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Suppress orthotopic colon cancer and its metastasis through exact targeting and highly selective drug release by a smart nanomicelle

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ABSTRACT

The treatment of metastatic cancer is a huge challenge at the moment. Highly precise targeting delivery and drug release in tumor have always been our pursuit in cancer therapy, especially to advance cancer with metastasis, for increasing the efficacy and biosafety. We established a smart nanosized micelle, formed by tocopherol succinate (TOS) conjugated hyaluronic acid (HA) using a disulfide bond linker. The micelle (HA-SS-TOS, HSST) can highly specifically bind with CD44 receptor over-expressed tumor, and response selectively to high GSH level in the cells, inducing disulfide bond breakage and the release of the payload (paclitaxel, PTX). To predict the antitumor efficacy of the micelles more clinically, we established an orthotopic colon cancer model with high metastasis rate, which could be visualized by the luciferase bioluminescence. Our data confirmed CD44 high expression in the colon cancer cells. Highly matching between the micellar fluorescence and bioluminescence of cancer cells in intestines demonstrated an exact recognition of our micelles to orthotopic colon tumor and its metastatic cells, attributing to the mediation of CD44 receptors. Furthermore, the fluorescence of the released Nile Red from the micelles was found only in the tumor and its metastatic cells, and almost completely overlapped with the bioluminescence of the cancer cells, indicating a highly selective drug release. Our micelles presented an excellent therapeutic effect against metastatic colon cancer, and induced significantly prolonged survival time for the mice, which might become a promising nanomedicine platform for the future clinical application against advanced cancers with high CD44 receptor expression.

1. Introduction

1.36 million people are diagnosed to suffer from colorectal cancer (CRC) each year, making CRC to be the third most commonly found cancer in the world [1]. Advanced colon cancer is often associated with the occurrence of tumor metastasis [2]. Currently, colectomy combined with chemotherapy was the most used treatment for both early and late stage CRC. A moderate number of patients after the treatment could be clinically remissive for years. However, 30–50% of all cases exhibited cancer recurrence after the period of remission, generally accompanied by metastasis, which induced high death rate of the patients. Chemotherapy drugs such as irinotecan, 5-fluorouracil, leucovorin, and oxaliplatin were frequently used to improve the prognosis of CRC patients with recurrence and metastasis [3,4]. Still, the median overall survival remained about only 2 years [3–5]. The treatment of metastatic cancer is still a huge challenge at the moment [6].

Cell differentiation 44 (CD44), a multistructural and multifunctional cell surface molecule, is frequently found to be overexpressed on colon cancer cells and plays an important role in colon cancer progression and metastasis [7,8]. CD44 is also regarded as a biomarker of cancer stem cell in colon primary tumor [9–12]. In addition, metastatic colon cancer cells, even different from primitive tumor cells, were still strictly related to the phenotype CD44 positive expression [13–15]. Hyaluronic acid (HA) is one of the chief components of extracellular matrix, playing a significant role in cell proliferation and migration [16]. Many researchers have reported that CD44 was the major receptor of HA in cancer cells [8] and frequently employed HA as carrier systems to shuttle drug molecules into the tumor with high CD44 expression [8,17–21]. However, still low targeting delivery efficiency and the lack of the specific drug release in the tumor as well as its metastatic cells have limited the therapeutic effect of those strategies.

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In this study, a smart nanosized micelle (HA-SS-TOS, HSST) was synthesized by D-α-Tocopherol Succinate (TOS) conjugated HA using a disulfide bond as the linker for targeting therapy of colon cancer. HSST micelles are expected to specifically bind with CD44 receptors overexpressed on colon tumor and its metastatic cells, and then selectively response to the high level of glutathione (GSH) in the cancer cells, so as to obtain a controlled release of a chemotherapy drug (Paclitaxel, PTX) by the breakage of disulfide bond. In order to predict antitumor effect of the micelles more clinically, we generated an orthotopic colon cancer model with high metastasis rate that could be visualized by luciferase bioluminescence of the cells. The progression and migration of orthotopic colon cancer cells during the treatment can be monitored by determining the density of bioluminescence. We hypothesized that PTX loaded HSST micelles (PTX@HSST) were able to efficiently eliminate colon tumors and their metastasis cells by a highly specific delivery into the cells and the selective PTX release. So, CD44 receptor expression on colon cancer cells was examined and the specific accumulation of HSST micelles and selective PTX release in orthotopic primary and metastatic tumors was investigated. Our study indicated that PTX@HSST presented a fast and dramatically precise distribution in primary orthotopic colon tumor and its metastatic sites after intravenous injection, and thus provided a promising therapeutic strategy against colon tumors and its metastasis in the future clinical application.

2. Materials and methods

2.1. Materials

Hyaluronic acid (HA, molecular weight: ~5700 Da) was purchased from Freda Biochem Co., Ltd. (Shandong, China). N-Hydroxysuccinimide (NHS), 1-Ethyl-3 (3-dimethylaminopropyl) carbodiimide (EDC), cystamine, adipicdihydrazide, Nile Red (NR) and D-α-Tocopherol Succinate (TOS) were purchased from Aladdin Reagent Database Inc. (Shanghai, China). Indocyanine Green (ICG) was purchased from TCI Development Co., Ltd. (Japan). Taxol and Paclitaxel (PTX) were gifted from Zhejiang Hisun Pharmaceutical Co., Ltd. (Taizhou, Zhejiang, China). CD44 antibody (ab51037) was purchased from Abcam Co, Ltd. (Shanghai, China). All of the other chemicals were of analytical or chromatographic grades.

