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Chitosan oligosaccharides with degree of polymerization 2–6 induces apoptosis in human colon carcinoma HCT116 cells

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ABSTRACT

Colon cancer is the third most common cancer, and yet there is a lack of effective therapeutic method with low side effects. Chitosan oligosaccharides (COS) is derived from chitosan after chitin deacetylation, and attracts more interests due to smaller molecular weight and soluble property. Previously, COS, mainly absorbed through intestinal epithelia, has been reported to exhibit many bioactivities, especially its anti-tumor effect. Recent references pay little attention to molecular weight distribution which is crucial for understanding its biological behavior. Here, we studied reducing sugar content and degree of polymerization (DP) of COS. 86.73% reducing sugar exists in COS sample and the content of chitosan fractions with 2–6 is 85.8%. COS suppressed the growth of HCT116 cells *in vitro* and *in vivo*, and the inhibition rate of tumor weight *in vivo* was high up to 58.6%. Moreover, the morphology observation, flow cytometry analysis and mRNA expression were applied to study the apoptosis related mechanism. COS treatment promoted mitosis, late stage apoptosis and S cell cycle arrest in HCT116 cells, and enhanced the mRNA expression of BAK and reduce BCL-2 and BCL-x_L. These findings may provide an important clue for clinical applications of COS as anti-tumor drug or pharmaceutic adjuvant in the future.

1. Introduction

In 2012, 8.2 million people worldwide died from cancer, an increase of 40% compared to that of the last decade. In particular, colon cancer has become the third leading cause of tumor-related deaths in the world, and its incidence is rapidly rising in China. Therefore, more effective treatments are urgently needed [1,2]. Although several conventional chemotherapeutic agents are used in the treatment of colon cancer, limited effectiveness, toxicity and eventual resistance remain serious problems [3]. Following three major solid tumor treatments of operation, radiotherapy and chemotherapy, biological response modifier (BRM) has demonstrated higher biological activities and fewer side effects [4]. Natural products and their synthetic analogs become a critical issue as potential cancer chemoprevention agent [5]. Common anti-tumor effects of natural products include inhibition of cell proliferation and of signaling pathways, apoptosis induction, cytoskeleton integrity destabilization, cell cycle modulation, cell migration and metastasis inhibition. Thus, these compounds are important tools in chemical and pharmacological investigation [6,7].

As the degradation product of chitin, COS has been reported to have favorable immunomodulatory, antioxidant, anti-microbial and anti-tumor properties in biochemical and medical fields. Maeda and Kimura found that low-molecular-weight chitosan inhibited the growth of sarcoma 180-bearing mice by the activation of intestinal immune functions [8]. Mattaveewong and colleagues induced colitis-associated colorectal cancer in C57BL/6 mice, and oral COS administration reduced tumor size in a dose-dependent manner [9]. Unfortunately, these references failed to explore the reducing sugar content and DP. In our study, the reducing sugar content and DP were analyzed. Then, we measured *in vivo* anti-tumor activity of COS against HCT116 on a mouse xenograft model. Moreover, we studied the promising anti-tumor mechanism by various methods, especially its apoptosis-induced effects.

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2. Materials and methods

2.1. Materials and reagents

Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Gibco by Thermo Fisher Scientific Inc. (USA). Dimethyl sulfoxide (DMSO) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were obtained commercially from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum (FBS) was purchased from BeiJing YuanHeng ShenMa Biological Technology Research Institute, China. Human colon carcinoma cell HCT116 was kindly offered by Department of Pharmacology and Toxicology, Beijing Institute of Radiation Medicine, Beijing, China. The average molecular weight of COS used in this study is 1000 Da, and degree of deacetylation is 95% [10]. All other reagents were of the highest available quality.

2.2. Analysis of reducing sugar content and degree of polymerization of COS

0.03 g COS sample was dissolved in 2.0 mL H_2O, and was mixed with 4.0 mL 98% H_2SO_4 dropwise in the ice-water bath. The mixture was hydrolyzed at 90 °C for 2 h. Then neutralize the reagent with NaOH solution and dilute with H_2O to 50.0 mL. The reducing sugar content in COS was measured by 3,5-dinitrosalicylic acid colormetric method (DNS method) [11] using T6 spectrophotometer (Persee General Instrument Co., Ltd, Beijing, China) at 540 nm. The concentration of reducing sugar was calculated according to a standard curve plotted with graded concentrations of glucosamine hydrochloride standard.

The DP of COS was analyzed by ion chromatography (ICS 5000^+, ThermoFisher co. Ltd, USA) coupled with anion-exchange column (Thermo DionexCarbopac™ PA200, ThermoFisher co. Ltd, USA) after samples were dissolved in sodium azide solution (20 μg/mL) with pulsed amperometric detector. Chitobiose to chitohexamer with DP from 2 to 6 were prepared with NaN_3 solution, and stored at 4 °C before use.

2.3. Cytotoxicity assay

The inhibitory effect of COS against HCT116 cells was evaluated by MTT assay [12]. HCT116 cells were seeded in 96-well plates at a density of 3000 cells/well. After 24 h, cell culture medium was replaced with 200 μL medium containing graded COS concentrations (10, 5, 2.5 1.25, 0.625, and 0.3125 mg/mL). The control group was treated with culture medium. Each sample was tested in triplicate. After 72 h incubation at 37 °C, proliferation of HCT116 cells was analyzed by MTT assay. The inhibition ratio was calculated as the following equation: Inhibition Ratio (%) = $(1\text{-}A_1/A_0) \times 100\%$, where A_1 is the absorbance of the test group and A_0 is the absorbance of the control group.

