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Original Articles

Subtype-specific binding peptides enhance the therapeutic efficacy of nanomedicine in the treatment of ovarian cancer

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ABSTRACT

Currently, epithelial ovarian cancer is viewed as a heterogeneous disease with five major histological subtypes. Clear cell carcinoma represents a specific histological subtype of epithelial ovarian cancer that demonstrates more aggressive clinical behavior and drug resistance compared with other subtypes. Nevertheless, clear cell carcinoma is treated in the same manner as the other subtypes without any particular consideration to its unique clinical characteristics. To improve the therapeutic efficacy of the current liposomal doxorubicin approach for the treatment of clear cell carcinoma, we aimed to develop a novel peptide-conjugated liposomal doxorubicin to actively target this subtype. Two phage clones (OC-6 and OC-26) that specifically bound to clear cell carcinoma were isolated from a phage peptide display library after biopanning procedures. The peptide sequences were translated and aligned (OCSP-6 for OC-6, and OCSP-26 for OC-26, respectively). Peptide-conjugated nanoparticles demonstrated better tumor endocytosis and time-dependent gradual increase of intracellular drug uptake than non-targeting liposomal nanoparticles. Furthermore, peptide-conjugated liposomal doxorubicin better controlled tumors than did non-targeting liposomal doxorubicin. The current work may pave a new way for the development of drugs that target each subtype of epithelial ovarian cancer in the future.

Introduction

In the traditional view, cancer cells that survive chemotherapy and acquire drug resistance gave rise to a population of drug-resistant cancer cells. To date, this issue has been discussed based on the mechanisms by which cancer cells become resistant, such as drug inactivation, changes in cellular targets, the suppression of drug accumulation and activation [1].

Nanoparticles (NPs) have been exploited for selective drug delivery to tumor cells, cancer stem cells, and/or to the supportive cancer cell microenvironment, i.e., stroma or tumor vasculature [2]. The enhanced permeability and retention (EPR) effect has become the gold standard for the development of nanomedicine. All NP-based drugs utilize the EPR effect as a guiding principle [3]. In theory, the use of NPs should present a pharmacokinetic advantage by prolonging the half-life of drugs in systemic circulation [4]. However, despite this pharmacokinetic advantage and advent of numerous nanotechnology publications and patents, success remains limited to only a few nanomedicines [5].

Ovarian cancer is the seventh most common cancer in women, with approximately 239,000 new cases and 140,200 estimated deaths worldwide [6]. In the United States, 21,980 women are expected to be diagnosed with and 14,270 women are expected to die of **ovarian cancer** in 2014 [7]. Epithelial ovarian cancer accounts for approximately 90% of all ovarian cancers [8]. Epithelial ovarian cancer comprises at least five histologically and clinically distinct subtypes, yet patients with these different diseases are currently all treated with the same platinum and taxane-based chemotherapeutic regimen. An increased knowledge of subtype-specific differences that correlate with treatment responses and drug resistance will yield novel treatment strategies for each distinct disease [9].

Clear cell carcinoma (CCC) accounts for 4–12% of epithelial ovarian cancer in Western countries. CCC exhibits distinctly different clinical behavior from that of other epithelial ovarian cancers; it is more aggressive and drug resistant [10]. Therefore, the development of a more effective new treatment strategy for CCC is imperative.

Pegylated doxorubicin-encapsulated liposome therapy (DOXIL, Tibotec Therapeutics, a division of Ortho Biotech Products, L.P., Bridgewater, NJ; CAELYXR, Schering-Plough Corporation, Kenilworth, NJ, USA) has been approved for the treatment of recurrent ovarian

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cancer. However, the therapeutic efficacy showed only a marginal advantage over topotecan in a pivotal phase III clinical trial [11]. Moreover, the uncontrolled, passive release of free drug might result in the suboptimal pharmacokinetics or reduced efficacy of Doxil [12].

To potentiate the therapeutic efficacy, liposomes can be surface-functionalized with targeting ligands to enhance the selective targeting of tumors. The grafting of targeting ligands to the liposome surface can further enhance tumor targeting and facilitate intracellular uptake after the liposome reaches the tumor microenvironment [13]. Combinatorial libraries displayed on microorganisms have been successfully used to discover cell surface-targeting peptides and have thus become an excellent strategy to identify tumor-specific targeting ligands [14].

In this study, we aimed to identify cancer cell-targeting peptides from selecting peptide-displaying phages from complex peptide display phage libraries. We describe the identification of two novel ovarian cancer cell-specific targeting peptides, SP-6 and SP-26, both of which exhibited high binding efficiency to the selection cells and harbored clinical potential as a drug delivery guider in the treatment of ovarian cancer.

