Evaluation of anti-HER2 scFv-conjugated PLGA–PEG nanoparticles on 3D tumor spheroids of BT474 and HCT116 cancer cells

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Abstract
Three-dimensional culture cells (spheroids) are one of the multicellular culture models that can be applied to anticancer chemotherapeutic development. Multicellular spheroids more closely mimic in vivo tumor-like patterns of physiologic environment and morphology. In previous research, we designed docetaxel-loaded pegylated poly(D, L-lactide-co-glycolide) nanoparticles conjugated with anti-HER2 single chain antibodies (scFv–Doc–PLGA–PEG) and evaluated them in 2D cell culture. In this study, we continuously evaluate the cellular uptake and cytotoxic effect of scFv–Doc–PLGA–PEG on a 3D tumor spheroid model of BT474 (HER2-overexpressing) and HCT116 (HER2-underexpressing) cancer cells. The results showed that the nanoparticle formulation conjugated with scFv had a significant internalization effect on the spheroids of HER2-overexpressing cancer cells as compared to the spheroids of HER2-underexpressing cancer cells. Therefore, cytotoxic effects of targeted nanoparticles decreased the size and increased necrotic score of HER2-overexpressing tumor spheroids. Thus, these scFv–Doc–PLGA–PEG nanoparticles have potential for active targeting for HER2-overexpressing cancer therapy. In addition, BT474 and HCT116 spheroids can be used as a tumor model for evaluation of targeting therapies.

Keywords: PLGA–PEG, docetaxel, active targeting, anti-HER2, cancer cell spheroids, scFv

Classification numbers: 2.04, 2.05, 4.02

1. Introduction
Anticancer-drug-loaded nanoparticle formulations have become an important research area in cancer therapy. Nanocarriers for targeted drug delivery can improve the therapeutic effects and reduce the side effects of the anticancer drugs. Particularly, active targeting drug delivery systems, also called ligand-mediated targeting carriers, are ligands such as peptides, monoclonal antibodies, antibody fragments etc conjugated directly on the surface of nanoparticles to enhance specific retention and internalization in the targeted disease cells by overexpressed surface molecules or receptors [1–4]. Overexpression of HER2/neu protein is detected in many tumors including invasive breast, colorectal, ovarian, pancreatic, stomach and prostate cancers. Therefore human epidermal growth factor receptor 2 (HER2/neu) is one of the major targets for the design of targeted anticancer drugs [5–7]
and high affinity ligand-functionalized polymer nanoparticles for drug delivery [1]. Application of the biodegradable polymer poly(lactic-co-glycolic) acid has shown immense potential as a drug delivery carrier. Polymer- (poly(D, L-lactide-co-glycolide-) coated polyethylene glycol possesses many advantages such as biocompatibility, biodegradability and serum stability for formulating highly stable drug delivery systems [8]. Therefore, it is often used in the literature for nanoparticle formation [9–11].

In previous research we designed docetaxel-loaded pegylated poly(D, L-lactide-co-glycolide) nanoparticles conjugated and unconjugated with anti-HER2 single chain antibody (scFv) and evaluated in 2D cell culture. The results showed that nanoparticle formulations conjugated with scFv had a significant effect on HER2-overexpressing cancer cells as compared to HER2-underexpressing cancer cells [12]. Preclinical models closely resembling the original cancer and predicting clinical outcome have been investigated by pharmaceutical investigators before evaluating in vivo models. Therefore, three-dimensional (3D) culture systems can be applied as a preclinical effective tool for the development of anticancer drugs and treatments [13, 14]. Multicellular tumor spheroids are formulated by mimicking the 3D network of the cellular–matrix and cell–cell interactions. Tumor spheroids are similar to many features of in vivo tumor-like development patterns within human tumor tissue, especially avascular tumor nodules in terms of morphology and growth kinetic properties [15–17], and they more closely mimic the physiologic environment of living organisms compared to conventional monolayer culture systems [18].

The objective of this study is to evaluate the internalization and cytotoxicity of docetaxel-loaded PLGA nanoparticles (Doc–NPs) conjugated and unconjugated with single chain anti-HER2 antibody using a three-dimensional multicellular spheroid model of BT474 (HER2-overexpressing) and HCT116 (HER2-underexpressing) cancer cells.

