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Enhanced antitumor and anti-metastasis efficiency via combined treatment with CXCR4 antagonist and liposomal doxorubicin

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ABSTRACT

Metastasis is the main cause of cancer treatment failure and death. However, current therapies are designed to impair carcinoma metastasis mainly by impairing initial dissemination events. CXCR4 is a G-protein coupled receptor that exclusively binds its ligand CXCL12, which can stimulate cells to metastasize to distant sites. As the antagonist of chemokine receptor CXCR4, Peptide S exhibited anti-metastasis effect. In order to enhance treatment efficiency through destroying primary tumors and inhibiting their metastases, we combined PEGylated doxorubicin-loaded liposomes (DOX-Lip) with anti-metastasis Peptide S for tumor therapy for the first time. DOX-Lip exhibited similar cytotoxic activity compared to free DOX *in vitro*, and Peptide S showed no toxic effect on cell viability. However, the Peptide S sensitized CXCR4-positive B16F10 melanoma cells to DOX-Lip (5 μM) when cocultured with stromal cells (50.18 ± 0.29% of viable cells in the absence of Peptide S vs 33.70 ± 3.99% of viable cells in the presence of Peptide S). Both Peptide S and DOX-Lip inhibited the adhesion of B16F10 cells to stromal cells. We further confirmed that the inhibition of phosphorylated Akt (pAkt) by Peptide S played a key role due to the fact that activation of pAkt by DOX-Lip promoted resistance to chemotherapy. Migration and invasion assays showed that DOX-Lip enhanced anti-metastasis effect of Peptide S *in vitro* because of the cytotoxicity of doxorubicin. *In vivo* studies also showed that the combined treatment with DOX-Lip and Peptide S not only retarded primary tumor growth, but also reduced lung metastasis. Both the DOX-Lip and DOX-Lip + Peptide S exhibited even more outstanding tumor inhibition effect (with tumor growth inhibition rates of 32.1% and 37.9% respectively). In conclusion, our combined treatment with CXCR4 antagonist and liposomal doxorubicin was proved to be promising for antitumor and anti-metastasis therapy.

1. Introduction

As the primary cause of cancer-associated death, metastatic cancers are mostly incurable because of its systemic nature and the resistance of disseminated tumor cells to existing therapeutic agents [1]. 90% of mortality from cancer is attributable to metastases rather than the primary tumors from which these malignant lesions arise [2]. Conventional chemotherapy is a major therapeutic approach for the treatment of cancers which can kill most active primary or circulating tumor cells directly. For many types of cancers, cytotoxic and cytostatic drugs have achieved some success in treating primary tumors [3]. In addition, nanocarriers have been widely used to deliver chemotherapeutic drugs. Various PEGylated liposomes were studied in our lab [4–6], which could prolong circulation time and enhance the accumulation of liposomes in tumor tissues through enhanced permeability and retention (EPR) effects [7] and liposomal formulations have already been approved for human use like Doxil® and DOX-SL®. However, current cytotoxic agents and rationally designed targeted compounds often displayed only limited activity against the corresponding metastatic lesions [8]. Possible reasons for unsatisfied therapeutic effect could be due to the fact that slowly growing micrometastases can resist the effects of cytotoxic agents which principally target cells in their active growth and division cycle [9], and the neoplastic cells within metastases are intrinsically more drug-resistant than the cells in the corresponding primary tumors [10]. What's more, some researches had proved that treatment of antitumor drugs might increase the invasiveness of tumor cells *in vitro* [11]. Therefore, monotherapy of drug loaded nano-formulations could be ineffective to metastasized tumors, and chemotherapeutics might be an inducement of tumor metastasis.