Materials and methods

Cell lines and cultures

Ovarian cancer cell lines (OC-3, SKOV-3, ES2-luc and TOV-21G) and a human normal ovarian surface epithelium cell line OSOE were used in this study. The OC-3 cell line was a primary culture derived from a human surgical sample of clear cell adenocarcinoma of the ovary[1]. The SKOV-3 and ES2 cell lines were purchased from the American Type Culture Collection (ATCC). The ES2/luciferase (ES2-luc) cell line was a kind gift from T.C. Wu. The TOV-21G cell line was obtained from BCRC. The OC-3 cell was grown in DMEM/F12 (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS, Gibco) and 1 mM MEM non-essential amino acid (NEAA, Gibco) at 37 °C in a 5% CO_2 incubator. The ES2-Luc line was grown in RPMI 1640 (Gibco) containing 10% FBS, 1 mM NEAA at 37 °C in a 5% CO_2 incubator. The SKOV-3 line was grown in McCoy's 5A (Sigma-Aldrich, St. Louis, MO, USA) containing 10% FBS at 37 °C in a 5% CO_2 incubator. The TOV-21G line was grown in a 1:1 mixture of MCDB 105 medium (Sigma-Aldrich) and Medium 199 (Gibco) containing FBS 15% at 37 °C in a 5% CO_2 incubator. The culture medium for OSOE cells was the same as that used for OC-3 cells.

Phage display biopanning procedures

OC-3 cells were first incubated with a UV-treated inactive control helper phage (insertless phage). The phage-displayed peptide library (New England BioLabs, Ipswich, MA, USA), which initially contained 1×10^{11} plaque-forming units (pfu), was then added. The bound phages were eluted with a lysis buffer (150 mM NaCl, 50 mM Tris–HCl, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, pH 7.4) on ice. This eluted phage pool was then amplified and titered in an Escherichia coli ER2738 culture (New England BioLabs). Recovered phages were used as an input for the next round of panning, as described previously [15,16]. The fifth-round phage elute was titrated on LB/IPTG/X-Gal plates for phage clone identification.

Identification of phage clones by ELISA and sequencing

Ninety-six-well ELISA plates (Falcon, CA, USA) were seeded with OC-3 cancer cells. The same titer of individual phage particles were placed into the cell-coated plates with control phage and incubated, followed by incubation with horseradish peroxidase (HRP)-conjugated mouse anti-M13 monoclonal antibody (mAb) (Pharmacia, Uppsala, Sweden) and incubation with the peroxidase substrate o-phenylenediamine dihydrochloride (Sigma-Aldrich) thereafter. The reaction was stopped, and the absorbance was measured at 490 nm using an ELISA reader. Phage clones with a signal intensity twice as high as that of the control phage of higher were further analyzed using DNA sequencing. Initially, 30 phage clones met the selection criteria. The DNA sequences of the purified phages were determined using the dideoxynucleotide chain termination method and an automated DNA sequencer (ABI PRISM 377; Perkin-Elmer). The DNA was sequenced with the primer 5′-CCCTCATAGTTAGCGTAACG-3′, which corresponds to the pIII gene sequence. The phage-displayed peptide sequences were translated and aligned using a Genetic Computer Group program [17].

Flow cytometry analysis

The ovarian cancer cells (OC-3, ES2-Luc, SKOV-3) or a normal control cells (OSOE) were collected using PBS containing 10 mM EDTA, and the cells then were incubated with different titers (2×10^9 or 2×10^{10} pfu/mL) of each individual phage clone at 4 °C for 1 hour. After washing, the phage-bound cells were incubated with anti-M13 mAbs (GE Healthcare) at 4 °C for 1 hour and then treated with PE-conjugated goat anti-mouse IgG antibody at 4 °C for 30 minutes. The cells were washed and analyzed by flow cytometry (Becton Dickinson) on a FACSCalibur using the CellQuest software [18].

Cytotoxicity assay by Annexin V/PI flow cytometry

The number of apoptotic or necrotic cells was determined by flow cytometry after double staining with Annexin V-FITC and propidium iodide using an assay kit from BD Pharmingen. Briefly, 2 mL of cells (1×10^6 cells/mL) were added to each well of 12-well plates and treated with liposomal doxorubicin (LD) or targeting peptide conjugated with liposomal doxorubicin (OCSP-6-LD or OCSP-26-LD) for 8 h. The cells were then harvested, counted, washed in PBS, re-suspended in binding buffer (10 mm Hepes/NaOH pH 7.4, 140 mM NaCl, and 2.5 mM CaCl2), and stained with FITC-conjugated annexin V (Pharmingen, Becton Dickinson Co., San Diego, CA, USA). After staining, the cells were incubated for 15 min in the dark at room temperature. The cells were re-washed with binding buffer and analyzed by flow cytometry (FACSCalibar; Becton-Dickinson) using the Cell Quest software.

