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Original article

Growth-inhibition of S180 residual-tumor by combination of cyclophosphamide and chitosan oligosaccharides *in vivo*

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ABSTRACT

Chitosan oligosaccharides (COS), hydrolyzed products of chitosan, have recently been reported to have various biological activities. Herein, the present study was undertaken to assess the ability of COS to potentiate the antitumor effect of cyclophosphamide (CTX) as well as alleviating the CTX-associated toxicities *in vivo,* in a residual-tumor; a model which is closer to clinical surgery. Sarcoma 180 (S180) residual-tumor mice were divided into 6 groups ($n = 6$); including control, CTX, COS 40 mg/kg, COS 80 mg/kg, and combination groups (CTX + COS 40, CTX + COS 80). Animals were killed 18 days post-intraperitoneal administration and the tumors were weighed. The spleens were harvested to determine lymphocytes proliferation and NK cell activities; blood cells were evaluated by flow cytometry, and the expression levels of TNF-α were measured using ELISA. Notably, the combined therapy (CTX + COS80) showed the most effective reduction of the tumor weight, the highest inhibition of tumor growth, and proliferation, when compared with control as well single CTX group. Additionally, COS was able to recover the CTX-induced decreases in the lymphocyte proliferation, splenocyte NK cell activity, TNF-α concentration, and abnormal $CD4^+/CD8^+$ T lymphocyte subset. The increase in infiltrating T cells and macrophages best explain the immunostimulatory effect of COS. Results herein highlighted the therapeutic potential of COS as adjuvant treatment during tumor chemotherapy.

1. Introduction

Cancer has become a major concern for human health, owing to the high mortality associated with the disease. For patients with solid tumor, immunosuppressive microenvironment exists around tumor tissue, preventing immune cells from developing anti-tumor effect. Surgical resection is the primary treatment for solid tumors, however, in most cases; surgery can only excise visible primary and metastatic cancers; leaving persistent residual tiny tumors, which lead to tumor recurrence and treatment failure [1]. After surgical resection, the damaged tissue and tumor microenvironment constitute available pathways for immune cells to tumor tissue. Although, most patients respond to chemotherapy treatments, they likely develop resistance to the conventional chemotherapeutic agents [2]. These limitations highlight the requirements for new anticancer agents and/or combined therapies that can alternatively supplement and/or improve the efficacy of chemotherapeutics currently available [3].

Among the anticancer drugs found between 1940 and 2014, approximately 49% of the approved drugs were derived, either in a whole or in a part, from natural products [4]. In the past, carbohydrates were considered as a food source to maintain a healthy diet [5]. As the biological roles of carbohydrate were advanced in recent years, there is an increasing interest on the effects of different polysaccharides/oligosaccharides on biological activities. Chitosan oligosaccharides (COS), the degradation products of chitin or chitosan, have various biological effects, including anti-inflammatory [6], antimicrobial [7],

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immunomodulatory [8], antioxidant [9] and anticancer activities [10]. The anticancer activity has attracted much attention and various reports have demonstrated both the *in vitro* [11–13] as well as the *in vivo* [10,14] effects of COS.

Currently, drug combinations have been widely used for treating cancers. Since the toxicity of chemotherapeutic agents still remains a major obstacle for application; using natural compounds (such as carbohydrate) as a potential adjuvant therapy may reduce drug-drug interaction toxicities. The potential synergism achieved can mitigate chemotherapeutic-associated pitfalls, such as drug resistance, reduce dosages, and probably the toxicity [15]. Zhao et al. [16] found that a combination therapy of sitagliptin and COS might achieve a better glycemic control efficacy in elderly patients with type-2 diabetes mellitus (T2DM) compared to single usage, through affecting the levels of resistin, C-reactive protein (CRP), and TNF-α, and increasing the level of adiponectin [17]. found that chitosan and Agaricus blazei Murill (ABM) might be able to reduce the weights of tumors and 5 mg/kg/day chitosan + 246 mg/kg/day ABM; targeting VEGF-VEGFR signaling, can be used as anti-hepatocarcinoma treatment in a xenograft model of hepatoma.

Cyclophosphamide (CTX) is a cell cycle-dependent DNA and protein alkylating agent and commonly used to treat many malignancies, has been associated with severe toxicities, such as diarrhea, nausea, bone marrow suppression and immunosuppression. Chen et al. [18] reported the capability of Fuzheng Qingjie, a polyherbal Chinese Medicine, to potentiate the anticancer effect of CTX. They found that CTX-induced immune suppression and body weight loss were prevented without obvious toxicity accumulation in H22 tumor-bearing mice. Recently, our research team evaluated the anti-proliferative effects of COS *in vitro* on ten tumor cell lines, including human gastric adenocarcinoma, human lung adenocarcinoma, human kidney carcinoma, human colon carcinoma, and human breast tumor cells with IC_{50} values ranged between 48.6 ± 7.0 to 1329.9 ± 93.4 μg/mL [19]. The COS produces relatively broad-spectrum anticancer effect, without particular sensitivity to any type of tumor cells. Additionally, COS with high purity and low degree of polymerization demonstrate excellent anticancer effect both in S180 and HCT116-xenograft model [19,20]. In our latest study, we further assessed the immunomodulatory effect of COS [21]. To illustrate whether COS can potentiate the anticancer effect of CTX and effectively alleviate the CTX-associated toxicities in a residual-tumor model in mice. The anticancer effect, lymphocytes proliferation, NK activity of lymphocyte and blood T lymphocyte subpopulation were evaluated. Furthermore, cell proliferation and infiltrating immune components of xenograft tumor were also revealed with an immunohistochemistry protocol using different biomarkers.

