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Human epidermal growth factor receptor-2 antibodies enhance the specificity and anticancer activity of light-sensitive doxorubicin-labeled liposomes

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ABSTRACT

Antibody-mediated targeting therapy has been successful in treating patients with cancers by improving the specificity and clinical efficacy. In this study, we developed a human epidermal growth factor receptor-2 (HER2) antibody-conjugated drug delivery system, using near-infrared (NIR) light-sensitive liposomes containing doxorubicin (DOX) and hollow gold nanospheres (HAuNS). We demonstrated the specific binding and selective toxicity of the system to HER2-positive tumor cells in co-cultures of HER2-positive and -negative cells. Furthermore, the HER2-antibody-mediated delivery of targeted liposomes was confirmed in a double-tumor model in nude mice simultaneously bearing HER2-positive and -negative tumors. This induced a >2-fold increased accumulation in the tumors with positive expression of HER2 than that with non-targeted liposomes (no HER2-antibody conjugation). The combination of targeted liposomes with NIR laser irradiation had significant antitumor activity *in vivo* with the tumor inhibition efficiency up to 92.7%, attributed to the increased accumulation in tumors and the double efficacy of photothermal-chemotherapy. Moreover, targeted liposomes did not cause systemic toxicity during the experiment period, attributable to the reduced dose of DOX, the decreased accumulation of liposomes in normal tissues, and the low irradiation power. The targeted liposomes provide a multi-functional nanotechnology platform for antibody-mediated delivery, light-trigged drug release, and combined photothermal-chemotherapy, which may have potential in the clinical treatment of cancer.

1. Introduction

Over the last few decades, antibody therapy has achieved clinical success in the treatment of hematological malignancies and solid tumors through improving the efficacy and decreasing the toxicity [1,2]. Recently, antibody-mediated molecular-targeting therapy by linking antibodies to biologically active molecules to form antibody-drug conjugates has been developed rapidly [3]. To date, three such conjugates have received market approval and more than 30 are currently in clinical trials. Antibody-targeting by conjugating antibodies on the surface of nanoparticles for effective delivery to target sites (tumors) to improve the therapeutic index and minimize off-target side-effects in normal tissues has also been widely exploited [4]. However, such progress is slow and there is only a few of formulations can be used for clinical trial. For example, liposomes, the best mainstream drug delivery system, have been extensively used in the clinic such as imaging for tumors and sites of infection, vaccine and gene delivery, and treatment of infections, cancer, lung disease, and skin conditions [5,6]. Although liposomes bearing various targeting ligands (such as antibodies, peptides, affibody etc.) appear to be promising candidates, the clinical benefit associated with targeting are under intense debate. There are only a few antibody-targeted liposomes become clinical trials [7]. The slow progress to the clinic is related to the complexity of manufacture and quality control for targeted nanomedicines compared to those traditionally seen for small-molecule therapeutics and non-targeted formulations [8].

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Recently, we developed a novel smart liposome formulation containing doxorubicin (DOX), a toxic anticancer molecule, and hollow gold nanospheres (HAuNS) that have plasmon absorption in the near-infrared (NIR) and display strong photothermal conduction [9]. In previous studies, we explored the potential utility of HAuNS as a biosafe photothermal conducting agent [10] for a variety of biomedical applications [11–14]. In the liposome formulation, HAuNS are attached to a thermosensitive membrane and DOX is encapsulated into the interior phase. The liposomes have the smart property of sensitivity to NIR light. Under an NIR laser, they have shown strong photothermal conversion, inducing a triggered release of DOX, and consequently provide a combined photothermal-chemotherapy. This leads to a remarkable higher antitumor efficacy as compared with the photothermal therapy or chemotherapy alone. However, our finding also revealed that the liposomes have minor systemic toxicity to the mice after intravenous injection, that could cause a maximal body weight loss of ~8% during treatment. But most of the loss weight could gradually recovered and each mouse has only 2.5% body weight loss at the end of three-week experiment. The systemic toxicity may have been due to the low specific accumulation and so an increased distribution of the liposomes in normal tissues.

In this study, we have improved the liposome formulation by conjugating HER2 (human epidermal growth factor receptor-2) antibodies on the surface to increase the targeted delivery to tumors. Based on NIR light-sensitive liposomes, we set out to establish a multifunctional nanotechnology platform for antibody-mediated targeted delivery, light-trigged drug release, and combined photothermal-chemotherapy by developing controllable methods for producing antibody-targeted liposomes, including the stable preparation of HER2 antibodies, a simple procedure for conjugating antibodies on liposomes, and reliable quality control. We hypothesized that targeted liposomes can achieve a significantly enhanced accumulation in tumors with high HER2 expression. As a result, the doses of liposomes and NIR laser irradiation can be reduced to obtain a safe and efficient photothermal-chemotherapy. HER2 is a member of the epidermal growth factor receptor family [15], which is frequently overexpressed in breast [16], gastric [17], and ovarian cancer [18], and plays important and complex roles in cell growth, survival, and differentiation. Therefore, HER2 is an important biomarker and therapeutic target for numerous human cancers [19].