In vivo homing and competitive peptide inhibition experiments

OC-3 cells (5×10^6) were injected s.c. into the dorsolateral flanks of severe combined immunodeficient (SCID) mice (4–6 weeks old). Mice bearing size-matched lung cancer xenografts (approximately 300 mm^3) were selected and injected i.v. with 2×10^9 pfu of the targeting phages (OC-6 and OC-26) or control phages. After perfusion, the xenograft tumors and mouse organs (brain, heart and lung tissues) were retrieved and homogenized. The phages bound to each tissue sample were retrieved via the addition of ER2738 bacteria and titrated on IPTG/X-Gal agar plates.

For the *in vivo* peptide competitive inhibition experiments, the phages OC-6 or OC-26 were injected along with 100 μg of synthetic targeting peptide (OCSP-6 or OCSP-26) or the control peptide. The phages bound to the lung (as a normal tissue) or tumor were then retrieved via the addition of ER2738 bacteria and titrated on IPTG/X-Gal agar plates.

Synthesis of targeting peptides and the preparation of targeting peptide-conjugated liposomal doxorubicin

The targeting peptides OCSP-6 (LTPPGRLSSWPL) and OCSP-26 (SVTLSLRLPFPS) were synthesized by the Core Facility of the Institute of Cellular and Organismic Biology at Academia Sinica and purified to > 95% purity using reverse-phase high-performance liquid chromatography.

The procedures used to prepare the targeting peptide-conjugated liposomal doxorubicin were described in our previous study [16,17]. Briefly, the peptides were coupled to NHS-PEG-DSPE [Nhydroxysuccinimido-carboxyl-PEG (MW, 3400) derived distearoylphosphatidyl ethanolamine] at a 1:1.5 molar ratio. The coupling reaction was performed with the free amine group in the amino terminus of the peptide to produce peptidyl-PEG-DSPE and confirmed via the quantitation of the remaining amino groups with trinitrobenzenesulfonate reagent (Sigma-Aldrich). The Peptidyl-PEG-DSPE was then transferred to the preformed PEGylated liposomal doxorubicin after co-incubation at a temperature above the transition temperature of the lipid bilayer. The synthetic targeting peptides and targeting peptide-conjugated nanoparticles were analyzed by MALDI-TOF mass spectrometry [19].

Endocytosis and intracellular drug accumulation of targeting peptide-conjugated nanoparticles in human ovarian cancer cell lines

To evaluate endocytosis, OC-3 cells were plated and grown to ~80% confluence on coverslips. The cells were then incubated with either liposomal SRB (LipoSRB) or targeting peptide-conjugated liposomal SRB (OCSP-6-LipoSRB or OCSP-26-LipoSRB) at 37 °C in complete medium. After 1 hour of incubation, the cells were washed with PBS, stained with DAPI, and then examined using a Leica confocal microscope (TCS-SP5-AOBS). The images were processed with the Leica Application Suite Advanced Fluorescence software.

To assess the intracellular uptake of doxorubicin, tumor cells were grown to 90% confluence on a 24-well plate, and 2.5 mg/mL of free doxorubicin (FD), equipotency liposomal doxorubicin (LD) or targeting peptide-conjugated liposomal doxorubicin (OCSP-6-LD or OCSP-26-LD) in complete culture medium was added. The cells were incubated for 1, 2, 4, 8, 24, and 48 hours at 37 °C. At each indicated time point, the cells were washed with PBS, and drugs coated on the cell surface were removed by adding 0.1 M Glycine (at pH 2.8) for 10 minutes. The cells were then lysed with 200 μL of 1% Triton X-100. To extract doxorubicin, 300 mL of IPA (0.75 N HCl in isopropanol) was added to the lysate and shaken for 30 minutes. After the lysate was centrifuged at 12,000 rpm for 5 minutes, the total amount of doxorubicin was determined by measuring the fluorescence at $\lambda_{Ex/Em}$ = 485/590 nm using a spectrofluorometer (SpectraMax M5, Molecular Devices). The concentration of doxorubicin was calculated by interpolation from a standard curve.