2. Materials and methods

2.1. Materials

Poly(D, L-lactide-co-glycolide) (PLGA, lactide-glycolide 50:50), bifunction poly(ethylene glycol) 2-aminoethyl ether acetic acid (NH2–PEG–COOH), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) and N-hydroxysuccimide (NHS) were obtained from Sigma-Aldrich (St Louis, MO, USA). Docetaxel anhydrous was purchased from Shanghai Bioman Pharma (Shanghai, China). Monoclonal mouse anti-hexahistidine antibodies were purchased from Abcam (Cambridge, MA, USA); anti-mouse secondary antibodies conjugated with Alexa Fluor 546 were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Molecular and cellular biology buffers were purchased from Sigma and all other organic solvents were analytical grade from Fisher Scientific.

Human breast cancer cell line BT474 and human colon cancer cell line HCT116 (American Type Culture Collection) were obtained from the Institute of Biotechnology (VAST). The cell lines were cultivated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin at 37 °C in a humidified incubator with 5% CO2.

2.2. Methods

2.2.1. Synthesis of scFv–Doc–PLGA–PEG nanoparticles. The Doc-encapsulated PLGA–PEG copolymer nanoparticles (Doc–NPs) and the conjugation of anti-HER2 scFv to Doc–NPs was synthesized and characterized in our previous study [12]. Briefly, 10 mg of PLGA–PEG copolymer and 1 mg of Doc were dissolved in acetone. The mixtures were poured into Millipore water solution with solvent:water = 1:5. Nanoparticles were formed and gently stirred at room temperature for 4–5 s to evaporate the organic solvent. Then, 2 ml of Doc–NP solution (4 mg ml−1) was incubated with 200 μl of 4 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 200 μl of 10 mM NHS for 15 min at room temperature with gentle stirring. The activated particles were covalently linked to 100 μl of scFv (1 mg ml−1) for 2 s at room temperature and gently vortexed. The Doc–NPs conjugated with anti-HER2 scFv were purified from unconjugated proteins by ultrafiltration. The average size of nanoparticle derivatives was analyzed by dynamic light scattering (DLS). The zeta potential of NPs was evaluated using the electrophoretic mode of a Zetasizer 3000 HS (Malvern Instruments, UK). A transmission electron microscopy (TEM) system (JEOL JEM-1010, USA) was used to determine the shape and surface morphology of nanoparticles produced.

2.2.2. Formation of spheroids. The spheroid model is formed using the hanging drop method. After culturing, cells of BT474 and HCT116 reached growth phase (about 80% of culture dish) and were trypsinized and resuspended at a concentration of 1 × 10⁶ cells/ml. Twenty microliters of the cell solution was dropped on the lid of a 100 nm tissue culture plate and incubated for 4 days at 37 °C and 5% CO2 to form spheroids.

2.2.3. Penetration analysis of scFv–Doc–PLGA–PEG. The cellular uptake of Doc–NPs and scFv–Doc–NPs by BT474 and HCT116 cellular spheroids was observed by fluorescence microscopy. Tumor spheroids were grown in a 96-well plate for 4 days before experiments. The spheroids were then incubated with 100 μg ml−1 of Doc–NPs or scFv–Doc–NPs for 1 h at 37 °C. After washing twice with phosphate-buffered saline (PBS), samples were incubated with monoclonal mouse anti-hexahistidine primary antibody for 1 h at 4 °C and then washed three times with PBS 1x. Samples were incubated with Alexa Fluor 546 goat anti-mouse secondary antibody for 1 h at room temperature and washed with PBS 1x three times. Images from transmitted light, tomographic
scanning and the 3D image were analyzed using a Nikon Ti E Eclipse microscope equipped with a Ti-TIRF illumination unit and confocal system.

2.2.4. Spheroid growth and necrotic score. To investigate the effect of nanoparticle formulations on size growth and necrotic score formulation, tumor spheroids of BT474 and HCT 116 cells grown in 96-well plate were then incubated with 200 μl of Doc, Doc–NPs or scFv–Doc–NPs (15 μg ml⁻¹ calculated by Doc) for 5 days at 37 °C. Then spheroids were imaged using an Olympus IX83 Inverted microscope, and the images were analyzed using Olympus cellSens™ microscope imaging software.

3. Results and discussion

3.1. Characteristics of nanoparticles

Docetaxel-loaded nanoparticles were prepared by the nanoprecipitation method. Transmission electron microscopy showed that the morphology of nanoparticles was spherical (figure 1) with a mean size and zeta potential of 105 nm and −25 mV, respectively. Conjugation of Doc–NPs with
anti-HER2 single chain antibody fragments by covalent coupling via crosslinkers EDC and NHS resulted in an increase of mean size and zeta potential of targeted nanoparticles (scFv–Doc–NPs) to 135 nm and −28 mV respectively. The scFv–Doc–NPs bound specifically to BT474 cells (HER2 positive) but no MDA-MB-231 cells (HER2 negative) were investigated by flow cytometry and their internalization by the targeted cancer cells was revealed by confocal fluorescence scanning microscopy [12]. Moreover, the scFv–Doc–NPs showed stronger cytotoxicity on BT474 cells than MDA-MB-231 cells in a 2D cell model.