2. Materials and methods

2.1. Materials

For the preparation of HER2-antibody (HER2ab), the pET-44b plasmid and *E. coli* BL21 (DE3) were from Novagen Inc. (Madison, WI). The pMD18-T simple vector and restriction endonucleases (Nde I and Xho I) were from Takara Biotech Co., Ltd (Dalian, China). DNA primers were synthesized by Sangon Biotech Co., Ltd (Shanghai, China). Isopropyl β-D-thiogalactopyranoside (IPTG) and protein molecular-weight markers were from MBI Co., Ltd (Hanover, Md). DNA molecular weight standards were from BioAsia Co., Ltd (Shanghai, China). 3,3′,5,5′-Tetramethylbenzidine (TMB) was from Merck (Germany).

Sodium citrate (>99%), cobalt chloride hexahydrate (99.99%), sodium borohydride (99%), and chloroauric acid trihydrate (American Chemical Society reagent grade) were from Thermo Fisher Scientific (Waltham, MA) and were used as received. Octadecyl-3-mercaptopionate (OMP) was from Chemical Industry Co. (Japan). Doxorubicin·HCl (DOX) was a gift from Zhejiang Hisun Pharmaceutical Co, Ltd, (Taizhou, Zhejiang, China). 1,2-Dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC), hydrogenated soybean phosphatidylcholine (HSPC), and cholesterol (Chol) were from Lipoid (Germany). PKH67 green fluorescent cell linker and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindotri-carbocyanine iodide (DiR) were from Invitrogen (Carlsbad, CA, USA). N,N-Disuccinimidyl carbonate (DSC) and octadecylamine (ODA) were from Shanghai Chemical Reagent Co. Ltd. (Shanghai, China). Fluorescein isothiocyanate (FITC), rhodamine B isothiocyanate (RITC), and DSPE-PEG_{2000}-NH_2 were from Sigma–Aldrich Inc., (St Louis, MO). DyLight649 goat anti-rabbit IgG was from Liankebio Co, Ltd, (Hangzhou, China). Horseradish peroxidase-conjugated anti-rabbit antibody was from Pufei Co., Ltd (Shanghai, China). The HercepTest™ kit (K5240) was from DakoCytomation Co., Ltd (Denmark). All other solvents were of analytical or chromatographic grade.

2.2. Cell culture and animals

SKOV3 (human ovarian carcinoma), BT474 and SK-Br-3 (human breast carcinoma), and A549 (human lung adenocarcinoma) cells were from the Institute of Biochemistry and Cell Biology (Shanghai, China). The cells were maintained in RPMI 1640 medium containing 10% fetal calf serum (Life Technologies, Inc., Carlsbad, CA) at 37 °C in a humidified atmosphere containing 5% CO_2. New Zealand rabbits and Balb/c nude mice were from the Experimental Animal Center at Zhejiang University, and housed under controlled conditions with standard commercial diets. All animal studies were carried out according to Institutional Animal Care and Ethical Committee-approved protocols.

2.3. Cloning and expression of the HER2 extracellular domain

The cDNA encoding the extracellular domain (amino-acids 23–652) of human HER2 (HER2-ECD) was cloned from SK-Br-3 cells by reverse transcription-polymerase chain reaction (RT-PCR) using specific primers according to the DNA sequence of HER2 (accession: NM_004448.2). The RT-PCR products were directly cloned into the pMD18-T simple vector and then subjected to DNA sequencing to confirm the desired sequence. The DNA fragment digested with restriction endonucleases of Nde I and Xho I was subsequently cloned into the expression plasmid pET44b. *E. coli* BL21 (DE3) transformed with the expression plasmid pET44b-HER2-ECD was grown to an optical density of 0.7 and induced at 30°C with 1 mM IPTG for 4 h. The bacteria were harvested by centrifugation at 5000 rpm for 10 min and re-suspended in phosphate-buffered saline (PBS)/5 mM EDTA. After sonication, the pellet (containing inclusion bodies) and supernatant were separated by centrifugation at 10000 × g for 15 min and then analyzed by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The inclusion bodies were washed with 2% Triton X-100 and dissolved in 8 M urea. The recombinant HER2-ECD was purified in a Ni-NTA column and eluted with 200 mM imidazole. Finally, the protein was renatured by serial dialysis and the purity was checked with 12% SDS-PAGE. The protein concentration was determined by the bicinchoninic acid method.

2.4. Preparation and activity of HER2 antibodies

Two male New Zealand rabbits were immunized by subcutaneous injection of a mixture of 1 mg recombinant HER2-ECD and 1 mg complete Freund's adjuvant. Every 10 days, 0.5 mg of the antigen mixed with the same volume of incomplete Freund's adjuvant were given three times. Blood samples were collected from the marginal vein of the ear before each immunization, and the sera were stored at −20 °C. The polyclonal antibody was precipitated by salting-out with 50% ammonium sulfate saturation, and then the precipitate was washed 3 times with 33% ammonium