2. Material and methods

2.1. Drugs, chemicals, and reagents

Cyclophosphamide (CTX) was obtained from Pude Pharmaceutical Company (Shanxi, China). Anti-mouse CD3, CD4, and CD8 antibodies were provided by Biolegend (San Diego, USA). Antibodies were supplied by Google Biological Technology Company (Wuhan, China). The average molecular weight of COS used in this study was 1000 Da [19–21]. All other chemicals and solvents were of analytical grade and purchased from Sinopharm Chemical Reagent Company (Beijing, China).

2.2. Analysis of COS

The structure of COS was identified using ^{13}C NMR spectroscopy (Avance 400 MHz, Bruker, Germany). The degree of deacetylation (DD) was measured by alkaline titration method [22]. A 0.1 g COS was dissolved in 15 mL of 0.1 mol/L standard hydrochloric acid aqueous solution, left at 25 °C for 15 min (when COS was completely dissolved). A 0.1 mol/L sodium hydroxide solution was then used to titrate excessive hydrochloric acid with methyl orange as indicator. Three replicates were performed.

DD was calculated according to the following formulas:

$$W_{NH2} = \frac{(c_1V_1 - c_2V_2) \times 16.02 \times 10^{-3}}{m_{dry}}$$

$$DD(\%) = \frac{203.2 \times W_{NH2}}{16.02 + 42.04 \times W_{NH2}} \times 100$$

where c_1 (mol/L) and V_1 (mL) are the concentration and volume of hydrochloric acid aqueous solution, c_2 (mol/L) and V_2 (mL) are the concentration and volume of sodium hydroxide solution, m_{dry} (g) is the mass of dried COS, and DD (%) is the degree of deacetylation of COS.

2.3. Animals

Kunming (KM) specific pathogen-free (SPF) female mice (weighing 18–20 g) were purchased from Vital River Laboratory Technology Co. (Beijing, China). The animals were housed in rodent facility at room temperature with a 12 h light-dark cycle for acclimatization, with free access to food and water. *All experimental procedures were in accordance to Beijing Medical Experimental Animal Care Commission. This work was approved by the Laboratory Animal Ethics Committee of Beijing Institute of Radiation Medicine (Beijing, China; Reference no., BIRMSPF-120125A).*

2.4. Transplanted residual-tumor model

S180 cells were inoculated in abdominal cavity to four mice. Seven days later, the animals were sacrificed after isoflurane inhalation (5%) and immersed in alcohol (75%) for 1 min. Ascites were harvested using sterile syringe and transferred to sterile centrifuge tube. S180 cells were suspended with stroke-physiological saline solution at a concentration of 10^{10}/L. A 0.2 mL S180 cell suspension was injected subcutaneously in the dorsal region of KM mice [19]. After the live tumor cells were transplanted, solid tumor gradually appeared. When the average tumor volume reached approximately 300 to 400 mm^3, a tumor surgical extirpation was performed [23]. The volume of the remained tumor was approximately 40 to 100 mm^3 after anesthetizing mice with intraperitoneal sodium pentobarbital (0.5%) injection [24]. The animal model described above, simulated the incomplete resection resulted in residual existence of tumor focus following surgical removal of tumor tissue.

2.5. Treatments

The S180-residual mice were randomly divided into six groups; six animals per each group. Since the dosage of 40 and 80 mg/kg had better effect than 20 mg/kg in our previous study [21], we have chosen 40 and 80 mg/kg in this work to study the combination effect with CTX. So the control (received normal saline) and animals treated with CTX 20 mg/kg, COS 40 mg/kg, COS 80 mg/kg, CTX 20 mg/kg + COS 40 mg/kg, and CTX 20 mg/kg + COS 80 mg/kg are allocated as treated group. CTX was administered into the tail vein of the tumor-bearing mice, once every couple of days, whereas COS administered daily by intraperitoneal injection for 18 days. The first treatment began 24 h post-surgery and this is considered as day 1. On day 18th, the mice were sacrificed and the tumors were removed, weighted, and then fixed in formalin (10% w/v in phosphate-buffered saline, (PBS), pH 7.4). At the same time, blood and splenocytes samples were collected; serum was obtained by centrifugation after withstanding and stored at −80 °C.

2.6. Antitumor activity

The evaluation of antitumor activity was based on tumor volume (TV) measurements calculated as follows: $TV = 0.5 \times ab^2$, where a and