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Comprehensively priming the tumor microenvironment by cancer-associated fibroblast-targeted liposomes for combined therapy with cancer cell-targeted chemotherapeutic drug delivery system

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ABSTRACT

Cancer-associated fibroblasts (CAFs) not only support tumorigenesis and tumor metastasis by reciprocal cellular cross-talk with cancer cells, but also remodel the extracellular matrix (ECM) and architecture of tumor microenvironment. This leads to poor tumor penetration of traditional chemotherapeutic nanomedicines and resulting drug resistance. In this study, we use a novel tumor stroma-targeted nanovehicle (FH-SSL-Nav) to specifically eradicate CAFs, promote tumor penetration of nanomedicines and cut off the stroma's support to cancer cells. FH-SSL-Nav exhibited excellent and comprehensive tumor microenvironment modulation including downregulation ECM deposition, decreasing interstitial fluid pressure (IFP) and facilitating blood perfusion. As a result, more chemotherapeutic drug delivery systems penetrated deep into tumor spheroids *in vitro* and tumor tissues *in vivo*. Furthermore, chemotherapeutic drug resistance induced by microenvironment was partly reversed by FH-SSL-Nav. In a human Hep G2 xenograft nude mouse model, FH-SSL-Nav greatly improved the tumor suppression of cancer cell-targeted liposomal doxorubicin (7pep-SSL-DOX) with low dose and low toxicity. Since Nav and DOX exhibited no synergy against Hep G2 cells, it was clear that the improved antitumor efficacy was basically due to the comprehensive tumor microenvironment priming by FH-SSL-Nav.

1. Introduction

Exploiting the enhanced permeability and retention (EPR) effect and high affinity moieties for tumor cell surface, versatile passive targeting nanoparticles and active targeting delivery system for tumor cells have been fabricated and investigated for tumor diagnosis and therapy [1]. Great innovation has been achieved to materialize and amplify tumor targeting over the past three decades [2]. Nevertheless, some drawbacks are emerging which limit the tumor inhibition effect of traditional cancer cell-targeted drug delivery system or nanodrug *in vivo*, among which the main bottlenecks are inefficient EPR effect [3], poor tumor penetration [3–6] and easily acquired drug resistance [7]. Tumor microenvironment, constitutive of cancer cells, stromal cells, immune cells, abnormal blood vessels and extracellular matrix (ECM), is responsible for the aforementioned bottlenecks of cancer cell-targeted chemotherapeutic drug delivery systems [8–10]. Owing to poor blood perfusion, elevated interstitial fluid pressure (IFP), dense ECM and accumulated solid stress in tumor microenvironment, cancer cell-targeted drug delivery systems fail to penetrate tumor tissues (<5 cell diameters) and localize only in perivascular areas, thereby greatly limiting their therapeutic efficacy [11–14]. In addition, chemical factors and growth factors secreted by stromal cells result in drug resistance to chemotherapy [15].

To circumvent the limitations, two main strategies have been applied: tumor microenvironment stimuli-triggered structure optimization of nanocarriers and tumor microenvironment modulation [16–19]. Small size [20] and cell penetrating peptides (CPPs) [21] are two main methods for the first strategy. Numerous platforms based on particle size shrinking triggered by tumor microenvironment [9,22–24] and CPPs decoration [25–27] have achieved favorable tumor penetration. However, because of the tumor heterogeneity [28] and non-selectivity of CPPs [29], the application of both methods were limited. On the

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other hand, several approaches have been developed to modulate the tumor microenvironment, including vascular normalization, direct ECM degradation or inhibition of ECM synthesis, chemotherapeutics-induced tumor priming, as well as reducing IFP [10]. Nonetheless, most of these modalities can only regulate single one or two aspects of tumor microenvironment, and are difficult to be precisely controlled, such as the narrow therapeutic window of time and concentration required in intratumoral vascular normalization [5]. What's more, tumor vascular normalization also may weaken the specific EPR effect, leading to less tumor tissue accumulation and permeability of nanoparticles [30]. Besides, physical stimuli-triggered tumor permeation enhancement has also applied for better drug delivery, such as ultrasound [31] and photo-thermal [32]. However, most physical stimuli have a limited penetration in biological tissues, especially light [33]. Therefore, novel strategies are urgently needed to regulate the microenvironment completely for increasing tumor accumulation and deep tumor penetration.

Cancer-associated fibroblasts (CAFs), which constitute the major population of tumor stroma, are found to be barriers for oncotherapy in several aspects. On one hand, as accomplice of cancer cells, CAFs participate in tumor-stroma biological interaction by secreting numerous cytokines to mediate signaling pathways for promoting tumor proliferation, angiogenesis and resistance [34–36]. On the other hand, through surrounding tumor cells and secreting ECM, CAFs create a protecting physical barrier and a dense scaffold for cancer cells, contributing to elevated IFP and weakened blood perfusion. As a result, nanoparticles and cancer cell-targeted delivery systems are hard to reach the cancer cells, leading to physical resistance of cancer cells and negating the effect of cancer cell-targeted chemotherapeutic drug delivery systems. Therefore, it might be a promising strategy to exhibit comprehensive tumor microenvironment modulation, deep tumor penetration of nanoparticles and weakened stroma's support to cancer cell by specifically and thoroughly eradicating CAFs.