2.2. Cell culture

CT26 (mouse colon carcinoma) and NIH-3T3 (mouse embryo fibroblast) cell lines were purchased from Institute of Biochemistry and Cell Biology (Shanghai, China). Parental CT26 (p-CT26) cells were obtained from a CT26 neoplasia mouse. CT26-Luc (Luciferase-expressing mouse colon carcinoma) cell line was purchased from the Company Limited of Science light Biology Science & Technology (Shanghai, China). HT29 (human colorectal adenocarcinoma) and LOVO (human colorectal adenocarcinoma) cell lines were purchased from Cell library of the Chinese Academy of Sciences (Shanghai, China). CD44-silencing CT26 cells (siCD44 CT26 cells), were obtained by a siRNA transfection using lipofectamine^{2000} (GeenPharma no. NM_ 001039150). CT26 cells were incubated with siRNA complexes for 24 h. Then, CD44 silence efficiency was measured by western blotting.

HT29, LOVO, p-CT26, CT26 and CT26-Luc cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (Life Technologies, Inc., Carlsbad, CA) and 100 U/mL penicillin and 100 U/mL streptomycin. NIH-3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Synthesis and characterization of HSST conjugates

The primary amino groups of cystamine (CYS) were coupled with the carboxyl groups of sodium hyaluronic acid (HA) through amination reaction. Briefly, HA (MW = ~5700 Da) in PBS solution (10 mg/mL) was mixed with EDC and NHS, and the mixture was stirred at room temperature for 60 min to activate the carboxyl groups of HA. Then, cystamine dihydrochloride was added, followed by the incubation for 8 h. The resulting solution was exhaustively dialyzed (MWCO: 3500 Da) in distilled water to remove the unreacted agents. CYS conjugated HA (HA-CYS) were lyophilized and stored at 4 °C for further use.

HA-SS-TOS (HSST) was synthesized by conjugating D-α-Tocopherol Succinate (TOS) to HA-CYS. Briefly, TOS was dissolved in DMSO at room temperature and mixed with NHS and EDC for 60 min to activate the carboxyl groups of TOS. Then, HA-CYS that had been dissolved in formamide was added to the above solution and stirred for 24 h at room temperature. Subsequently, HSST was precipitated from the solution by adding frozen ethanol, and the precipitate was further dissolved in distilled water. The product solution was dialyzed (MWCO: 3500 Da) in an excess amount of water/ethanol (1:1, v/v) for 1 day and distilled water for another 2 days. Finally, the solution was filtered through a 0.45 μm pore-sized membrane, lyophilized and stored at 4 °C for further use.

For PTX loading, a solution of PTX in ethanol (25 mg/mL) was dropwise added to the aqueous solution containing HSST (5 mg/mL) at a weight ratio of PTX/micelle (1/10) under the constant stirring. Then, the solution was dialyzed against distilled water overnight (MWCO: 3500 Da). The unloaded PTX was removed by centrifugation at 4000 rpm for 10 min and further filtered through a microporous membrane with 0.45 μm pores. PTX loaded HSST micelles (PTX@HSST) were obtained and the amount of PTX in the micelles was determined using an HPLC method [22].

The morphology of the micelles was examined using transmission electron microscopy (TEM; JEOL JEM-1230 microscopes at 120 kV, JEOL, Japan). The stability of PTX@HSST in pure water, PBS (PTX@HSST-PBS, pH7.4), and PBS with 10% fetal bovine serum (FBS) (PTX@HSST-10% FBS) 4 or 37 °C were measured using dynamic light scattering (DLS) by a Zetasizer (3000 HS, Malvern Instruments Ltd.).

2.4. CD44 receptor expression and cellular uptake

Briefly, CT26 and NIH-3T3 cells were incubated with primary antibody (anti-CD44 antibody) for 1 h at 4 °C, washed with PBS for three times and then incubated with secondary antibody (Alexa Fluor 680-conjugatedgoat anti-mouse IgG, Invitrogen). After repeatedly rinsed with PBS, the cells were observed by confocal laser scanning microscopy (CLSM, Nikon A1R, Japan).

To quantify CD44-mediated cell uptake, CT26 and NIH-3T3 cells were incubated with PTX@HSST (5 μg PTX/mL) for different time. For blocking experiment, the cells were pre-treated with HA (5 mg/mL) for 0.5 h, and then incubated with PTX@HSST. The cells were rinsed with PBS for three times and lysated using a lysis buffer. PTX was determined using a HPLC method [23]. The concentration of PTX was normalized against protein content as measured by BCA assay.

2.5. In vitro cytotoxicity

The anticancer effect of PTX@HSST was investigated by MTT method according to the manufacturer's suggested procedures. CT26 and NIH-3T3 cells were treated with free PTX (Taxol), PTX@HSST or blank HSST micelles (as a control) for 48 h. HT29,