Although many developed nano-formulations have focused on delivering apoptosis-inducing therapies to the bulk tumor, tackling cancer metastasis will require the development of novel platforms which are more specific in targeting residual cancer cells that have migrated away from the bulk tumor, rather than only debulking the main tumor mass. Cancer metastasis involves the invasion of tumor cells to blood or lymph vessels, intravasation into the vessel, extravasation from the blood vessel in another location, and invasion into the tissue to form a secondary tumor [12]. Current strategies are focused on different stages of tumor metastasis, such as anti-angiogenic agents,

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modulating protease activity, suppressing cancer cell invasion, and so on. Among various biomolecules involved in cell invasion and metastasis, chemokine receptors play a critical role [13]. As a highly conserved transmembrane G-protein coupled receptor expressed in tumor cells, CXCR4 activated by CXCL12 can induce invasion and metastasis of many malignant tumors [14], including colorectal cancer [15], ovarian cancer [16], melanoma [17], and others. Blocking CXCL12/CXCR4 interaction may be promising therapeutics for metastases because CXCL12/CXCR4 axis triggers a variety of responses such as cell proliferation, chemotaxis and gene transcription. Small molecule inhibitors of CXCR4, like AMD3100 or blocking antibodies, are being investigated and has achieved certain anti-metastasis effect so far [18]. Recently, a new family of peptides was designed as CXCR4 antagonist, which showed anti-metastasis effect to a certain extent. Peptide S (Arg–Ala–[Cys–Arg–His–Trp–Cys]) was one of them [19]. However, these anti-metastatic therapies were designed more likely to impair initial dissemination events, and they could hardly suppress tumor growth.

Collectively, these findings further reinforced the importance of developing new approaches to inhibit the survival of primary tumor cells and the metastases. Katsuhisa et al. [20] investigated the effectiveness of the combination therapy of adriamycin (ADR) and anti-metastasis agent rh-SOD against highly metastatic clone *in vitro*. Interestingly, some studies have shown that CXCR4 inhibitor AMD3100 could disrupt tumor–stroma interactions and make them more sensitive to chemotherapeutic drugs [21,22]. These studies suggested that combined treatments of CXCR4 inhibitors and chemotherapeutic drugs might be a potential therapeutic strategy for anti-metastasis treatment. However, current researches only focused on the antitumor effects *in situ*, while the anti-metastasis efficacy and mechanism of these combination treatments were still unclear.

To explore a new strategy for enhancing antitumor and anti-metastasis efficiency, we first combined the anti-metastasis agent Peptide S with PEG_{2000} modified liposomal doxorubicin (as illustrated in Fig. 1). Then we tested whether Peptide S could sensitize tumor cells to liposomal doxorubicinvia CXCL12/CXCR4 axis, and the probable mechanisms were also investigated. The anti-invasion effect induced by Peptide S was then evaluated *in vitro*. Finally, the *in vivo* antitumor and anti-metastasis efficacy was studied to prove that combination therapy of liposomal doxorubicin and Peptide S could enhance anti-metastasis effect by destroying local tumor cells while Peptide S prevented cell invasion and metastasis at the same time.

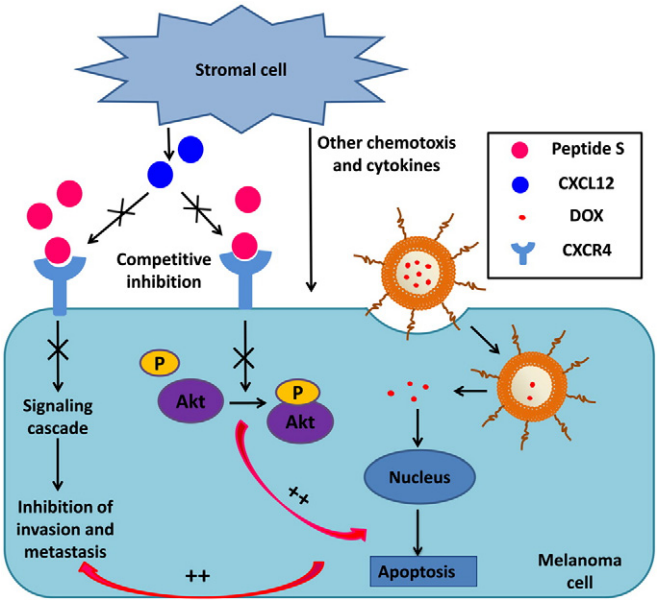

Fig. 1. Schematic illustration of combined treatment with Peptide S and DOX-Lip.

