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Follicle-stimulating hormone polypeptide modified nanoparticle drug delivery system in the treatment of lymphatic metastasis during ovarian carcinoma therapy

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HIGHLIGHTS

- The FSHP modified nanoparticle drug delivery system was assessed in treating lymphatic metastasis of ovarian cancer first time.
- The PTX concentration in the lymph nodes in the FSHP-NP-PTX group was higher than that in the other groups.
- FSHP-NP-PTX exhibits the most significant inhibiting effect on cell proliferation both in vitro and in vivo.

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ABSTRACT

Objective. Traditional chemotherapy drugs have an obvious drawback of nonspecific biodistribution in treating ovarian cancer. Follicle-stimulating hormone receptor (FSHR), a G-protein coupled receptor which is mainly expressed in reproductive system, is an important drug target in developing novel therapeutics.

Methods. Using a polypeptide of follicle-stimulating hormone (named as FSHP), a conjugated nanoparticle, FSHP-NP was developed to target FSHR in lymphatic metastasis of ovarian cancer. FSHP-NP was tested for recognition specificity and uptake efficiency on FSHR-expressing cells. A paclitaxel (PTX)-loaded FSHP-NP (FSHP-NP-PTX) was further developed and its anti-tumor effect was determined in vivo and in vitro.

Results. Taking NuTu-19 cells as an example, FSHP-NP-PTX displayed significantly stronger anti-cell proliferative and anti-tumor effects in a dose- and time-dependent manner when compared with free PTX or naked PTX-loaded nanoparticles (NP-PTX) in vitro. In vivo examinations showed that the size and weight of the lymph nodes were reduced in the FSHP-NP-PTX group.

Conclusion. FSHR as a novel therapeutic target in ovarian cancer and delivery of PTX via conjugated nanoparticle (FSHP-NP) might represent a new therapeutic approach in ovarian cancer.

Introduction

According to the newest data, ovarian cancer ranked the leading cause of death among gynecologic malignancies in developed countries [1]. The large mortality is partly because that ovarian cancer is usually diagnosed at an advanced stage, which, mostly occurs during cancer metastasis [2–4]. The 5-year survival rate of ovarian cancer is decreased with the increasing number of lymph node metastasis, from 68.7% in patients without lymphatic metastases, to 28.0% in patients with two or more metastasis [5]. It is crucial to find an effective method to treat ovarian cancer patients, especially those developing lymphatic metastases.

Routine treatment or prevention for patients bearing ovarian cancer with lymph node metastases is a combination of pelvic lymphadenectomy plus adjuvant chemotherapy. However, the conventional treatment has inevitable drawbacks, such as failure to clear all lymph nodes in surgery. Besides, most chemotherapy drugs, including paclitaxel (PTX), the most commonly used drug in chemotherapy of ovarian cancer, would concentrate in the liver and spleen, but not the targeted site [6]. Nanoparticles are often used in drug delivery systems to improve the transmembrane targeting specificity [7,8]. Research on targeted drug delivery systems has focused on improving the specificity, and a nanoparticle drug delivery system was suggested to reach targeted administration and reduce the side effects at the same time [9]. In addition, active drug targeted delivery systems modified by small molecules like nanoparticles on the surface could enable the binding specificity, ensuring that the specified drug carriers go into

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the target sites [10–12]. As a result, active targeting system combined with nanoparticles can be a promising tool in drug delivery.

Follicle-stimulating hormone receptor (FSHR) is distributed mainly in the genital system [13] and is known to be negative or none expressed in other important tissues. Many studies have proved that FSHR was expressed in ovarian epithelium and ovarian cancer [14], and FSHR mRNA was expressed positively in 100% of ovarian epithelial inclusions, specifically 100% of cystadenomas, 94% of borderline tumors, and 60% of carcinomas [15]. Considering the high expression of FSHR in ovarian cancer patients, FSH polypeptide (FSHP) modified nanoparticle administration system was developed to specifically target FSHR. In this study, we proposed to develop a nanoparticle administration system coated with a specified target towards lymphatic metastasis of ovarian cancer and to examine whether these modified nanoparticles had any therapeutical effect on lymph node metastases.

Materials and methods

Preparation and characterization of nanoparticles

Unmodified nanoparticles (NP) were prepared from Male-PEG-PLA and MePEG-PLA (East China University of Science and Technology, Wuhan, Hubei, China), which were obtained through a kind of emulsion/solvent evaporation technique as described previously [16]. After evaporation at 40 °C, the nanoparticles were collected by centrifugation at 15,000 rpm for 45 min. For coumarin-6 (Sigma-Aldrich, USA) loaded NP, coumarin-6 was added in copolymer solution before primary emulsification. Likewise, for PTX-loaded NP (NP-PTX), PTX (1 mg, Xi'an Sanjiang Bio-Engineering, Co., Ltd., China) was dissolved in the mixture before primary emulsification.

FSH peptide β81–95 (QCHCGKCDSDSTDCT) was synthesized and purified by high-performance liquid chromatography (HPLC). To prepare FSH peptide modified nanoparticles (FSHP-NP), the mixture of FSH peptide and nanoparticles (1:1) was magnetically stirred for 8 h at room temperature, and then eluted with 0.01 mol/L HEPES buffer through a Sepharose CL-4B column to remove the unconjugated peptide.

