing an attractive target for anti-cancer therapy [19,20]. Interestingly, the amount of these targets expressed in the stroma is relatively high when compared to targets expressed at the cancer cell surface.

In this study, we present an innovative concept of an anti-cancer strategy aiming at forming a depot of chemotherapeutic-loaded lyophilisomes in the ovarian cancer ECM. Targeting the cancer ECM rather than cancer cells might be helpful to overcome hurdles observed in cell-targeting therapies as the cancer ECM is a relatively stable structure, unlike cancer cells that are characteristically genetically instable [21]. Due to intratumoral heterogeneity, cell-targeting therapies may only affect subpopulations of cancer cells, and leave other cancer cells and cancer-promoting cells (e.g. cancer-associated fibroblasts, endothelial cells, and macrophages) unaffected [22]. Release of chemotherapeutics from a depot of drug-loaded lyophilisomes in the cancer ECM may affect all cells in its vicinity including cancer cells, cancer stem cells and cancer-associated stromal cells. Collagens have been used as micro-environmental anchors for targeted anti-cancer therapy [23,24], but collagen is also abundantly present in normal tissues. Therefore, in this study we focus on CS-E as a much more cancer-specific molecular target. We describe the construction and evaluation of a lyophilisome-based drug delivery system specifically targeting highly sulfated CS-E in the ovarian cancer stroma.

2. Materials and methods

2.1. Patient material

Study approval was given by the Regional Committee for Medical Research Ethics and performed according to the Code for Proper Secondary Use of Human Tissue (Dutch Federation of Biomedical Scientific Societies, www.federa.org). Cryosections (5 μm) of advanced stage high grade serous ovarian cancer were used for immunofluorescent analysis of antibody-functionalized lyophilisome specificity. Paraffin embedded sections (4 μm) of lung, cervical, breast, renal cell, endometrial, and colon cancer were used for immunohistochemical analysis of CS-E expression.

2.2. Production of antibody-functionalized lyophilisomes

2.2.1. Modification of GD3G7 antibodies for sortase-mediated conjugation

The single chain antibody GD3G7 was previously selected against embryonic glycosaminoglycans and showed specificity for CS-E [19]. For site-selective conjugation of GD3G7 at the carboxy terminus, leaving the antigen-binding parts of this antibody intact, the LPETG sortase A-recognition motif was introduced. To this end the GD3G7 reading frame was cloned in plasmid pHENIX-LPETG-His-VSV to yield pHENIX-GD3G7-LPETG-His-VSV. Expression of the fusion protein in *E. coli* strain ER2566 was induced with isopropyl β-D-thiogalactoside (IPTG) as described previously [25]. GD3G7-LPETG-His-VSV was released from the periplasmic space via osmotic lysis using 200 mM Tris-HCl, pH 8.0, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 20% (w/v) sucrose containing protease inhibitors. Purification by nickel-NTA affinity chromatography was performed as described (NTA-Ni Sepharose®, IBA Life sciences) [26].

2.2.2. Introduction of DBCO functionality to GD3G7 by sortagging

pGBMCS-SortA, a gift from Dr. Fuyuhiko Inagaki [27] was transfected into *E. coli* ER2566 for standard protein expression. Bacterial expression was performed as described and IPTG-induced cells were lysed by sonication at 4 °C using a Bandalin Sonopuls HD2070 sonicator. His-tagged sortase was purified with NTA-Ni Sepharose as described above.

To equip the GD3G7 antibody with a bio-orthogonal chemical click handle (Fig. 1), 16 μM GD3G7-LPETG-His-VSV was incubated overnight at room temperature with 4 mM amino-PEG_4-DBCO (Click Chemistry Tools, Scottsdale, USA) in the presence of 40 μM sortase A in 50 mM Tris, 150 mM NaCl, 10 mM $CaCl_2$, pH 7.5. Reaction product was cleared from unreacted GD3G7-LPETG-His-VSV, cleaved G-His-VSV tags and sortase A by depletion on nickel-NTA beads. Free amino-PEG_4-DBCO was removed by filtration in PBS over a 10 kDa centrifugal filter device (Amicon® Ultra-4, Merck Millipore) using standard protocols. Routinely, filters were washed five times to obtain highly purified product.

The sortase mediated reaction was evaluated by applying bioorthogonal click chemistry between DBCO and azide. The sortagged product was incubated with azido-cyanine-7.5 (Lumiprobe GmbH, Hannover, Germany) for 1 h at 4° C, followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 10% gels and gel imaging at 800 nm (Odyssey® CLx imaging system). Thereafter, gels were stained for presence of proteins with 0.1% (w/v) Coomassie Brilliant Blue R-250 solution (MP Biomedicals, Santa Anna, CA) in 50% (v/v) methanol and 10% (v/v) acetic acid in water.

2.2.3. Preparation of lyophilisomes and introduction of azide functionality

Lyophilisomes were prepared from bovine serum albumin (BSA; PAA Laboratories, Linz, Austria) as described previously [10]. Briefly, droplets of 20 μl 2.5 mg/ml BSA (containing 10% FITC-labeled BSA (Sigma-Aldrich, St. Louis, MO, USA)) in 0.01 M acetic acid were snap frozen in liquid nitrogen. Capsules were formed using an annealing and lyophilization regimen [11]. Large structures were removed by centrifugation (60g).

Lyophilisomes were prepared for click chemistry by introducing azide groups to the surface of lyophilisomes. Lyophilisomes were suspended in PBS containing 0.1% tween20 (v/v) (PBST; pH 8.0), sonicated (Cycle 0.5; Amplitude 20; 10 cycles) with a Sartorius labsonic P sonicator (Göttingen, Germany), mixed with 100 times molar excess NHS-PEG_4-azide (Jena Bioscience, Jena, Germany) and incubated under rotation at room temperature overnight. Next, lyophilisomes were washed three times with PBST and centrifuged at 17,000*g* for 5 min to remove free NHS-PEG_4-azide, and stored in PBST at 4 °C.

Modification of lyophilisomes with PEG_4-azide was analyzed using flow cytometry. Lyophilisomes (2.5 μg) with or without PEG_4-azide were incubated with 1 μg/ml DBCO-IR dye 680RD (LI-COR Biotechnology, Bad Homburg, Germany), which binds only