3.2. Analysis of cellular uptake

The human breast cancer cell line BT474 (HER2 over-expressing) was chosen for cytotoxicity studies. The cell line

Figure 3. Confocal fluorescence microscopy images of BT474 and HCT116 spheroids treated with scFv–Doc–NPs for 30 min with (a) transmitted light, (b) bright field, (c) the 3D image and (d) tomographic scanning of an intact BT474 spheroid.
comes from breast epithelial tissue, making it a good HER2-overexpressing cytotoxicity model [19]. Another cell line that was used in this study is the human epithelial colorectal carcinoma cell line HCT 116. This cell line has low expression of HER2 receptor [20] and the cells represent a model of low-level HER2 expression. To visualize cellular uptake of targeted nanocarriers, many studies had linked fluorescent factors on the surface of nanoparticles [21]. In this study a six-histidine tag fused with anti-HER2 scFv antibodies can be used to study the binding activity and cellular uptake of
scFv–Doc–NPs such that it did not change their characterization in vitro.

The size of spheroids that is suitable for the full exchange of oxygen and nutrients is about 100–500 μm. However, small spheroids (<100 μm) do not expose the complexity of tumor tissue with slow growth, while larger spheroids (>500 μm) with rapid development result in the limited diffusion of oxygen and nutrients [22]. In this study, the average

Figure 5. HCT116 spheroids. (a) The effect of nanoparticle formulations on central necrosis after incubation for 5 days: (A) control, (B) Doc, (C) Doc–NPs and (D) scFv–Doc–NPs. (b) Diagram of growth size after treatment for 5 days.
sizes of BT474 and HCT116 spheroids are 193.91 μm and 268.47 μm, respectively (figure 2).

Spheroids of two cell lines were treated with 100 μg of scFv–Doc–NPs; the fluorescence intensity through an anti-His tag antibody with Alexa Fluor 546 observed by confocal scanning microscopy in BT474 spheroids was stronger than in HCT116 spheroids (figure 3). In particular, when an intact BT474 spheroid was scanned at specific depths the tomographic scanning images (figure 3(D)) were taken every 2 μm from the top to the bottom and the 3D image (figure 3(C)) was reconstructed using tomography. These results showed that the permeability and cellular uptake of scFv–Doc–NPs for the BT474 spheroid were more effective than those for the HCT116 spheroid.

3.3. The effects on spheroid growth and necrotic score

BT474 and HCT116 spheroids treated with 100 μg of Doc–NPs (both non-targeted and targeted) and observed for 5 days were imaged with an Olympus IX83 inverted microscope with an interval of 1 day, and the images were analyzed using Olympus cellSens™ microscope imaging software. All images of spheroids were converted to simplified threshold images under the same conversion conditions and then the edges of the spheroids were recorded using a selection tool. Diameters of the spheroid edges were measured initially as pixels, and converted to micrometers by comparison to a reference length. The results showed that scFv–Doc–NPs have a more potent inhibitory effect than the other aqueous solution forms at a dose of 15 μg Doc in both BT474 (figure 4) and HCT116 spheroids (figure 5) through spheroid growth and formation of necrotic score. Targeted nanoparticles decreased the size and increased the necrotic score of HER2-overexpressing tumor spheroids—BT474—during 5 days of treatment (images (D) and (E) of figure 4(a)). However, both Doc–NPs and scFv–Doc–NPs have the same effect on HER2-underexpressing tumor spheroids—HCT116 (images (C), (D) and (E) of figure 5(a)). Thus, the toxicity of Doc–NPs conjugated with anti-HER2 scFv was more effective for BT474 spheroids than HCT116 spheroids.

4. Conclusion

BT474 and HCT116 spheroids developed by the hanging drop method can be used as a tumor model for evaluation of HER2-targeted therapies before further in vivo applications. Encapsulation of docetaxel in a nanopolymer formulation conjugated with anti-HER2 scFv improved permeability and cytotoxicity in the HER2-overexpressing multicellular spheroid model. Thus, this drug delivery system has been proposed as a potential approach to improve the efficacy of nanoparticles in active targeting for HER2-overexpressing cancer therapy.

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