The morphology of nanoparticles was examined by transmission electron microscope (TEM) (H-600, Hitachi, Japan) following negative staining with sodium phosphotungstate solution. Particle size and zeta potential were determined by dynamic light scattering analysis using Nicomp Zeta Potential/Particle Sizer (model 380 XLS, Nicomp™, Santa Barbara, CA, USA). X-ray photoelectron spectroscopy (XPS) was used to analyze the surface chemistry of the nanoparticles and was carried out on a PHI-5000C ESCA system (Perkin Elmer, USA). Data analysis was carried out by AugerScan 3.21 software (RBD enterprises, USA). Determination of the contents of coumarin-6 and PTX was achieved by analyzing nanoparticles dissolved in methanol through a HPLC system. The drug loading capacity (DLC) and entrapping efficiency (EE) of coumarin-6 and PTX were calculated by the following equations:

$$\text{DLC}\ (\%) = \frac{\text{Amount of coumarin} - 6 \text{ in nanoparticles}}{\text{Nanoparticles weight}} \times 100\%$$

$$\text{DLC}\ (\%) = \frac{\text{Amount of PTX in nanoparticles}}{\text{Nanoparticles weight}} \times 100\%$$

$$\text{EE}\ (\%) = \frac{\text{Amount of coumarin} - 6 \text{ in nanoparticles}}{\text{Total amount of coumarin} - 6 \text{ in dispersion}} \times 100\%.$$

$$\text{EE}\ (\%) = \frac{\text{Amount of PTX in nanoparticles}}{\text{Total amount of PTX in dispersion}} \times 100\%.$$

Analysis of the FSHR expression

Immunocytochemical analysis

SKOV-3 cells were purchased from the cell bank of the Chinese Academy of Sciences (Shanghai, China). Fischer 344 rat derived epithelial ovarian cancer cell line NuTu-19 was a generous gift from Professor He Wang (Sichuan University, China), provided by the Comprehensive Cancer Center (Michigan University, USA). SKOV-3 cells and NuTu-19 cells were fixed with 4% paraformaldehyde and then incubated in 0.25% Triton® X-100 (Beyotime, China) for 15 min, respectively. Then the cells were incubated in turns with FSHR antibody and peroxidase-conjugated anti-rabbit IgG. The control group was performed with non-immune rabbit serum. The staining reaction was done with diaminobenzidine and hematoxylin.

RT-PCR analysis

Total RNA was extracted by using Trizol Reagent (Invitrogen, CA, USA), according to the manufacturer's instructions. Isolated RNA was reverse-transcribed by an RT system (Toyobo, Japan) and amplified with a PCR system (Tiangen, Beijing, China). The primer sequences used were as follows: FSHR (rat) forward (5′-ACGCCATTTTCACCAAGAAC-3′), FSHR (rat) reverse (5′-TGGGCTTGCATTTCATAACA-3′); GAPDH (rat) forward (5′-TACATGTTCCAGTATGACTC-3′), GAPDH (rat) reverse (5′-TGTGAGGGAGATGCTCAGTG-3′). Amplification was conducted in 20 μL reactions, containing 1 μL diluted cDNA, 1 μL forward and reverse primers, 7 μL ddH_2O and 10 μL PCR mixture. The PCR cycling conditions were: 1 cycle of 95 °C for 3 min followed by 37 cycles of 15 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C and a final extension of 3 min at 72 °C. An aliquot of the PCR product was subjected to electrophoresis on 2% agarose gel containing ethidium bromide and products were visualized under UV light (Peiqing Science & Technology Co., Ltd., Shanghai, China) and photographed.

Evaluation of uptake efficiency of FSHP-NP

The uptake efficiency was qualitatively analyzed by fluorescent microscopy (Olympus, Japan) and quantitatively determined via flow cytometry (Becton Dickinson, USA) as previously described [16]. Briefly, NuTu-19 and SKOV-3 cells were placed into a 6-well plate at a density of 1×10^5 cells/mL. Twenty-four hours later, nanoparticles (NP and FSHP-NP) were incorporated with different doses of coumarin-6 (1 μg/mL, 10 μg/mL and 100 μg/mL) for 60 min at 37 °C or incorporated with 10 μg/mL coumarin-6 for 30, 60 and 90 min at 37 °C. After that, cells were washed three times with HBSS (4 °C) and observed under a fluorescent microscope (Olympus, Japan). Quantitative analysis was conducted using a flow cytometry. After being incorporated with coumarin-6 (10 μg/mL for 60 min) and washed with HBSS (4 °C) for 3 times, cells were digested by trypsin–EDTA acid. Then, cells were resuspended in 0.5 mL PBS and being used for analysis of fluorescence intensity using a FACSCalibur flow cytometry (Becton Dickinson, USA).

In vitro cytotoxicity of FSHP-NP-PTX

To evaluate the cytotoxicity of FSHP-NP-PTX, NuTu-19 cells were shifted to 96-well plate at the density of 2.5×10^4 cells/mL and incubated overnight. Then the cells were incubated in serum-free medium with different concentrations of PTX, NP-PTX, or FSHP-NP-PTX (0.01 μM, 0.1 μM, 1 μM and 10 μM) for 48 h. Meanwhile, time-gradient experiments were also performed by incubating same concentrations of (0.1 μM) PTX, NP-PTX, and FSHP-NP-PTX for 24 h, 48 h and 72 h, respectively. Cell proliferation was tested by *Sulforhodamine B* (SRB, Sigma-Aldrich, USA) colorimetry at 570 nm wavelength. The inhibiting rate (%) = $[(OD_{[CG]} - OD_{[EG]}) / OD_{[CG]}] \times 100\%$.

In vivo distribution of nanoparticles

All animal studies were approved by the Ethical Committee of Fudan University. Fischer 344 pathogen-free female rats, 6–8 weeks old, were purchased from Vital River Laboratory Animal Technology Co., Ltd., China. Fischer 344 rats were housed in 12 h of light/12 h of dark cycles