HCT116-Luc cells, referred to HCT116 stably transfected with Luciferase (Luc), were planted into 96-well plate as described above. After exposure to COS (10, 5, 2.5 1.25, 0.625, and 0.3125 mg/mL) for 72 h, 150 μg/mL of D-luciferin substrate (PerkinElmer®, USA) was added into each well. *In vitro* bioluminescence imaging was performed by IVIS Spectrum® system. Bioluminescent photon flux was measured and analyzed by Living image® software in 12 × 8 grid template manner.

2.4. Apoptosis morphology of HCT116-GFP-H_2B_9 induced by COS

HCT116 cells transfected with genes of Green Fluorescent Protein (GFP) and histone (H_2B_9) are referred to as HCT116-GFP-H_2B_9. Cell suspension was planted into six-well plate at the density of 2×10^5/mL. Twenty-four hours later, graded concentrations of COS were added into each well at the final concentrations of 0, 100, 500, and 1000 μg/mL. After 72 h of treatment, the morphological pictures were captured under fluorescence microscope (Gentimes Technologies Inc., Shanghai, China).

2.5. Flow cytometry analysis of apoptosis and cell cycle distribution

HCT116 cells were plated in a six-well plate at the density of 10^5 cells per well. Cells were allowed to adhere and proliferate for 24 h prior to exposure to COS. After cells were exposed to COS at different concentrations (0, 100, 500, or 1000 μg/mL) for an additional 72 h. Non-adherent cells and adherent cells were collected together. Cells in each centrifuge tube were then pelleted. Pelleted cells were re-suspended in 100 μL staining buffer containing Annexin V and propidium iodiede (PI) dyes and incubated for 30 min at room temperature prior to flow cytometry analysis [13]. Cells were washed and resuspended in 100 μL PBS. All analyses were performed on Millipore guava easyCyte ™ flow cytometer (Millipore company, USA). Guavasoft 2.7 was used for acquisition and analysis.

HCT116 cells, treated as mentioned above, were harvested, washed, and resuspended with phosphate buffered saline (PBS), and fixed with ethanol at 4 °C for 30 min. Then, cells were washed and resuspended in PBS containing 0.1 mg/mL RNase A for 30 min at 37 °C. After centrifugation, cells were cultured with 1 mL PBS with 500 μg/mL PI dye for another 5 min. The stained cells were analyzed by flow cytometer.

2.6. qRT-PCR

2×10^5 cells were treated with vehicle or various concentrations of COS for 72 h and lysed by Trizol reagent [14]. Total RNA was extracted according to the manufacturer's instructions. cDNA was synthesized using the HiFiScipt gDNA Removal cDNA Synthesis Kit (Beijing Cowin Biotech., China). The SYBR Green Premix ExTaq PCR kit (Beijing Cowin Biotech., China) was applied to qRT-PCR analysis (Mx3005P, Agilent Technologies., USA). Primers used in the analysis are listed in Table 1.

2.7. In vivo experiment

Three to five-week old female nude mice (Nu/Nu), weighing approximately 20 g, were obtained from Vital River Laboratory Animal Technology Co., Beijing, China. The animals were kept under standard conditions with free access to food and water. All studies were performed in accordance with the Guide for Care and Use of Laboratory Animals, as proposed by the Committee on Care Laboratory Animal Resources, Commission on Life Sciences and National Research Council. 5×10^6 HCT116 cells in 0.2 mL PBS were injected subcutaneously into the right flank of each mouse [15]. Animals were randomized into the control group, the positive group treated with fluorouracil (5-FU) at a dose of 30 mg/kg twice a week by intravenous injection, and two COS-treated groups at doses of 40 mg/kg and 10 mg/kg in every two days intraperitoneally. Tumor sizes were measured every two days by a digital caliper. Tumor volume was calculated using formula $V\ (mm^3) = (ab^2)/2$, where a is the length and b is the width of the tumor tissue. At

Table 1
Primers used for qRT-PCR.

Gene name	Primers
β-actin	Forward 5′- TCTACAATGAGCTGCGTGTG-3′ Reverse 5′- GGTCAGGATCTTCATGAGGT-3′
BCL-2	Forward 5′- GACTGAGTACCTGAACCGGC-3′ Reverse 5′- GTTGACTTCACTTGTGGCCC-3′
BCL-x_L	Forward 5′-GGGCATTCAGTGACCTGACA-3′ Reverse 5′-AAGAGTGAGCCCAGCAGAAC-3′
BAK	Forward 5′- CAGGAACAGGAGGCTGAAGG-3′ Reverse 5′- ATAGGCATTCTCTGCCGTGG-3′
CYCS	Forward 5′-ACTCTTACACAGCCGCCAAT-3′ Reverse 5′-AGGCAGTGGCCAATTATTACTCA-3′
NF-κB	Forward 5′-TGCATACTCCACAGCACCTG-3′ Reverse 5′-TTCTGTTGCCACCTTTCGGT-3′