In our recent study, we found that a novel tumor stroma-targeted nanoliposome system, FH-SSL-Nav, could target CAFs *in vitro* and *in vivo* [37]. Navitoclax (Nav) is a targeted high-affinity small molecule, which could specifically induce apoptosis in CAFs at a very low dose, but lack similar effect in normal tissue cells [38]. FHKHKSPALSPVGGG peptide (FH for short), is a small peptide isolated by phage display, and have been proved to show an extremely high binding affinity to Tenascin C (TNC) [39]. TNC is a tumor-specific extracellular matrix highly expressed in most solid tumors, and mainly secreted by CAFs. The CAF eradicating of FH-SSL-Nav *in vivo* has been confirmed in Hep G2 tumor-bearing nude mice model. HAIYPRH (7pep) is a seven-amino-acid peptide that identified by phage display to specifically recognize and bind to the human transferrin receptor (hTfR), which is overexpressed in numerous cancers [40]. The cancer cell targeting effect of 7pep-modified nanocarriers have been verified in several studies [41,42].

The aim of this study was to verify the hypothesis that CAFs eradicating effect of FH-SSL-Nav could proceed to modulate the tumor microenvironment comprehensively and be beneficial for tumor penetration of chemotherapeutic drug-loaded nanoparticles. The 7pep-modified and doxorubicin-loaded sterically stabilized liposomes (7pep-SSL-DOX, cancer cell-targeted delivery system) and nonmodified liposomes (SSL-DOX) were used to evaluate the tumor penetration promoting effect of FH-SSL-Nav in co-culture models and Hep G2 xenograft nude mouse model. In addition, the combination therapy outcome was also investigated *in vitro* and *in vivo*.

2. Materials and methods

2.1. Materials

DSPE-PEG_{2000}-NHS and DSPE-PEG_{2000} were purchased from NOF Corporation (Tokyo, Japan), and egg phosphatidylcholine (EPC) was from Lipoid GmbH (Ludwigshafen, Germany). Cholesterol (Chol) and Sephadex G-50 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nav was kindly provided by Biochempartner Biomart Co., Ltd. (Shanghai, China). Fluorescent probe DiR, DiD, DiI and DiO were all purchased from Biotium Inc. (Hayward, CA), and Pentahydrate (bisbenzimide) (Hoechst 33,258) was from Molecular Probes Inc. (Eugene, OR). DMEM medium, penicillin and streptomycin were supplemented by Macgene Biotech Co., Ltd. (Beijing, China). Fetal bovine serum (FBS) was obtained from Gibco. Low melting point agarose (LMP) was provided by Biodee Biotech (Beijing, China). All other chemicals and reagents were of analytical or HPLC grade

2.2. Liposomes preparation

The CAF-targeted liposomal Nav (SSL-Nav and FH-SSL-Nav) with optimized particle size around 90 nm were prepared by thin lipid film hydration followed by probe sonication as we reported previously [37], and cryogenic transmission electron microscopy (Cryo-TEM) was used to observe the morphology. Ammonium sulfate gradient was used to prepare the DOX-loaded tumor cell-targeted liposomes (SSL-DOX and 7pep-SSL-DOX), and the formulation characterizations are described in Supplementary material.

The same method was used for preparation of coumarin-6-loaded liposome (SSL-Cou6), DiR-loaded liposomes (SSL-DiR, 7pep-SSL-DiR) and DiD-loaded liposomes (SSL-DiD) in the following studies.

2.3. In vitro cellular uptake

LX-2 cells were seeded into 12-well plates at a density of 1.5×10^5 cells/well and cultured at 37 °C overnight. Prior to the experiment, cells were washed twice with cold PBS to remove the remaining complete culture medium. Free Nav, SSL-Nav and FH-SSL-Nav were diluted with serum-free DMEM medium into 20 μg/mL, and then added into each well. In addition, the drug-free culture medium was applied as the blank control. Competition inhibition group was pre-incubated with free FH peptide (0.2 mg/mL) for 1 h and then treated with a mixture of FH-SSL-Nav and free FH peptide. After incubated at 37 °C for 3 h, cells were washed with cold PBS, ruptured with 200 μL RIPA lysis buffer, and then dissolved with 300 μL anhydrous methanol. The concentrations of Nav were quantified by RP-HPLC with a mobile phase containing 1 mM ammonium acetate and 0.1% acetic acid in a mixture of methanol and water (95:5, v/v) and detected at 215 nm.

2.4. Tumor IFP measurement

Female BALB/c nude mice of 18–20 g were obtained from Peking University Health Science Center (Beijing, China), and kept under specific pathogen free (SPF) condition with free access to standard food and water. All care and handling of animals were performed with the approval of the Ethics Committee of Peking University. The armpit hepatocellular carcinoma was established by inoculating 1×10^7 Hep G2 cells in the right flank subcutaneous of each mouse. Tumor IFP was measured by "wick-in-needle" method using a tip sealed 21-gauge needle with a side hole and a pressure transducer (PowerLab, ADInstruments, Australia), as previously reported [17].

2.4.1. Reducing tumor IFP with consecutive dosing regimen

When tumor volume reached about 200 mm^3, the tumor-bearing mice were treated with three consecutive administration of PBS, SSL-Nav or FH-SSL-Nav *via* the tail veins at a dosage of 5 mg/kg Nav (200 μL), respectively, or free Nav through oral gavage at the same dosage. Tumor IFP measurements were carried out before and after treatment. After the final measurement, 20 μL whole blood was collected from the orbit of each mouse and complete blood counts were performed to evaluate the hematotoxicity of various Nav formulations.