2. Materials and methods

2.1. Materials

Inhibitory Peptide S of CXCR4 (Arg–Ala–[Cys–Arg–His–Trp–Cys]) was synthesized according to the standard solid phase peptide synthesis by ChinaPeptides Co., Ltd. (Shanghai, China). Soybean phosphatidylcholine was purchased from Shanghai Advanced Vehicle Technology L.T.D. Co. (Shanghai,China) and Cholesterol was purchased from Kelong Chemical Company (Chengdu, China). 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine-poly (ethylene glycol) 2000 (DSPE-PEG_{2000}) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Recombinant Murine SDF-1α/CXCL12 was obtained from PeproTech (New Jersey, USA). Cell culture inserts for 24-well plates (8.0 mm pores, Translucent PET Membrane) and BD Matrigel™ Basement Membrane Matrix were purchased from BD Biosciences (Franklin Lakes, NJ). Rabbit anti-CXCR4 polyclonal antibody and rabbit antibody against phospho-Akt (Ser473) was purchased from EnoGene (Nanjing, China). Horseradish peroxidase (HRP) -labeled goat anti-rabbit secondary antibodies were purchased from ZSGB-BIO (Beijing, China). Fluorescence probe Fluo-3 AM and Cell Counting Kit 8 were purchased from Dojindo (Beijing, China)

C57/BL6 mice (6–8 weeks old, 18–22 g) were purchased from the Experimental Animal Center of Sichuan University (Sichuan, People's Republic of China). All animal experiments for this study were approved by the Experimental Animals Administrative Committee of Sichuan University.

2.2. Cell lines and cell culture

Mouse metastatic melanoma cells (B16F10), human cervical carcinoma cells (Hela) and mouse fibroblast (L929) were obtained from State Key Laboratory of Biotherapy (Sichuan University) and were cultured in DMEM medium (GIBCO) supplemented with 10% FBS, 100 μg/mL streptomycin and 100 U/mL penicillin at 37 °C in a humidified incubator with 5% CO_2.

2.3. Expression of CXCR4 in B16F10 cells

The expression of CXCR4 in Hela cells and B16F10 cells was measured by Western blot studies. Approximately 5×10^6 cells were harvested, washed with cold PBS, and lysed in ice-cold lysis buffer containing protease inhibitors. The lysate was centrifuged at 14,000 rpm for 15 min at 4 °C to collect the supernatant proteins. Then appropriate total protein samples of different cells were separated by 12% SDS-PAGE and transferred to polyvinylidene difluoride membranes. After incubating with primary antibody against CXCR4, the membranes were incubated with HRP-labeled goat anti-rabbit secondary antibodies and detected by Immobilon Western HRP Substrate on a Bio-Rad ChemiDoc MP System (Bio-Rad Laboratories, USA).

2.4. Intracellular free Ca^{2+} detection

To monitor the effect of Peptide S on intracellular calcium concentration, we used Fluo-3 AM to examine the level of intracellular free Ca^{2+} of B16F10 cells. Fluo-3 AM is a fluorescent dye which could penetrate the cell membrane. After Fluo-3 AM into the cells, it can be cut into Fluo-3 by intracellular esterase and stay in the intracellular. Fluo-3 can be combined with calcium ions, and then produce strong fluorescence. After loading with the Fluo-3 dye for 30 min at 37 °C, B16F10 cells were washed with HBSS solution and exposed to CXCL12 with or without Peptide S. Detection of intracellular Ca^{2+} was carried by flow cytometer (Cytomics™ FC500, Beckman Coulter, Miami, FL, USA).